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DNA Polymerases at the Eukaryotic Fork - 20 Years Later

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Abstract

Function of the eukaryotic genome depends on efficient and accurate replication of anti-parallel DNA strands. Eukaryotic DNA polymerases have different properties adapted to perform a wide spectrum of DNA transactions. Here we focus on major players in the bulk replication, DNA polymerases of the B-family. We review the organization of the replication fork in eukaryotes in a historical perspective, analyze contemporary models and propose a new integrative model of the fork.

Keywords

replication fork; DNA polymerases; replication origins

1. The hypothesis of three DNA polymerases (Pols) at the fork

The need for a model accommodating three DNA polymerases at the fork first arose in 1990 when the list of eukaryotic DNA polymerases expanded to include the third B-family enzyme, Pol ϵ . Two papers important for the DNA replication field were simultaneously published that year. Three distinct DNA Pols (α , δ and ϵ) from HeLa cells have been characterized in Stuart Linn's laboratory [1]. Akio Sugino's laboratory described the *POL2* gene encoding for the third DNA Pol (Pol ϵ) essential for DNA replication in yeast [2]. Based on the biochemical properties of the three replicative Pols known at that time, Morrison and co-authors proposed a novel model of the replication fork in eukaryotes, where each Pol had a specialized role (Fig. 1A). In this model, Pol α , the only Pol possessing the primase activity, initiates DNA synthesis at origins and primes Okazaki fragments on the lagging strand. Pol δ replicates the rest of the lagging strand, and Pol ϵ replicates the leading DNA strand. This assignment was suggested largely because Pol δ is conditionally processive depending on the presence of the proliferating cell nuclear antigen (PCNA), and Pol ϵ was believed to have a high intrinsic processivity (but see [3] and discussion below). Almost two decades later, this model was re-introduced as proven for eukaryotes (Fig. 1B)[4,5]. Indeed, it gained cumulative biochemical and, especially, genetic support, which was considered compelling. Now it is a predominant view on the eukaryotic fork. In the current review, we analyze evidence in favor of the three-Pol model of the fork and examine if all of the available experimental data could be explained by this model. We came to the conclusion that the role of Pol δ may be underestimated in the current model. In addition, a role is emerging for the fourth Pol of the B-family, Pol ζ , as an accessory polymerase in chromosomal DNA replication. We present a new version of Sugino's fork

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model that takes into account both the data consistent with and the data in conflict with the original model.

2. Introduction to the structure of the Pol players at the fork

In this section, we briefly describe the four B-family Pols that are critical for the DNA replication fork in our model. DNA Pols α , ϵ , δ and ζ all belong to the B-family. Their catalytic subunits, called in yeast Pol1, Pol2, Pol3 and Rev3, respectively, possess the same general domain arrangement on the primary amino acid sequence (Fig 2; [6,7]). In addition to the polymerase domain, all Pols have conserved 3'→5' exonuclease domains. Pol α and ζ , however, lack exonuclease activity, because the sequence of catalytic motifs in the Exo domains is destroyed. All Pols possess remnants of the uracil recognizing domain but do not sense uracil like their archael homologs [8]. The C-terminal end of Pols has two Zn-finger domains critical for the assembly of the holoenzymes [9–11]. The size of the catalytic subunits widely varies due to the presence of additional N-terminal (in Rev3 and Pol1) or C-terminal extensions (in Pol2 and Pol1), whose structure and roles are mainly unknown. A recent breakthrough was the discovery that the catalytic subunit of Pol ϵ is a fusion of two distinct, active and inactive, Pols of the B family [7]. Included in Fig. 2 for comparison is the schematic outline of phage RB69 DNA polymerase, whose crystal structure is often used to model eukaryotic DNA polymerases. It obviously lacks the complexity of the eukaryotic enzymes.

Functional B-family Pols in eukaryotes are multi-subunit complexes. Pol α is a four-subunit complex [12]. All four subunits are essential. The largest subunit is a catalytic polypeptide capable of accurate and robust but low-processivity DNA synthesis [13,14]. The current model, based partially on crystallography of yeast Pol α fragments, partially on low-resolution electron microscopy (EM) images, suggests that one larger domain has all the structural elements required for the DNA polymerase reaction and is connected by a flexible linker to the C-terminal Zn-finger domain responsible for interactions with the other subunits [11]. The smallest polypeptide in the four-subunit complex is the catalytic primase subunit. It is tightly associated with the larger accessory primase subunit that, in turn, interacts with p166. The second largest subunit has a unique iron-sulfur domain essential for the priming reaction in addition to the primase catalytic subunit and is also responsible for the association with the origin recognition complex [15,16].

