The Major Roles of DNA Polymerases Epsilon and Delta at the Eukaryotic Replication Fork Are Evolutionarily Conserved

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Abstract

Coordinated replication of eukaryotic genomes is intrinsically asymmetric, with continuous leading strand synthesis preceding discontinuous lagging strand synthesis. Here we provide two types of evidence indicating that, in fission yeast, these two biosynthetic tasks are performed by two different replicases. First, in Schizosaccharomyces pombe strains encoding a polδ-L591M mutator allele, base substitutions in reporter genes placed in opposite orientations relative to a well-characterized replication origin are strand-specific and distributed in patterns implying that Polδ is primarily involved in lagging strand replication. Second, in strains encoding a polε-M630F allele and lacking the ability to repair rNMPs in DNA due to a defect in RNase H2, rNMPs are selectively observed in nascent leading strand DNA. The latter observation demonstrates that abundant rNMP incorporation during replication can be tolerated and that they are normally removed in an RNase H2-dependent manner. This provides strong physical evidence that Polε is the primary leading strand replicase. Collectively, these data and earlier results in budding yeast indicate that the major roles of Polδ and Polε at the eukaryotic replication fork are evolutionarily conserved.

Introduction

Three DNA polymerases, Polε, Polδ, and Polε, are required for efficient genome replication in eukaryotes [1,2]. The Polε holoenzyme complex has both primase activity and DNA polymerase activity and is required to initiate each DNA synthesis reaction. The primase subunit first synthesizes a short RNA primer of ~10 nucleotides and the DNA polymerase subunit then extends this primer using dNTPs for a further 20–30 nucleotides, thus initiating DNA replication. Polδ or Polε then substitutes for Polε and perform the bulk of DNA replication by elongating these primers.

Genomic DNA is replicated faithfully during every cell cycle with an error rate of approximately 1 in $10^{-10}$ errors per base pair, ensuring that the genetic blueprint is transmitted largely unaltered through the generations. In eukaryotic cells, DNA replication is initiated bi-directionally from many replication origins. Because of the antiparallel structure of DNA, one strand (leading strand) is replicated continuously in the same direction of the replication fork, while the second strand (lagging strand) is synthesized discontinuously in the opposite direction to that of replication fork progression. The relatively small (200–1000 base) stretches of DNA synthesized during lagging strand replication are known as Okazaki fragments and are rapidly processed and ligated to complete lagging strand replication. The fidelity of replication is ensured by the nucleotide selectivity of replicases to achieve error rates of $10^{-4} - 10^{-5}$, by exonucleolytic proofreading during replication to increase fidelity about 100-fold, and by post-replication DNA mismatch repair to further increase fidelity and lower the mutation rate to $10^{-8} - 10^{-10}$ [3].

Polδ, Polε, and Polε all belong to the B family of DNA polymerases. The structure of the active site of B family DNA polymerases is highly conserved throughout evolution. As for most polymerases, the precise geometry of the polymerase active site ensures that mismatches are largely precluded from incorporation [4]. The importance of polymerase active site geometry to replication fidelity is illustrated by the fact that substitutions of conserved active site residues often reduce DNA synthesis fidelity. Relevant to the present study are substitutions in Saccharomyces cerevisiae Polδ and Polε (M644G and L612M, respectively) that increase error rates during DNA synthesis in vivo [5–8]. These biased error rates result in elevated spontaneous mutation rates in vivo [5–8]. These polymerases have particular value for studies of replication fidelity in vivo because their error rates are preferentially elevated for only one of two possible mismatches that could result in a particular base substitution in a cell. For example Polδ L612M preferentially generates T-dGTP rather than A-dCTP errors, and this preference yields strand specific A→G or C→T mutations during duplex DNA replication in vivo. These biased error rates result in asymmetric mutation profiles in a URA3 reporter gene that is replicated in only one direction due to its close proximity to an active origin. When present in each of the two possible URA3
Author Summary

It is important to understand the architecture of the DNA replication machinery and whether this is common to all organisms. Recent work in Saccharomyces cerevisiae has genetically assigned specific DNA polymerases to leading and lagging strand DNA synthesis, Polα and Polδ respectively. In this manuscript, we use a similar genetic assay to demonstrate that, in the highly evolutionarily diverged yeast Schizosaccharomyces pombe, Polδ is similarly responsible for lagging strand synthesis. Importantly, we establish a novel physical assay, the incorporation of rNMPs into newly replicated DNA, which demonstrates that Polδ is responsible for leading strand synthesis and does not contribute significantly to lagging strand replication. These data strongly support and consolidate the interpretation of previous genetic data and suggest that the division of labour between polymerases is conserved through evolution.

