

Role of DNA Polymerases in Repeat-Mediated Genome Instability

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SUMMARY

Expansions of simple DNA repeats cause numerous hereditary diseases in humans. We analyzed the role of DNA polymerases in the instability of Friedreich's ataxia (GAA)_n repeats in a yeast experimental system. The elementary step of expansion corresponded to ~160 bp in the wild-type strain, matching the size of Okazaki fragments in yeast. This step increased when DNA polymerase α was mutated, suggesting a link between the scale of expansions and Okazaki fragment size. Expandable repeats strongly elevated the rate of mutations at substantial distances around them, a phenomenon we call repeat-induced mutagenesis (RIM). Notably, defects in the replicative DNA polymerases δ and ϵ strongly increased rates for both repeat expansions and RIM. The increases in repeat-mediated instability observed in DNA polymerase δ mutants depended on translesion DNA polymerases. We conclude that repeat expansions and RIM are two sides of the same replicative mechanism.

INTRODUCTION

Expansions of DNA microsatellites are responsible for several dozens of hereditary neurological and developmental disorders in humans, including fragile X syndrome, Friedreich's ataxia, Huntington's disease, myotonic dystrophy, and others (reviewed in López Castel et al., 2010; Mirkin, 2007; and Orr and Zoghbi, 2007). These repeats are stably inherited when short, but become highly unstable when their length exceeds a threshold of ~150 bp. Hundred, and, in some cases, thousands of copies of the repeat could be added in a few generations (Ashley and Warren, 1995). Notably, the longer the repeat, the higher is its propensity to expand.

Molecular mechanisms of repeat expansions were studied in many model experimental systems, including bacteria, yeast, *Drosophila*, mice, and cultured human cells (reviewed in Kovtun and McMurray [2008]). These studies implicated DNA replication

(reviewed in Cleary and Pearson, 2005 and Mirkin, 2006), DNA repair (reviewed in McMurray, 2008, 2010), DNA recombination (reviewed in Wells et al., 2005), and transcription (reviewed in Dion and Wilson, 2009) in the expansion process. As a rule, expansions observed in these systems were relatively short-scale (addition of 10–20 repeats), differently from what was observed in human pedigrees. To bridge the gap between the model system and human data, we developed a system to analyze large-scale repeat expansions in budding yeast, *S. cerevisiae* (Shishkin et al., 2009). To this end, Friedreich's ataxia (GAA)_n repeats were cloned into an intron of the artificially split *URA3* gene. Expansions of the repeat that increased the intron's length beyond ~1.1 kb blocked *URA3* splicing, thus making cells 5-fluoroorotic acid (5-FOA) resistant. Using this approach, we indeed observed large-scale repeat expansions, up to hundreds of triplets. Consistent with data from human pedigrees, longer repeats in our system expanded with a higher propensity than shorter ones. Preliminary genetic analysis led us to propose that these expansions occur during replication through the repeat and possibly involve template switching at the replication fork (Shishkin et al., 2009).

Prima facie, a replication model for repeat instability implies that replicative DNA polymerases at the fork should be key players in the expansion process. Surprisingly, however, there is a lack of data on this topic, with the exception of just one study that investigated the effects of DNA polymerase mutations on the instability of (CAG)_n repeats (Schweitzer and Livingston, 1999). We therefore decided to analyze the rates and elementary steps of large-scale expansions of (GAA)_n repeats in yeast strains carrying defects in each of the three replicative DNA polymerases: alpha (α); delta (δ); and epsilon (ϵ), as well as in mutants with compromised translesion DNA synthesis (TLS). We found that the rate of repeat expansions increased in all replicative but not TLS polymerase mutants and most significantly in those affecting Pol δ and Pol ϵ . A mutation in the proliferating cell nuclear antigen (PCNA) precluding the K164 ubiquitylation, a signal critical for TLS, moderated these increases. An elementary expansion step for different (GAA)_n repeats in the wild-type strain fell between 44 and 63 repeats (122–189 bp), but it became much bigger, 80–85 triplets, in Pol α mutants. These results show a link between the size of an Okazaki fragment and the scale of expansions.

We have previously noticed that (GAA)_n repeats also elevate the rate of mutagenesis in cis at a significant distance from their location (Shishkin et al., 2009). Here we confirmed the phenomenon of repeat-induced mutagenesis (RIM) and analyzed the role of the DNA polymerase mutations in this process. Tellingly, the effects of various replication mutants on RIM were qualitatively similar to their effects on repeat expansions. Altogether, these data demonstrate the pivotal role of DNA replication in various forms of genome instability attributed to DNA repeats, including their expansions and mutagenesis.