Pol δ is a complex of four (three in budding yeast) polypeptides [17–19]. The largest catalytic subunit has DNA polymerase and 3'→5' exonuclease active sites, as well as sites for protein-protein interactions [20,21] and a PCNA binding motif [19]. The essential second subunit serves as a stabilizer for the catalytic subunit and as a matchmaker with the third subunit. In yeast, mutations abolishing interactions between the second and third subunit phenocopy the deletion of the third subunit gene [21]. The third subunit plays several important roles. It has a conserved PCNA-binding motif and a motif that mediates interaction with Pol α [22–24]. However, the corresponding gene, *POL32*, is dispensable for growth in budding yeast [19]. Deletion of this gene renders yeast unable to undergo ultraviolet (UV)-induced mutagenesis, similar to deletions of *REV1* and *REV3* genes encoding for translesion synthesis (TLS) polymerases. This suggests a role of the Pol32 protein in the regulation of error-prone TLS [6,25,26]. Pol32 interacts with Rev1 and can recruit Pol ζ via this interaction, which could explain the role of this subunit in induced mutagenesis ([27]; see also discussion below). The role of the fourth subunit is enigmatic. The deletion does not result in noticeable phenotypes in fission yeast [28], while the experiments with human enzyme suggested that it plays a regulatory role in Pol δ response to DNA damage [29].

Pol ϵ is a four-subunit complex [30]. A low-resolution cryo-EM structure of the yeast complex is available [31]. The largest catalytic subunit has robust DNA polymerase and proofreading

exonuclease activity. The second subunit mediates all protein-protein interactions within the holoenzyme and is essential. Mutations that weaken these interactions confer a mutator phenotype [32,33]. The third and fourth subunits are involved in the interaction with double-stranded DNA [31,34] but are not required for growth, although their absence results in an elevation of spontaneous mutagenesis [35,36]. It has also been shown that the fourth subunit is involved in chromatin remodeling [37]. Pol ϵ is additionally regulated by multiple accessory factors involved in origin recognition [30,38,39].

Pol ζ has been isolated from yeast in the active form as a two-subunit complex [40]. A heterodimer of the Rev3 protein and the second subunit encoded by the *REV7* gene has DNA polymerase activity and is uniquely proficient in the extension of mismatched primer termini [25,26]. The genetic data suggest that the role of Pol ζ as a key player in TLS is conserved between yeast and humans [41,42]. In addition, the human homolog of the second subunit of the yeast enzyme may have an additional role in regulating cell cycle progression. The human REV7 shows similarity to the spindle checkpoint protein MAD2 and was reported to interact with MAD2 *in vitro* [43].

Evolutionary scenarios that led to the creation of the four polymerases are very complex [7, 44]. For example, in addition to the fusion of two Pols to generate Pol α as discussed previously, the second subunits (so-called B-subunits) of Pol α , Pol δ and Pol ϵ share some similarity and have homologs in Archaea [45,46]. In Archaea, these polypeptides have 3'→5' exonuclease motifs and are subunits of a so-called D family of DNA polymerases [47]. Changes of critical catalytic residues in the phosphodiesterase motifs in the eukaryotic orthologs of B-subunits renders them inactive as nucleases. A crystal structure of the B subunit of human Pol δ complexed with a part of the third subunit reveals an OB-fold DNA binding domain and a surface for interaction with the other subunits [21]. The Zn-finger domain history is also non-trivial, involving duplication and the use of different ancestors for Pol2 and the other B-family Pols [7].

3. Lessons from simple organisms

The asymmetric nature of DNA poses topological problems for replication of the anti-parallel strands by a fork moving in one direction, so the replication of the two strands is inherently different [48]. In simple DNA replication systems, such as bacteriophage T4, one B-family DNA polymerase is sufficient for rapid synthesis of leading and lagging strands [49]. In the bacterium *Escherichia coli*, the fork is managed by a dimeric or even trimeric DNA Pol III [50,51]. Some bacterial species, like *Bacillus subtilis*, utilize two separate Pols for the leading and lagging DNA strands [52]. Replication of the mammalian virus SV40 requires only primase-associated Pol α and Pol δ for synthesis of both strands [53]. Even when the same Pol replicates both DNA strands, the accuracy of lagging strand synthesis is up to 10-fold higher than that of the leading DNA strand [54].