Results

Approach

Which DNA polymerase replicates which strand has only been determined in the budding yeast S. cerevisiae [9,10]. We thus wished to determine if this division of labour between the main replicative polymerases is conserved in the distantly related eukaryote, the fission yeast Schizosaccharomyces pombe. Our strategy was to establish the direction of replication for a specific locus, to create mutants in the genes encoding two replicative polymerases, Polδ and Polε, that exhibit specific and characteristic profiles of misincorporation, and to use these to assign each polymerase to one or the other strand (or both) based on the profile of misincorporation at the directionally replicated loci.

The catalytic subunits of Polδ or Polε are encoded by the *cdc6* (*pol δ*) and the *cdc20* (*pol ε*) genes, respectively. For clarity, here we simply refer to them as *pol δ* and *pol ε*. We employed recombination-mediated cassette exchange (RMCE) to create strains that harbor each specific mutant polymerase [17]. Mutant genes introduced into the genome by this method are flanked by lox (P and M3) sequences. Thus, we also created control strains (*pol δ*−) that have the gene encoding the wild-type polymerase flanked by the same lox sites.

Direction of DNA Replication at the ura4 Locus

The *Schizosaccharomyces pombe* ura4+ gene allows for both positive and negative selection. Selecting for loss of ura4+ function is achieved by growth on medium containing 5-fluoro-orotic acid (3-FOA), which identifies loss-of-function mutants. However, mutations in either the ura4 or the ura5 genes of *Schizosaccharomyces pombe* confer 3-FOA resistance, and it has been reported that greater than 50% of spontaneously arising 3-FOA resistant clones harbor mutations in *ura5* [18]. In wild type cells, ura4+ is located on chromosome III while ura5+ is located on chromosome II. Therefore, to efficiently identify mutations at a single chromosomal location that confer 3-FOA resistance, we created two artificial loci where *ura5+* was placed adjacent to ura4+ on chromosome III. These differ only in the orientation of the *ura4*::ura5 fragment (Figure 1A). We confirmed that this novel *ura5*::ura4 fragment does not function as a replication origin by demonstrating it would not support maintenance of plasmid sequence in cells. We also deleted the genomic *ura5*+ gene on chromosome II, so that resultant ura4+::ura5 strains have only one copy of the ura4+ and ura5+ genes.

The ura4+::ura5 locus is on Chromosome III, near two autonomous replicating sequences; *ars3003*/*3004*. Both the *ars3003* and *ars3004* sequences have been well characterized and are known to be highly efficient at initiating replication [19,20]. However, more than 50% of *Schizosaccharomyces pombe* intergenic regions have the potential to function as origins of replication [21]. Thus, to experimentally determine the direction of DNA replication at the ura4+::ura5 locus, we employed the method of directional 2-D gel electrophoresis [22]. DNA from an asynchronous population of cells is first digested with HindIII and BglII and fragments separated in the first dimension without ethidium bromide. The lane is then excised and digested with SphI, which cleaves within the HindIII-BglII fragment containing the ura4+ and ura5+ genes. This DNA is then subjected to the second dimension of electrophoresis in the presence of ethidium bromide and DNA in the gel is transferred to a membrane for Southern blot analysis with the ura4+ containing HindIII-SphI fragment. The results revealed the direction of DNA replication, as illustrated in Figure 1B. Most of detectable replication intermediates show the pattern consistent with DNA replication moving from right to left (Figure 1C, see red arrow bottom panel: its equivalent is similarly indicated in the top panel of Figure 1B). Thus, we conclude that a leftward replication fork replicates the ura4+ ura5+ locus in the majority of cells.