RESULTS

Characteristics of Primary Expansions of (GAA)_n Repeats in the Wild-type Yeast

Our approach to detect large-scale repeat expansions in yeast was based on the fact that introns longer than ~1.1 kb cannot be efficiently spliced in *S. cerevisiae* (Yu and Gabriel, 1999). We reasoned, therefore, that large-scale expansions of a repeat within the intron of an artificially split *URA3* gene would cause its inactivation, allowing yeast to grow on 5-FOA-containing media. This approach has indeed allowed us to detect large-scale expansions of the Friedreich's ataxia (GAA)_n repeats in our system: up to 300 triplets could be added to the starting (GAA)₁₀₀ repeat (Shishkin et al., 2009). Our original experimental system, however, appeared to have a caveat: introns carrying different (GAA)_n repeats were much shorter (700–850 bp) than the splicing threshold and also were not of a uniform length between different repeats. This caveat led to a situation in which only longer-sized expansions pushed the intron length beyond the splicing threshold (~1.1 kb) and were recovered on selective media. These longer-sized expansions could be secondary expansions that followed a primary expansion event. Consequently, we could not easily distinguish between the primary and secondary events and were unable to estimate an elementary expansion step for different repeats.

To address these problems, we adjusted the lengths of each intron carrying different (GAA)_n repeats by adding nonrepetitive sequences and making them sufficiently long (900–1,000 bp). As a result, the addition of a relatively few (25–30 copies) triplets to an initial (GAA)_n repeat during the primary expansion event increased the overall length of the intron beyond the splicing threshold (Figure S1). This design effectively eliminated the selective pressure in favor of longer, secondary expansions, making it possible to establish the rates and scales of the primary expansion events. *URA3* cassettes with 52, 78, 100, 123, or 150 (GAA)_n repeats with balanced introns were integrated into the chromosome III, ~1 kb downstream of *ARS306* (Figure 1A). In all cases, the (GAA)_n runs were located in the sense strand for transcription, as in the human *FXN* gene (Campuzano et al., 1996), and in the lagging strand template, i.e., in the orientation causing replication fork stalling (Krasilnikova and Mirkin, 2004).

Figure 1B shows the length distributions for expanded repeats originating from different-sized (GAA)_n runs. These distributions resulted from the PCR analysis of roughly 100 expansions for each initial repeat length. One can see that each distribution is relatively narrow, continuous, and unimodal. A combination of primary and secondary expansion events would result in much

broader, possibly multimodal distributions, which we observed for our original, unbalanced cassettes. Hence, we conclude that our data reflect primary expansion events.

Using the Kernell density plot method, the median values of added repeats for various (GAA)_n repeats were determined (Figure 1C). The median expansion step for the original 78, 100, 123, and 150 (GAA)_n repeats corresponded to 44, 54, 63, and 62 triplets, respectively. Notably, the primary expansion step reached a plateau of ~60 triplets for longer repeats (Figure 1C). Most importantly, the entire range of the primary expansion steps for different lengths repeats appeared to be quite narrow—44–63 triplets. Okazaki fragment size in *S. cerevisiae* was recently estimated biochemically (Smith and Whitehouse, 2012) and genetically (Waisserreiger et al., 2012) to be ~165 nt. Our expansion steps are in near-perfect agreement with this estimate.

The rates of the primary expansion events for various (GAA)_n repeats determined using the method of mutant accumulation (Drake, 1991) are shown in Figure S1. Note that we were unable to detect expansions for the (GAA)₅₂ repeat, indicating that its expansion rate is less than 10⁻⁸ per replication. Thus, large-scale expansions do not occur until the repeat's length exceeds Okazaki fragment size. For longer repeats, the rates of expansions increased exponentially, with their lengths varying from 4.5 × 10⁻⁶ per replication for the (GAA)₇₈ run to 1.4 × 10⁻⁴ per replication for the (GAA)₁₅₀ run. In other words, doubling the repeat's size increased its expansion rate by 30-fold. Overall, these data are in accord with what is known for families with Friedreich's ataxia (Montermini et al., 1997), where the expansions threshold falls between 40 and 60 repeats, while expansion rates drastically increase with the repeat's length.

Effect of Mutations in DNA Polymerase Genes on Repeat Expansions

To analyze the roles of different DNA polymerases in repeat expansions, we chose a particular repetitive run, (GAA)₁₀₀. Expansions were analyzed in yeast strains carrying mutations in the genes encoding for each of the three replicative DNA polymerases: α , δ , and ϵ . These were missense mutations, resulting in amino acid changes within the active sites of the corresponding DNA polymerases, directly affecting their efficiency and/or fidelity.

POL1 encodes the catalytic subunit of DNA polymerase in the Pol α -primase complex involved in the initiation of the DNA synthesis at origins and at the beginning of Okazaki fragments. Mutations *pol1-L868F* and *pol1-Y869A* are a leucine-to-phenylalanine and a tyrosine-to-alanine substitution, respectively, in the enzyme's catalytic domain. Mutation *pol1-L868F* leads to a slow growth combined with the strong mutator phenotype (Niimi et al., 2004), constitutively monoubiquitinated PCNA (Suzuki et al., 2009), and accumulation of long stretches of single-stranded DNA in the Okazaki initiation zone of the fork (Suzuki et al., 2009). Mutation *pol1-Y869A* also leads to a slow growth and sensitivity to hydroxyurea (HU) and increases the mutation rate, particularly in the mismatch repair-deficient strains (Pavlov et al., 2001).