4. Early insights into the roles of Pol δ and Pol ϵ

The first attempt to solve the puzzle of three polymerases at the fork was undertaken in the early 1990s by Alan Morrison in Sugino's lab at NIEHS. Morrison and co-authors generated Exo⁻ derivatives of Pol ϵ and Pol δ by altering the conserved ExoI motif FDIE (mutations *pol2-4* and *pol3-01*, respectively; [55,56]). The corresponding Pols are inaccurate because they can not proofread replication errors. The phenotypes of the corresponding yeast mutants are summarized in Table 1. The studies of the proofreading-deficient mutants provided the following information.

- i. The *pol2-4* and *pol3-01* mutations conferred a moderate and strong mutator effect, respectively. This was consistent with the involvement of both Pols in chromosomal

DNA replication. The substantially more severe mutator effect of *pol3-01*, however, was not expected from the “one strand – one DNA polymerase” model. Although various explanations were discussed (for example, [57–59]), the difference between the phenotypes of *pol3-01* and *pol2-4* mutants remains somewhat puzzling.

- ii. A combination of each of the *pol3-01* and *pol2-4* mutations with a DNA mismatch repair (MMR) defect (*pms1* mutation) resulted in a synergistic increase in mutation rates (Table 1, [56,60]). This indicated that the proofreading activities of Pol ϵ and Pol δ act in series with the MMR system, providing further evidence for the involvement of these activities in chromosomal replication. In accordance with the stronger effect of the *pol3-01* allele in MMR-proficient strains, the combination of *pol3-01* and *pms1* had more dramatic consequences than the combination of *pol2-4* and *pms1*. The double *pol3-01 pms1* mutation was lethal in haploid cells because of an extremely high level of spontaneous mutagenesis. The synergistic interaction of the two mutations was established by studying the diploid strains that can tolerate a higher mutation rate [56]. In contrast, the mutation rate in the *pol2-4 pms1* strains, although quite high, was still below the level that would result in lethality in haploid cells, so the haploid double mutants survived [60]. This, again, raised a question of whether Pol ϵ and Pol δ copy equal portions of the genome.
- iii. A combination of *pol3-01* and *pol2-4* mutations resulted in a synergistic increase in the mutation rate that was, similar to the *pol3-01 pms1* combination, incompatible with life in haploid cells [60]. Note that if the two Pols worked on different DNA strands, the interaction of the mutator effects of *pol3-01* and *pol2-4* would be expected to be simply additive. The synergistic interaction was interpreted as an indication of competition of the two exonucleases for the same pool of replication errors. An alternative explanation for the excessively high mutation rate in the *pol3-01 pol2-4* strain is that MMR system becomes saturated when the two proofreading defects are added up [60]. This, however, seems unlikely, because the mutator effect of *pol2-4* is small in comparison to the *pol3-01* effect. If the uncorrected errors resulting from the two proofreading defects simply sum up, the effect of the double *pol3-01 pol2-4* mutation would not be significantly different from the effect of *pol3-01* alone, and MMR is clearly not saturated in the *pol3-01* mutants [56]. As we discuss further below, the synergistic interaction of Pol ϵ and Pol δ proofreading defects is the observation that is most difficult to reconcile with the model wherein these two Pols replicate opposite DNA strands.
- iv. To address the question of whether Pol δ and Pol ϵ work on the opposite strands more specifically, Morrison and Sugino analyzed the spectra of spontaneous mutations arising in the *pol3-01* and *pol2-4* mutants using two different orientations of the reporter gene in respect to the nearest origin of replication [60]. The authors reasoned that if Pol δ synthesized both DNA strands of this reporter gene, the spectra of mutations generated in the proofreading-deficient mutant should be similar in the two orientations. A limited number of mutants sequenced indicated that this simple prediction is not completely met and there might be some strand specificity of Pol involvement at the fork.

Although not sufficient to confirm or refute the Sugino lab model, these experiments provided the foundation for those that followed. Particularly useful was the development of the theoretical basis for the analysis of synergistic interactions of mutation rates in yeast and the use of the mutational spectra analysis to decipher the role of DNA polymerases at the fork. As described in the following sections, this approach appeared to be most instrumental in shaping our current understanding of the eukaryotic replication fork.