Characterization of a polδ-L591M Mutant

We then created the polδ-L591M mutant using RMCE. *Schizosaccharomyces pombe* Polδ L591 is equivalent to *S. cerevisiae* Polδ L612. *polδ-L591M* cells grow as well as wild type cells (Figure 1D), demonstrating that this mutant of Polδ is proficient for DNA replication in vivo. In wild type and ura4+ ura5+ backgrounds, *polδ-L591M* showed a strong mutator phenotype (Figure 1E). Spontaneous mutation
rates are elevated ~100-fold in polδ-L591M (4.5 × 10⁻⁶/cell division) compared with that in polδ⁺ (4.7 × 10⁻⁸).

Mutational Bias in polδ L591M Strains

The elevated mutation rates indicate that most of the mutations seen in polδ-L591M cells reflect the error specificity of this mutant polymerase, rather than background mutations. As shown in Table 1, more than half of mutations were point mutations, consistent with elevated base misincorporation observed in vivo for the equivalent S. cerevisiae strain and in vitro for the corresponding mutant version (L612M) of S. cerevisiae Pol6 [5,6,23] and human (L606M) Pol6 [11,12]. In addition to point mutations, we observed a variety of duplication and deletion mutations. All of these deletions and duplications were observed at repetitive DNA sequences. More than half of the deletions were >100 bp, while the majority of duplications were <100 bp (Table S1). Possible mechanisms by which such mutations may arise are addressed in the Discussion.

Among the point mutations, transition mutations showed significant strand dependence for misincorporation. Figure 2 and Table 2 show the predicted mismatches formed during synthesis of the transcribed strand, which corresponds to lagging or leading strand synthesis in the Forward or Reverse strains, respectively (illustrated in Figure 2A). A:T to G:C changes can result from either AdoCTP mismatches or T:dGTP mismatches. Depending on which template strand is copied by the mutated polymerase, this will give a bias of mutation resulting in a spectrum dependent on the orientation of the DNA sequence (see Figure 2B). We observed that, for A:T to G:C changes, T:dG mispairing is 12.5-fold more frequent than AdoC mispairing in the Forward strain, while AdoC...
is more frequent in the Reverse strain. Since the misincorporation rate of the corresponding mutant S. cerevisiae and human polymerases are much higher for T:dG than for A:dC [6,12], the results in Figure 2 imply that Polδ preferentially replicates the lagging strand template. A similar bias was also observed for G:C to A:G mutations. G:T is ~3 fold higher than C:dA in the Forward strain while C:dA is ~3 fold higher in the Reverse strain. Comparing these data with the published in vitro results is also consistent with Polδ being responsible for replicating the lagging strand template. Strand dependence was not observed in polδ-591M cells reflecting base misincorporation by the mutant polymerase rather than sequence context or the transcriptional direction of marker genes. We did not observe strong hotspots for particular mutations, but the total number of occurrences is higher for some mutations, e.g., T to C at ura4 base pair 236 and 76 in the Forward background and C to T at ura4 190 and (−91) in the Reverse background (Figure 3).

Characterization of the polε-M630F Mutant

S. cerevisiae Polε M644G shows strong bias between A:dA and T:dG mispairs in vitro and the spontaneous mutation rates of the corresponding mutant cells are significantly higher than that of wild type and exhibit strand bias [8]. However, we found that the equivalent Schizo. pombe polε-M630G mutation is lethal, as was a polε-M630K mutation. Analysis of strains expressing Polε M630G or Polε-M630K from an ectopic integrated copy in a polε background (Figure S1) suggest this is largely due to catalytic inactivity, as mutation frequencies were not dramatically increased. Thus, we created strains harboring polε-M630F as an alternative. The decision to substitute to phenylalanine was based on earlier studies showing that Polε L868F is error prone in vitro and mutagenic in vivo [13], Polε M644F is error-prone in vitro with a weak bias in error rates [7], and Polε L979F is error prone in vitro [24] and mutagenic in vivo [14]. The Schizo. pombe polε-M630F that we created using the RMCE methodology grows slightly more slowly than polε, although the size of mutant colonies becomes comparable to that of wild type after prolonged incubation (Figure 4A). Strains harboring polε-M630F did not exhibit a substantial increase in spontaneous mutation rate in a mismatch repair proficient background. In strains wherein mismatch repair is inactivated by deleting the msh2 gene, polε-M630F increased the mutation rate by 4–5 fold (Figure 4C). However, upon sequencing ura5 and ura4 from 5-FOA resistant clones, a strand bias sufficient