POL3 encodes the catalytic subunit of Pol δ , which is the main replicative and repair DNA polymerase and the one well-suited for replication of the lagging strand (Kunkel and Burgers,

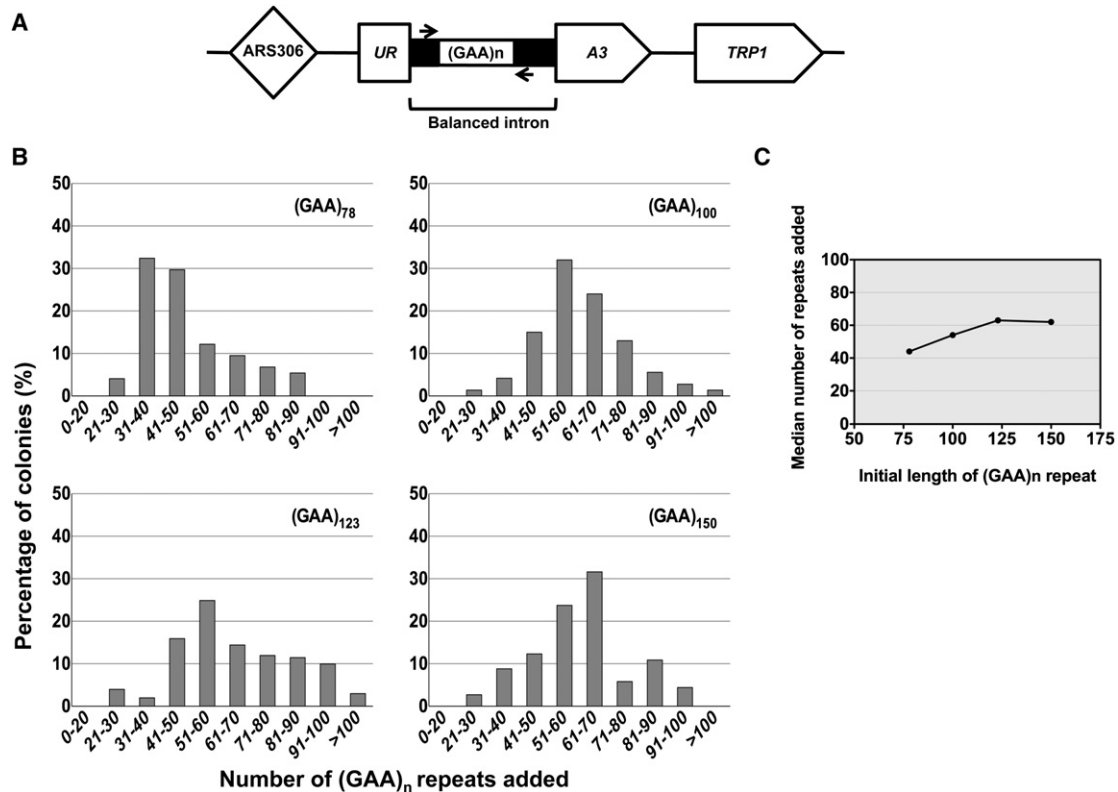


Figure 1. Yeast Model System to Monitor Repeat Expansions

(A) Selectable cassette for the detection of large-scale expansions of the (GAA)_n repeats. Arrows show positions of primers used for the PCR analysis of repeat lengths.

(B) Distributions of repeats added during the expansion of various (GAA)_n runs.

(C) Primary steps of repeat expansion for various (GAA)_n runs. Each point represents a median of the corresponding distribution shown above.

See also Figure S1.

2008). We studied two mutations, *pol3-Y708A* and *pol3-t*, both of which are thought to increase polymerase slippage and/or dissociation from the template. Mutation *pol3-Y708A* is an alanine substitution for a tyrosine within the binding pocket for the nascent base pair in the polymerase active site (Pavlov et al., 2001; Swan et al., 2009). It slows down yeast growth, increases its HU and methylmethane sulfonate sensitivity, and confers a mutator phenotype dependent on the TLS polymerase Polζ (Northam et al., 2006; Pavlov et al., 2001). A temperature-sensitive mutation *pol3-t* is an asparagine substitution for aspartate 643 in the vicinity of the polymerase active site (Tran et al., 1999). At semipermissive temperatures, it impairs yeast growth and grossly elevates deletions between direct repeats (Gordenin et al., 1992; Kokoska et al., 1998).