5. Current evidence supporting Sugino's lab model

Substantial evidence for Sugino's model was obtained by studying the mutational spectra in yeast strains carrying inaccurate variants of replicative Pols. This approach is based on the assumption that finding a mutation that represents the specific "signature" of a particular Pol reveals the participation of this Pol in the copying of this region of the genome. The main obstacle in the analysis of spontaneous mutational spectrum *in vivo* is the difficulty of assigning the strand where the mistake was made. For example, an A•T to G•C transition could result from an initial C incorporation opposite A or a T incorporation opposite G in the complementary DNA strand. One approach to solve this problem was the use of a purine base analog. The strand, into which such an analog is incorporated at each particular site, is unambiguously defined by the orientation of the purine•pyrimidine pair in the DNA. When the base analog-induced mutagenesis at specific base pairs was analyzed, the *pol3-01* and *pol2-4* mutations elevated the mutation frequency in different orientations of the reporter gene, which placed the proofreading activities of Pol δ and Pol ϵ on opposite DNA strands [61]. Similar conclusions were later drawn for plasmid DNA replication from the analysis of spontaneous mutation spectra in proofreading-deficient strains [62], which used the logic originally proposed by Morrison and Sugino [60].

The use of proofreading-defective mutants had limitations, because the Exo of one Pol can correct errors made by another Pol [63,64]. It would have been better to use mutations affecting the base selectivity of the Pol that would specifically allow tracing of the DNA synthetic activity of each Pol. Such mutants were not available until recently. Most mutator variants of replicative Pols were compromised in catalytic efficiency and were unable to compete with wild-type Pols (e.g., [65,66]). Finally and fortunately, in the last several years, mutations in the conserved Pol region II (S/AL/MYPS/NI) were found to lead to loss of fidelity without loss of Pol activity. Mutation of this motif was proven to be universally useful for Pol α [67,68], Pol ϵ [69,70], Pol δ [71,72] and Pol ζ [73,74]. Substantiating common knowledge that "everything new is just forgotten old", we acknowledge the seminal research of Linda Reha-Krantz who described mutator mutations in this motif in phage T4 a decade before its usefulness was appreciated [75,76].

The use of the Pol fidelity mutants was very fruitful. Using the Pol α fidelity mutant, it was established that Exo of Pol δ , but not of Pol ϵ , corrects errors made by Pol α [77]. This suggested that the Pols are regulated in such a manner that only Pol δ has access to the primer termini generated by Pol α on the lagging DNA strand. This is consistent with the clear role of Pol δ in Okazaki fragment maturation [58,78]. These studies, taken together with the demonstration that Exos of Pol ϵ and Pol δ operate on different strands, led to the inevitable conclusion that Pol ϵ is involved in the leading DNA strand replication [19]. Subsequently, analysis of error specificity of the inaccurate Pol δ and Pol ϵ variants *in vitro* revealed the types of errors that could be used to distinguish between the two DNA strands *in vivo*. For example, the inaccurate variant of Pol δ generated T•dGTP errors ~30 times more frequently than the A•dCTP pair, implying that the increase in A•T to C•G transitions *in vivo* can be attributed largely to errors during the copying of the T-containing strand. Elegant and scrupulous study of the mutational spectrum in Pol δ fidelity mutants confirmed the role of Pol δ in lagging strand replication and suggested its lesser charge for the errors in the leading DNA strand [79].

In an attempt to provide direct evidence for the involvement of Pol ϵ in the leading DNA strand replication, Pursell and co-authors employed an analogous Pol ϵ motif II mutant [69]. As in the case of Pol δ , the inaccurate variant of Pol ϵ showed asymmetric error rates for several mispairs *in vitro*. Quite strikingly, the spectrum of spontaneous mutations in the corresponding Pol ϵ mutant *in vivo*, was comprised almost exclusively of only one type of base substitution, A•T to T•A transversion. Moreover, the mutations were largely confined to two hotspots. The

difference in the rate of these mutations in opposite orientations of the reporter gene was consistent with Pol ϵ generating errors during leading and not lagging strand DNA synthesis. However, the absence of other types of mutations, despite the capability of this Pol ϵ variant to produce a wide spectrum of errors, is an argument against the role of Pol ϵ in copying extensive stretches of DNA. Another caveat is that the analysis was performed in an MMR-proficient yeast strain, and, therefore took into account only those *in vivo* errors that escaped MMR. While there could be several explanations for why these particular errors escaped repair, one possibility is that these errors were not generated in the context of the moving replication fork. Finally, the efficiency of MMR in yeast is known to be unequal on the leading and lagging strands [80], which could contribute to the mutational bias observed by Pursell and co-authors. Further studies using MMR-deficient strains and a reporter gene lacking strong mutation hotspots would help to better understand the role of Pol ϵ at the fork.