### Table 1. 5-FOA resistant mutants from polδ-L591M.

|                      | polδ-L591M forward | polδ-L591M reverse | polδ+ forward | polδ+ reverse |
|----------------------|--------------------|--------------------|--------------|--------------|
| Base substitutions   |                    |                    |              |              |
| 1 base deletions     | 71                 | 97                 | 83           | 101          |
| 1 base insertions    | 5                  | 37                 | 8            | 5            |
| Duplication          | 47                 | 45                 | 6            | 6            |
| Deletions            | 18                 | 13                 | 12           | 12           |
| Others               | 41                 | 30                 | 3            | 3            |
| **Total**            | **236**            | **229**            | **123**      | **131**      |

Summary of all 5-FOA resistant mutations seen in the polδ-L591M mutant and the polδ+ strains in the Forward and Reverse ura4“ura5” backgrounds. Single base changes for polδ-L591M are indicated above or below the corresponding sequence in Figure 2.

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Figure 2. Strand bias for polδ-L591M. A. Schematic of replication through the Forward and Reverse ura4“ura5” loci. Leading strand synthesis shown in red, lagging strand synthesis is shown in green. The transcribed strand is shown in blue for reference. B. Top: the relative numbers of AT>GC and GC>AT mutations identified, classified as resulting from either A:dC or T:dG mispairing (AT>GC) and G:dT or C:dA mispairing (GC>AT). Bottom. Schematic illustration of replication of a specific A:T base pair in both orientations. If the lagging strand polymerase, but not the leading strand polymerase, is prone to mispairing dG opposite T during incorporation, but not dC opposite A, then this A:T base pair will mutate to G:C more frequently in the forward orientation than the reverse orientation.

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Table 2. Strand bias of mutants from polö-L591M.

| Mutation | Mispair | in vitro** | Forward (lagging strand) | Reverse (leading strand) |
|----------|---------|------------|--------------------------|--------------------------|
| AT->GC   | A:dC    | 1          | 2                        | 10                       |
|          | T:dG    | 18-44      | 25                       | 4                        |
| GC->AT   | G:dT    | ~2         | 15                       | 8                        |
|          | C:dA    | 1          | 5                        | 27                       |
| GC->TA   | G:dA    | 7-14       | 9                        | 31                       |
|          | C:dT    | 1          | 4                        | 3                        |
| AT->TA   | A:dA    | 2-12       | 4                        | 7                        |
|          | T:dT    | 1          | 1                        | 3                        |
| AT->CG   | A:dG    | 0          | 4                        | 0                        |
|          | T:dC    | 4          | 0                        | 0                        |
| GC->CG   | G:dG    | 2          | 0                        | 0                        |
|          | C:dC    | 0          | 0                        | 0                        |
| AAT      | A:A     | 1          | 9                        | 10                       |
|          | A:T     | ~5         | 23                       | 13                       |
| AGC      | A:G     | ~17        | 1                        | 7                        |
|          | A:C     | 1          | 18                       | 7                        |

Strand bias seen in the polö-L591M mutant in the Forward and Reverse ura4^+:ura5^- backgrounds. Data from lines 1 and 2 are plotted in Figure 1F. *Expected mispairs during synthesis of the transcribed strand. **expected numbers based on in vitro analysis of Polö L612M from Nick McElheny SA et al. [6].