POL2 encodes the catalytic subunit of Polε, which participates in the leading strand synthesis (Kunkel and Burgers, 2008; Pavlov and Shcherbakova, 2010). Mutation *pol2-9* is a methionine-to-isoleucine substitution in the polymerase active site. At restrictive temperatures, this temperature-sensitive mutation impairs DNA synthesis, likely due to the mutant polymerase dissociating from its template (Araki et al., 1992).

The rates of repeat expansions in these replication mutants are presented in Figure 2A. One can see that Polε mutation

causes the strongest (18-fold) increase in the expansion rate of the (GAA)₁₀₀ repeat. Both Polδ mutations also strongly (~12-fold) increase repeat expansion rates. At the same time, the Polα mutations resulted in a much more modest (2- and 3-fold) increase.

We then analyzed the scale of repeat expansions in various DNA polymerase mutant strains. To this end, expansions of the original (GAA)₁₀₀ run in the wild-type and mutant strains were compared via PCR analysis of the expanded repeats. Figure 2B shows the length distributions of expanded repeats in various mutants, generated upon the analysis of roughly 75 expansions in each case. The length distributions are indistinguishable in the wild-type, Polδ, and Polε mutant strains. The median expansion step in all these cases is about 55 triplets, and the interquartile range is 44–65 triplets. In contrast, the distributions of expanded repeats in both Polα mutant strains are dramatically different: the median expansion step grew to 80 or 85 triplets (depending on the mutant), while its interquartile range varies from 65 to 90 triplets. Statistical analysis of these distributions using a two-sample Kolmogorov–Smirnov test confirms that the differences of the expansion scales between the wild-type and Polα mutants are highly significant (Figure S2). Summarizing, defects in the major leading and lagging strand DNA polymerases increase

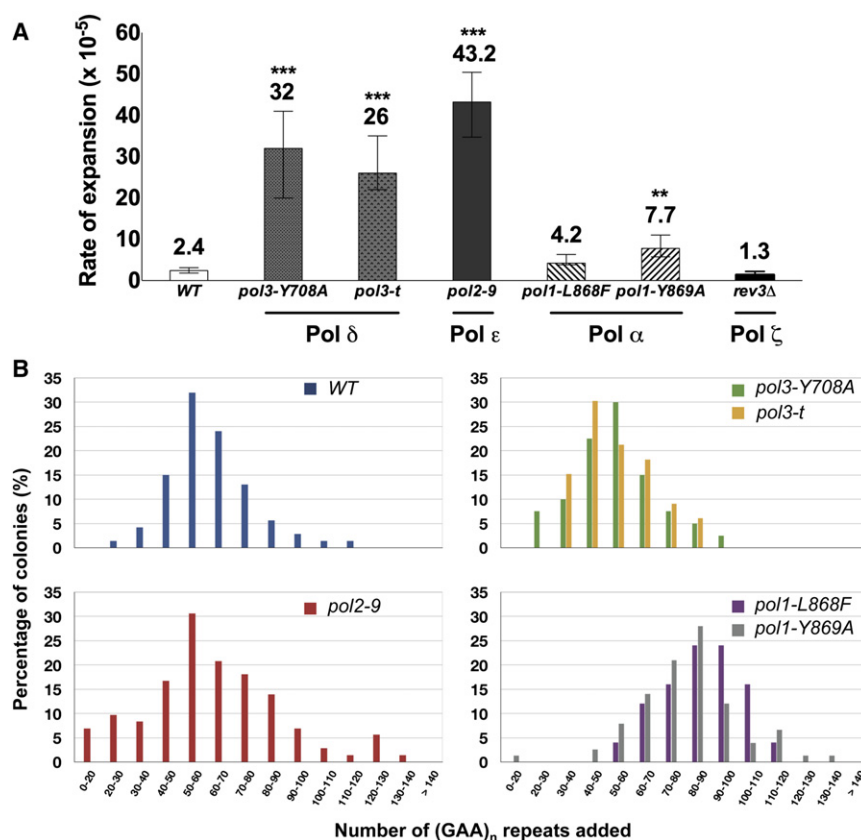


Figure 2. Characteristics of (GAA)₁₀₀ Repeat Expansions in Various Replication Mutants

(A) Rate of expansions. Error bars denote 95% confidence intervals.

(B) Scale of expansions.

See also Figure S2 and S3.

damage-induced mutagenesis. Recruitment of TLS polymerases to the fork is regulated by monoubiquitylation of PCNA at its lysine 164 residue. Mutation *pol30-K164R* is a lysine-to-arginine substitution, which abolishes ubiquitin modification, thereby preventing a switch from the replicative to TLS polymerase and damage-induced mutagenesis (Ulrich, 2011). One can see that neither Polζ knockout (Figure 2A) nor the PCNA mutation alone (Figure 3A) has an effect on repeat expansions.

As discussed above, the phenotypes of several DNA polymerase mutants studied by us are known to depend on TLS polymerases. We were wondering, therefore, whether elevated rates of repeat expansions in our polymerase mutants could be attributed to the TLS. To address this question, we looked at the expansion

the rate but not the scale of repeat expansions, while mutations in Polα, responsible for the proper initiation of Okazaki fragments, increase the scale but not so much the rate of expansions for the (GAA)_n repeats.

To analyze the pattern of the replication fork progression through our repeat-containing *URA3* cassette in these polymerase mutants, we carried out two-dimensional analysis of yeast replication intermediates (Figure S3). In the wild-type strain, the replication fork progression is smooth, as is evident from the shape of the Y-arc, with the exception of a stall site coinciding with the location of the (GAA)₁₀₀ repeat. In the *pol2-9* mutant, fork stalling at the repeat is grossly exacerbated; in fact, the stalling is so profound that the Y-arc past the repeat looks severely constricted. In the *pol3-Y708A* mutant, fork progression is also compromised, as is evident from the dramatic decrease in the amount of intermediates in which fork passed the repeat. The latter effect seems also to be true for *POL1* mutants, albeit it is less pronounced. Note that, in all DNA polymerase mutants, the Y-arcs look fuzzier in the first dimension of the electrophoresis, reflecting the accumulation of replication intermediates of similar shapes, but different molecular weights caused by replication fork indiscretions.

Next we analyzed the effects of two mutations, which affect the DNA damage tolerance on the repeat expansion rates. *REV3* encodes the catalytic subunit of Polζ that mediates TLS, allowing the replisome to bypass DNA lesions (Prakash et al., 2005). Inactivation of this polymerase ablates nearly all DNA

rates in double mutants, in which a replicative polymerase defect was combined with either the *pol30-K164R* mutation or *REV3* deletion. The surge in expansions caused by the *pol3-Y708A* mutation drastically decreased when combined with the *pol30-K164R* mutation, showing that TLS was indeed involved (Figure 3A). More specifically, Polζ was at least partially responsible for this expansion surge. In the case of *pol2-9*, addition of either *pol30-K164R* or *rev3Δ* mutations decreased expansion rate by 2-fold, indicating that TLS carried out by Polζ accounts for half of the expansion spurt. At the same time, TLS does not seem to be a factor in *pol3-t* as well as in the *pol1-Y869A* mutant, since expansion rates are only mildly and statistically insignificantly decreased in the double mutants compared to the polymerase mutants alone.

We believe, therefore, that the erratic progression of the replication fork through repeats accentuated in replicative polymerase mutants is a prime factor leading to their expansions. TLS may or may not be involved in the process, depending on the properties of the particular DNA polymerase mutation.

Effect of Mutations in DNA Polymerase Genes on Repeat-Induced Mutagenesis In Cis

We have previously found that, besides expanding, (GAA)_n repeats enticed mutations in cis in the body of the *URA3* cassette. This repeat-induced mutagenesis (RIM) was detected among 5-FOA-resistant colonies, in which the repeat lengths were not increased relative to those in the starting *URA*⁺ clones