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to infer which strand is copied by the mutant Polö was not observed (Table S2).

rNMP Incorporation into DNA by Polö M630F
Mutations at Polö M644 in S. cerevisiae affect the rate of rNMP incorporation into DNA [15]. We thus tested this possibility in Schizo. pombe. rNMPs incorporated into DNA are rapidly excised by the activity of RNase H2, whose catalytic subunit is encoded by the rnh201 gene of Schizo. pombe. Since increased rNMP incorporation increases alkali-dependent DNA fragmentation, we assayed for increased gel mobility of DNA from the endogenous ura4^+ locus using Southern blot analysis. As anticipated, genomic DNA prepared from polö-M630F was not particularly sensitive to alkali treatment when compared to genomic DNA from the polö^+ strain (Figure 5A, lanes 1 and 2). However, it becomes significantly sensitive compared to polö^+ when rnh201 is deleted (lanes 3 and 4). This indicates that Polö M630F incorporates rNMP into DNA at higher rate than wild type Polö and that these are largely removed by RNase H2 activity.

Based on this observation, we chose to test the strand specificity of rNMP incorporation using alkali treatment and subsequent probing for either the leading or lagging strand using the appropriate single-stranded probes. We prepared two pairs of probe across ars3003/3004 (Figure 5B). The top strand is detected by probe A and C, while the bottom strand is detected by probe B and D. As shown in Figure 5C, only one of each of the two strands from rnh201Δ polö-M630F was sensitive to alkali at each probed site. The alkali sensitive strand was the bottom strand on the left side of the origin, while the top was sensitive on the right side (Figure 5B and 5C). Since those probed sites are inferred to be copied by replication forks emerged at ars3003/3004, the alkali-sensitive strands correspond to the nascent leading strand products of replication. Similar results were obtained at another origin (Figure S2). These results strongly suggest that Polö replicates the leading strand template.

Table 3. Lack of strand bias of mutants from polö^+.

| Mutation | Mispair | Forward (lagging strand) | Reverse (leading strand) |
|----------|---------|--------------------------|--------------------------|
| AT->GC   | A:dC    | 1                        | 3                        |
|          | T:dG    | 0                        | 5                        |
| GC->AT   | G:dT    | 13                       | 22                       |
|          | C:dA    | 10                       | 17                       |
| GC->TA   | G:dA    | 20                       | 29                       |
|          | C:dT    | 12                       | 6                        |
| AT->TA   | A:dA    | 4                        | 2                        |
|          | T:dT    | 3                        | 3                        |
| AT->CG   | A:dG    | 1                        | 2                        |
|          | T:dC    | 7                        | 5                        |
| GC->CG   | G:dG    | 0                        | 0                        |
|          | C:dC    | 1                        | 4                        |

Lack of significant strand bias of mutations observed from the polö^+ strain in the Forward and Reverse ura4^+:ura5^- backgrounds.

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Discussion

An understanding of the fundamental mechanism of DNA replication is an important aspect of appreciating how replication and the errors made during replication influence evolution and human disease. While we have a breadth of knowledge about the proteins involved in eukaryotic DNA replication, including those that move with the active replisome, we do not have an unambiguous view of the architecture of the replication machine itself. Indeed, only recently has genetic data from the budding yeast S. cerevisiae linked the key replicative polymerases Polö and Polö to leading and lagging strand synthesis, respectively. While these assignments are consistent with a number of additional observations, such as the role of Polö in the maturation of lagging strands [25-27], it is important to provide additional evidence to reinforce these assignments, as well as to establish if they are evolutionarily conserved.