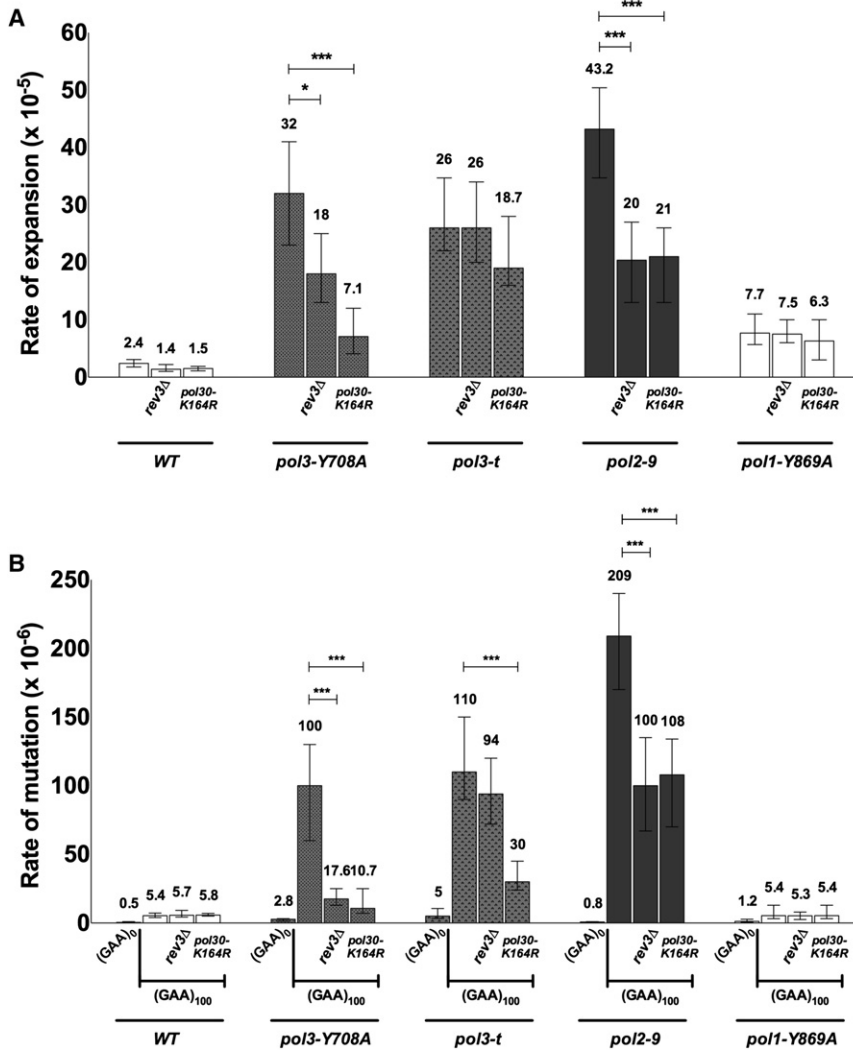


Figure 3. Effect of TLS Deficiency on the Instability of (GAA)₁₀₀ Repeat

(A) Repeat expansions.
(B) Repeat-induced mutagenesis.
Error bars denote 95% confidence intervals.
See also Figure S4.

which is modestly (2–4X) higher than in the wild-type. Finally, the rate of RIM is unaffected in the Pol α mutant. These effects of the polymerase mutations on RIM are in qualitative agreement with our data on expansions, where Pole mutants had the strongest, Pol δ the intermediate, and Pol α the weakest effects.

We investigated the role of TLS in RIM (Figure 3B). In the wild-type genetic background, TLS is not responsible for this mutagenesis, as it is not affected by either a *pol30-K164R* mutation or a *REV3* deletion. In contrast, RIM is primarily Pol ζ dependent in the *pol3-Y708A* mutant. The situation in the *pol2-9* mutant is intermediate between these two extremes. In the *pol3-t* mutant, RIM is Pol ζ independent, but requires ubiquitylation of PCNA, as it is strongly reduced by the *pol30-K164R* mutation. Again, these effects are qualitatively similar to what we described above for repeat expansions. The *pol1-L868F* mutation elevated the rate of mutagenesis more than 100-fold, even without the repeat, making it unfeasible for studying RIM.

Our preliminary analysis of mutations accumulating during RIM in the wild-type

and mutant strains is presented in Figure S4. Importantly, the mutations accumulate both upstream and downstream of the repeat relative to the direction of replication. They are detected at substantial distances from the repeat: up to 500 bp upstream and 1 kb downstream of the repeat. Finally, while the spectrum of mutations in the wild-type strain includes various base pair changes, the spectrum in the *pol3-Y708A* mutant shows a strong bias for C-to-G transversions, which is a clear-cut mutational signature of Pol ζ (Northam et al., 2010; Zhong et al., 2006).

DISCUSSION

We analyzed the roles of replicative and translesion DNA synthesis in repeat-mediated genome instability, including large-scale expansions and RIM. Defects in replicative polymerases strongly affected both processes. The role of TLS was prominent only when a replicative polymerase was compromised. The *pol3-Y708A* mutant best illustrates the latter situation as repeat expansions, and RIM almost completely depends on the TLS machinery.

(Shishkin et al., 2009). Furthermore, this mutagenesis depended on the repeat's length: a 2-fold increase in the repeat's length (from 78 to 150 triplets) resulted in a 10-fold increase in the mutation rate (Shishkin et al., 2009). It remained unclear, however, whether expansions and mutations occur in the course of the same process and what is the nature of the repeat-induced mutations.

We thus studied the effects of the same polymerase mutations (used to study expansions) on RIM. We chose a cassette with the (GAA)₁₀₀ repeat and calculated the rates of 5-FOA-resistant events, in which the repeat's length was not substantially changed. We also sequenced the whole *URA3* cassette from a significant fraction of such clones to assure that they do have mutations, which turned out to be the case. Our data in Figure 3B show that, in the wild-type strain, the (GAA)₁₀₀ repeat increases mutagenesis in cis by an order of magnitude compared to the repeatless situation. The (GAA)₁₀₀ repeat stimulates mutagenesis 230-fold in the Pole mutant, which is a 23X surge relatively to the wild-type. In Pol δ mutants, mutators in their own right, the repeat elevates mutation rates 22–36-fold,

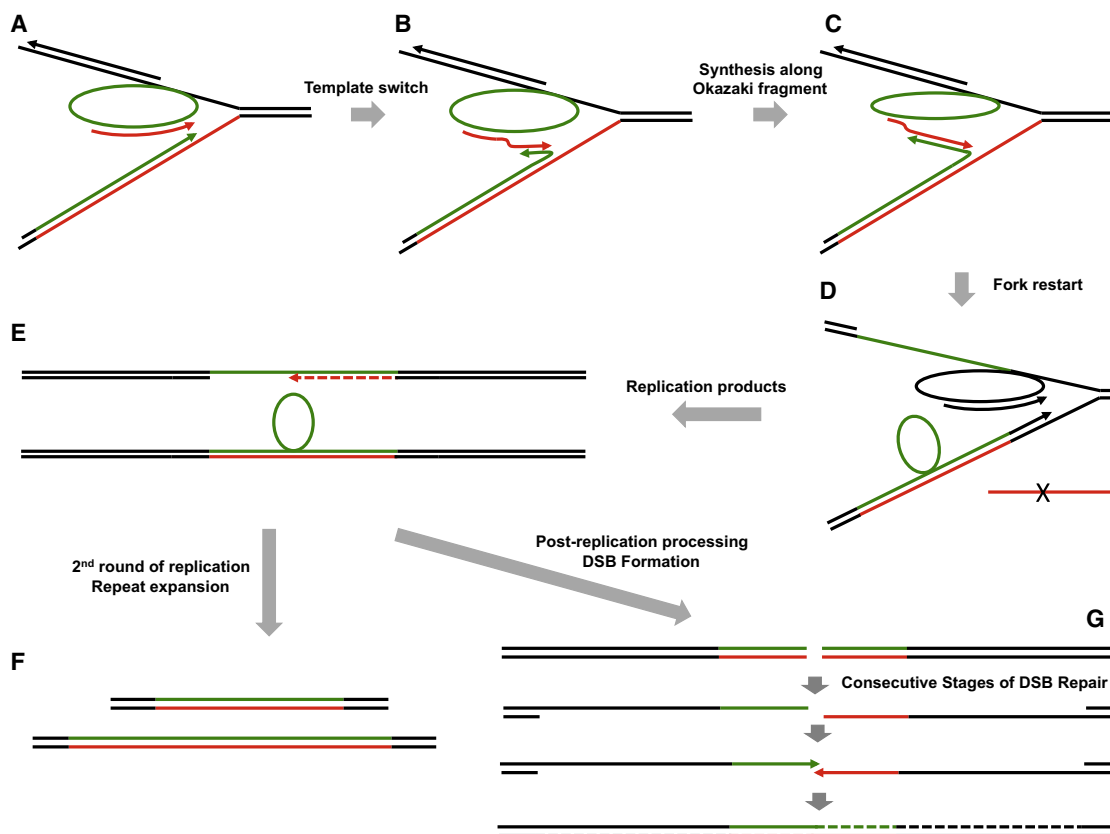


Figure 4. Model Depicting Different Outcomes of Template-Switching During Replication of an Expandable Repeat

(A) Progression of the replication fork through a long repetitive run.
 (B) Switching from template to nascent strand during replication of the repeat.
 (C) Extension of the leading strand along the repetitive Okazaki fragment.
 (D) Leading strand switch to its original template and fork restart.
 (E) Replication products containing repetitive loop-outs and gaps.
 (F) Repeat expansions after the second round of replication.
 (G) Postreplicative formation of a double-strand break within the repeat and its repair leading to RIM. Complementary strands of the repeat are shown in green and red; 3'-ends of growing DNA chains are shown by arrows.

Previous analysis of large-scale repeat expansions in yeast led us to propose the model for the repeat expansions based on template switching (Cherng et al., 2011; Shishkin et al., 2009). It stipulated that, while a replication fork progresses through a long repetitive run (Figure 4A), either leading or lagging strand DNA polymerase can inadvertently switch from its template onto the corresponding nascent strand (Figure 4B). This is followed by DNA polymerization along a nascent strand, which is presented as the leading strand extension in Figure 4C. When the extended strand returns to its original template, the fork can restart (Figure 4D). This scenario would result in the accumulation of extra repeats as a loop out in the nascent leading strand (Figure 4E), which can convert into expansions during the next replication round (Figure 4F).

An important prediction of this model is that mutations in the replicative DNA polymerases that destabilize the fork should favor template switching, increasing the rate of expansions. Our data are in excellent agreement with this prediction: both expansion rates and fork stalling at the repeat increased the

most by the *pol2-9* mutation followed by the two *Polδ* mutations and then *Polα* mutations.

Another prediction of our model is that template switching becomes much more feasible when the length of a repetitive run exceeds the size of an Okazaki fragment, i.e., when both leading and lagging strand DNA polymerases traverse through the repeat. The median size of an Okazaki fragment in yeast corresponds to ~165 nt (Smith and Whitehouse, 2012). Indeed, we could never detect large-scale expansions for the $(GAA)_{52}$ run, which is shorter than an average Okazaki fragment.