Lagging Strand Synthesis by Polö
To investigate the role of Schizo. pombe Polö during DNA replication, we created strains that replicate using a Polö L591M mutant protein. We showed that Polö L591M is highly mutagenic and induced various types of mutations in Schizo. pombe. Strand dependence in transition mutations allowed us to conclude that the main role of Polö is during lagging strand synthesis (Figure 2 and Table 2). However, the mutational bias seen in this mutant is weaker than would be predicted from the in vivo and in vitro studies of the equivalent S. cerevisiae mutant. Because we used mismatch repair proficient cells for this study (the double mutant was lethal), the mutation spectra we observed here reflect mispairs that have escaped mismatch detection and repair. This may influence our interpretations. For example, bacterial MutS protein has variable affinity for different mismatches, with G:T being one of the best substrates [28,29]. Thus, the specificity of mismatch repair might have partially masked the bias of misincorporation induced by Polö L591M. It is also possible that the mutation spectra were affected by spontaneous base damage that results in mismatches that escape mismatch repair. These caveats mean that, while our results are consistent with a function of Polö as a lagging strand polymerase, we cannot exclude the possibility that Polö partly participates in leading strand synthesis or that Polö (or indeed
Figure 3. Mutation spectra. A. ura4 and B. ura5. The promoter region of ura5 (lower case) and the ORF’s of both rad4 and ura5 (upper case) are shown. The position of each mapped mutation is indicated. Mutations arising in the “forward” background (transcribed strand replicated by lagging strand synthesis) are shown above the sequence, whereas the mutations arising in the “reverse” background (transcribed strand replicated by leading strand synthesis) are shown below the sequence.

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other polymerases) may partially replicate the lagging strand [10,30].

In addition to point mutations that were expected from \textit{in vitro} studies of \textit{S. cerevisiae} and human polymerases, we also observed significantly enhanced formation of deletions and duplications in \textit{pol} \textit{d-L591M} cells (Table 1). All deletions and duplications occurred at repetitive DNA sequences. The majority of duplications involved <100 bases (Table S1), reminiscent of the mutation spectra for \textit{S. cerevisiae} \textit{rad27} mutants. Jin \textit{et al.} showed that duplication rates were enhanced by mutations in the Polδ

Figure 4. Mutation rate and dNMP incorporation by \textit{pol}-M630F. A. Cell growth of wild type cells and \textit{pol}+ (wild-type \textit{pol} flanked by lox sites) and \textit{pol}-M630F strains. Serial dilutions of cells were spotted on YEA plates, incubated (30°C) for 2 or 3 days and photographed. B. Schematic of the wild type \textit{ura4}+ locus and the two versions of the modified \textit{ura4}+ : \textit{ura5}+ loci. C. Spontaneous mutation rates for \textit{pol}+, \textit{msh2Δ}, \textit{pol}-M630F and \textit{pol}-M630F \textit{msh2Δ} double mutant cells in the \textit{ura4}+ (wild type), Forward and Reverse backgrounds. Error bars are standard deviations. doi:10.1371/journal.pgen.1002407.g004
exonuclease domain [25] and *S. cerevisiae* pol δ-L612M cells require functional Rad27 for viability [31]. These studies are consistent with our observations and add support to the premise that Polδ is involved directly in lagging strand synthesis in *Schizosaccharomyces pombe*.

The size of deletions we observed was relatively larger than that of duplications. More than half of the deletions were loss of >100 bp of sequence. Cai et al. have observed that exonuclease deficient *E. coli* DNA polymerase II generates similar deletions flanked by direct repeat sequences [32]. They proposed a model in which a mismatch made by a mutator polymerase during replication of the first direct repeat promotes primer relocation to the second direct repeat. Furthermore, we observed a low frequency of inversions flanked by inverted repeat sequences and most of these inversions were associated with deletion, duplication, and/or gene conversion. These events can be explained by template switching. Taken together, these observations suggest that a mismatch formed during DNA replication can cause various kinds of genome rearrangements. Interestingly, chromosome abnormalities such as chromatid breaks are substantially elevated in *Pold1*<sup>+/L604G</sup> and *Pold1*<sup>+/L604K</sup> mouse cells [33].

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**Figure 5. Strand bias for rNMP incorporation.** A. Alkali sensitivity of genomic DNA. Genomic DNA from the indicated strains was either digested with EcoRI or left undigested, then treated with alkali and separated by alkaline agarose gel electrophoresis. DNA was revealed with ethidium bromide following neutralization and then processed for Southern analysis and probed with a *ura4* probe that reveals both DNA strands. B. Schematic of the loci either side of *ars3003/3004* indicating the positions of the EcoRI sites, plus the location and strand specificity of the probes used. C. Alkali sensitivity of each strand, either on the left of *ars3003/3004* (probes A and B) or on the right (probes C and D). Strains were either *polε*<sup>+</sup> or *polε*-M630F (<sup>–</sup>) with or without concomitant deletion of *rnh201*, as indicated. The membrane from Figure 3D was stripped and hybridized with the indicated single stranded probes. Probe A and C hybridize with the top strand, probes B and D hybridize with the bottom strand.

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Leading Strand Synthesis by Polε

To investigate a role of Polε during normal DNA replication, we utilized the observation that S. cerevisiae Polε M644G increased rNMP incorporation [15,34]. We first demonstrated that Schizo. pombe polε-p60-M630F cells incorporate rNMP into DNA at higher frequency than polε cells (Figure 5A). This property of the mutant polymerase made it possible to determine the strand that is copied by the mutant Polε. Incorporation of rNMP in the leading strand was strikingly higher in polε-M630F mutant cells compared to polε cells (Figure 5B and Figure S1). This result strongly suggests that Polε synthesizes the leading strand. On the other hand, we failed to observe a significant difference in rNMP incorporation in the lagging strand. This suggests that Polε has, at most, a limited role in lagging strand synthesis.

Schizo. pombe cells that harbor polε-M630G were not viable, while the corresponding mutation does not cause lethality in S. cerevisiae. Interestingly, the N-terminal catalytic domain of Polε can be entirely deleted in both yeasts [35,36], while a catalytically dead Polε, that retains the full-length protein, is inviable. Our mutation frequency analysis of cells expressing Polε-M630G in a polε background (Figure S1) suggest the inviability of polε-M630G is because the corresponding protein is catalytically dead, rather than because it increases the mutation burden beyond that which is sustainable.

Incorporation and Repair of rNMPs in DNA

In addition to supporting a role for Polε in leading strand replication, the results in Figure 5 extend to Schizo. pombe two important conclusions derived from earlier studies in S. cerevisiae, namely that large numbers of rNTPs can be incorporated into the nascent leading strand during replication without strongly affecting growth (Figure 4A) and the rNMPs that are stably incorporated into the Schizo. pombe genome by a eukaryotic replicase are efficiently repaired in a RNase H2-dependent manner. In S. cerevisiae, unrepaird rNMPs in DNA promote formation of short deletions between short, tandemly repeated DNA sequences, by a mechanism that is unaffected by mismatch repair status [34] and is initiated by topoisomerase 1-dependent cleavage of rNMPs [37]. Many of the deletions occur in a manner that depends on the orientation of the reporter gene in relation to the closest origin of replication [15], indicating that they result from rNMPs incorporated into the nascent leading strand by Polε. The characteristics of the Schizo. pombe polε-p60-M630F strains described here offer the opportunity to determine if these consequences are conserved in fission yeast, and to also test whether mating type switching, which depends on rNMPs in DNA [38], is affected by increased rNMP incorporation by replicases and/or by RNase H2 or topoisomerase status.

DNA Replication in Schizo. pombe

In this study, we examined roles of Pol6 and Polε during normal DNA replication in Schizo. pombe using two different methods. The first method was a genetic analysis of mutation spectra asymmetry in pol6 mutant cells. The second was a physical rNMP incorporation assay using polε mutant cells. The combination of these analyses indicates that genomic DNA is replicated in Schizo. pombe in similar manner as has been suggested for S. cerevisiae. Because Schizo. pombe and S. cerevisiae are highly diverged in evolutionary terms [39,40] our results strengthen the interpretation that replication in all eukaryotes follows similar rules. We also add a physical assay to the previous genetic data, increasing the likelihood that the interpretation of the genetics is indeed correct. We mainly examined DNA replication at the genomic ura4 locus, because replication initiation at this locus is known to be highly efficient (Figure 1B). However, a similar result was obtained for a second independent locus using the physical method for assigning Polε activity (Figure S1). Thus, it is reasonable to suggest that DNA replication occurs in similar manner throughout the genome. However, it remains possible that cells utilize these two polymerases in a different manner in some specific situations or at some specific loci.

Materials and Methods

Schizo. pombe Strains, Media, and Methods

Schizo. pombe cells were grown in yeast extract (YE) medium. Standard genetic and molecular procedures were employed as described previously [41]. To examine cell growth on plates, serial dilutions of cells were spotted on YE agar plates, and incubated at 30°C.