Our model postulates the existence of a link between the expansion step and the size of an Okazaki fragment (Figure 4C). Thus mutations affecting the size of an Okazaki fragment in yeast should affect the expansion step accordingly. In the wild-type yeast, the primary expansion step for various $(GAA)_n$ repeats appeared to be 44–63 triplets, which is close to an average Okazaki fragment size. Remarkably, the primary expansion step got much bigger: 80–85 triplets in *Polα* mutants. Both the activity and fidelity of *Polα* are compromised in these mutants, the net result

of which is delays in accumulation of RNA/DNA hybrid primers and concomitant increase of the single-stranded DNA region at the fork (Okazaki initiation zone) together with the size of an Okazaki fragment. For *pol1-L868F* mutant, an increase in size of an Okazaki initiation zone over the wild-type has indeed been demonstrated (Suzuki et al., 2009). We propose, therefore, that a larger expansion step detected in $\text{Pol}\alpha$ mutants is likely due to the increased average size of an Okazaki fragment in those mutants, in agreement with our predictions.

Overall, our data are in excellent agreement with the template-switch model for repeat expansions. Note, however, that the finer mechanisms of template switching remain to be elucidated. For example, *pol30-K164R* mutation in the PCNA appears to have no effect on repeat expansions in the wild-type yeast. This is intriguing, since polyubiquitination of K164 in the PCNA is important at least for one form of template switching driven by the Rad5 protein (Ulrich, 2011). We found that TLS leads to an increased rate of expansions in some strains with defective replicative DNA polymerases. The fork progression impairment in mutants of replicative DNA polymerases is known to recruit $\text{Pol}\zeta$ (Northam et al., 2006, 2010; Suzuki et al., 2009). We speculate, therefore, that the template switching could be easier when the primer ends are handled by $\text{Pol}\zeta$. That said, expansions in the *pol3-t* strain do not involve translesion DNA synthesis (Figure 3). One explanation could be that the altered catalytic subunit of DNA polymerase δ acquires some properties of polymerase ζ that promote template switching.

Besides expanding, $(\text{GAA})_n$ repeats induced mutagenesis at a distance in cis (RIM), a phenomenon which also depended on the repeat's length (Shishkin et al., 2009). Here we show that RIM is detected up to 1.0 kb away from the repeat, and this is probably an underestimation attributable to the size of our selectable *URA3* cassette. Studying the role of polymerase mutations in RIM shed light on its mechanisms. The effects of the polymerase mutations on mutagenesis mirrored their effects on expansions: the rate of RIM was elevated the most in the $\text{Pol}\delta$ mutant followed by $\text{Pol}\delta$ mutants and then $\text{Pol}\alpha$ mutants. Furthermore, RIM did not depend on translesion DNA synthesis in the wild-type genetic setting, but became $\text{Pol}\zeta$ dependent in *pol3-Y708A* mutant, again similar to the expansion data. We conclude, therefore, that RIM results from the same replication problem that leads to repeat expansions. That said, for the very long $(\text{GAA})_n$ repeat ($n \geq 230$), RIM becomes independent of DNA replication (Tom Petes, personal communication).

As shown in Figure 4E, two replication products result from template switching within the repeat: one contains a large (Okazaki fragment-size), repetitive loop-out in the nascent leading strand, while another, unless filled, has a repetitive single-stranded gap within the repeat. These single-stranded segments may also fold onto the surrounding repetitive duplex, forming triplex DNA structures (Krasilnikova and Mirkin, 2004). We hypothesize that postreplicative processing of these complex structures can produce double-strand breaks. We have, in fact, previously demonstrated the formation of double-stranded breaks at $(\text{GAA})_n$ repeats in the course of DNA replication (Kim et al., 2008). Double-stranded break (DSB) repair, which involves extensive end-resection, strand reannealing, and repair DNA synthesis, is known to be error-prone (Hicks et al., 2010). We

believe that this scenario could be responsible for RIM (Figure 4G). It nicely explains two groups of our data: (1) the remarkable similarity in the effects of polymerase mutations on expansions and mutagenesis and (2) the fact that we observe RIM both upstream and downstream of the repeat with regard to the replication direction. While this idea seems plausible, other mechanisms accounting for RIM cannot be ruled out and warrant further investigation.

EXPERIMENTAL PROCEDURES

Strains and Plasmids

S. cerevisiae CH1585 strain (*MATa leu2- Δ 1 trp1- Δ 63 ura3-52 his3-200*) was used to create all mutants employed in this study. Mutations in essential replication genes were introduced by the POP-IN/POP-OUT approach, as described in the Supplemental Information.

Fluctuation Assay and Rates

Fluctuation assays were carried out as previously described (Shishkin et al., 2009).

Two-Dimensional Electrophoretic Analysis of Replication Intermediates

Replication analysis was carried out as previously described in Shishkin et al. (2009).

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Extended Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2012.10.006>.

LICENSING INFORMATION

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