Generating DNA Polymerase Mutant Strains

The cdc6 and cdc24 genes were amplified by PCR and cloned into pUC19. cdc6-L591F and cdc24-M630F mutant genes were constructed by PCR-mediated site-directed mutagenesis and sequenced to ensure that only the desired mutation was introduced. Both wild-type and mutant genes were introduced into Schizo. pombe at their native loci by recombination-mediated cassette exchange (RMCE) [17].

Determining Spontaneous Mutation Rates

Spontaneous mutation rates were determined by fluctuation assay as described previously [42]. Briefly, 11 independent single colonies were suspended in 5 ml YEP (YE+polypeptide) medium and grown to saturation at 30°C. Cells were diluted appropriately and plated on YE or YE containing 0.1% 5-fluoroorotic acid (5-FOA). Colonies were counted after 4 days incubation at 30°C. Mutation rates were calculated by the method of median [43]. Genomic DNA from a single 5-FOA resistant colony was isolated and the ura5-ura4 construct was amplified by PCR to be sequenced.

2-D Gel Analysis

Directional 2-D gel analysis was performed as described previously [22] with modifications. Genomic DNA was extracted and digested with HindIII and BflI as described in [44]. After the first dimension electrophoresis, DNA was digested with SpeI in a gel slice and subjected to the second dimension electrophoresis. Replication intermediates were detected by Southern blot.

Detecting Alkali-Sensitive Sites in Genomic DNA

Genomic DNA was extracted from exponentially growing cells and purified by Qiagen genomic-tip 100/G. 5 µg of undigested or EcoRI digested DNA was incubated in 0.3 M NaOH at 55°C for 2 hours and subjected to 1% alkaline agarose gel electrophoresis [15]. Gels were neutralized and stained with ethidium bromide, followed by Southern blot.

Southern Blotting

Southern blotting was performed according to [45]. DNA fragments of interest were amplified by PCR from Schizo. pombe genomic DNA and used as templates to obtain labeled probes. Radioactive nucleotides were incorporated into DNA using Ready-To-Go DNA Labeling beads (GE Healthcare) or strand specific primers and TaKaRa Ex Taq (TAKARA BIO).
Supporting Information

**Figure S1** Ectopic expression of polymerase epsilon mutants. A. Schematic of the loci where pol2 or mutant versions are expressed from the cdc20 (Pol2) promoter following integration downstream of ura4+. B. Mutation frequencies of indicated strains, either with or without mismatch repair. C. 1. Protein levels of wild type GFP-ura4 tagged Pol2 expressed from the cdc20 locus. 2–4, protein levels of GFP-tagged pol2 and indicated mutants expressed at the ectopic locus in a Pol2 background.

**Figure S2** Strand Bias for rNMP Incorporation at ade6 locus. A. Schematic of the loci either side of ars3045 (Heicheinger et al, 2006; EMBO J. 25, 5171–5179) indicating the positions of the Bom1H sites, plus the location and strand specificity of the probes used. C. Alkali sensitivity of each strand, either on the left of ars3035/3036 (probes E and F) or on the right (probes G and H). Strains were either pol2 (+) or pol2-M630F (−) with or without concomitant deletion of mhr201, as indicated. Probe E and G hybridize with the top strand, probes F and H hybridize with the bottom strand.

**Table S1** Size of deletion or duplication seen in the pol2-L591M mutant in the ura4+ura5+ backgrounds.

**Table S2** Lack of significant strand bias of mutations observed from the pol2-M630F strain in the Forward and Reverse ura4+ura5+ backgrounds. *Expected mispairs during synthesis of the transcribed strand. **Expected numbers based on in vitro analysis of Pol2 M644F from Pursell ZF et al. [7].

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**Author Contributions**

Conceived and designed the experiments: AMC TAK IM. Performed the experiments: IM. Analyzed the data: AMC TAK IM. Contributed reagents/materials/analysis tools: IM. Wrote the paper: IM AMC TAK.

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