Despite these uncertainties, these results, along with the ample evidence for the role of Pol δ in lagging strand replication, were considered to provide final proof of Sugino's lab model of the replication fork (Fig 1A, B)[4,5]. Indeed, when Pols are tracked genetically, the results are always consistent with the model of Pol α and Pol δ synthesizing the lagging DNA strand and Pol ϵ the leading strand. A limitation of the genetic mutational approach, however, is that it registers rare events happening once in 10,000 – 1,000,000 cells. The results of such studies need to be interpreted with caution, as these rare events may not necessarily reflect the way replication typically occurs.

6. What is not consistent with the “one strand – one DNA polymerase” model?

Several observations are difficult to reconcile with the strict division of the synthetic activity of Pol ϵ and Pol δ between two different DNA strands.

Strikingly, the deletion of the first part of the *POL2* gene, encoding for the N-terminal, active Pol (Fig. 2) is not lethal in yeast [81–83]. Thus, yeast can survive without the DNA polymerase activity of Pol ϵ . However, the mutants display severe growth and replication defects [84], and mutants with single amino acid substitutions in the active site of Pol ϵ are inviable [65,82]. These observations suggest that Pol ϵ is normally a component of the replication machinery, but in the absence of Pol ϵ , Pol δ can be a substitute for it, comprising an “alternative” fork [4,85]. In contrast, deletion of the catalytic subunit of Pol δ is lethal, so, apparently, Pol ϵ can not compensate for the absence of Pol δ .

The second observation not easily reconciled with the current fork model is that inactivation of the proofreading activity of Pol δ has a stronger effect on the genome-wide mutation rate than an analogous defect in Pol ϵ (Table 1), [56,86]. Consequently, a combination of the proofreading defect of Pol δ with a MMR defect is lethal in haploids, while strains simultaneously lacking the proofreading activity of Pol ϵ and MMR survive [56,60]. There are also examples of amino acid substitutions in the DNA polymerase domain of Pol δ having more severe consequences for growth and mutagenesis in comparison to analogous mutations in Pol ϵ (Table 1; [65]).

The third conflicting observation is the competition of the exonucleases of Pol δ and Pol ϵ for the same pool of replication mistakes (Table 1), which does not quite agree with these Pols working on separate DNA strands. If the latter were true, the interaction of the mutator effects of Exo[−] mutations would have been additive.

The fourth argument against the strict division of labor comes from the studies of DNA damage-induced mutagenesis. Lesions in the template stall replicative Pols, which signals for a switch to a specialized TLS polymerase. The TLS Pols bypass the lesion, often in a way that generates mutations. All induced mutagenesis in yeast vanishes with the deletion of *REV1* or *REV3* genes,

encoding for TLS Pols. Strikingly, damage-induced mutagenesis also disappears when the third subunit of Pol δ , Pol32, is absent [23,87,88]; or when the interaction between Pol32 and the second subunit of Pol δ , Pol31, is abolished [21,23]. The Pol32 subunit was recently shown to interact with Rev1 and subsequently Pol ζ [27]. These observations suggest that Pol δ may be responsible for the recruitment of TLS Pols, as proposed by Chris Lawrence (see discussion in [6], regardless of the DNA strand where the lesion resides. Indeed, if Pol δ operated only on the lagging strand (Fig. 3A) the absence of Pol32 would eliminate only half of the induced mutations. In contrast, if Pol δ worked on both strands (Fig. 3B), the suppression of induced mutagenesis would be complete. This is exactly what is observed. Of course, Pol δ and its Pol32 subunit could be required for TLS at a step subsequent to the initial DNA polymerase switch at the lesion site. This unidentified step could proceed similarly on both strands irrespective of what Pol synthesized this strand before the encounter with a lesion. In this case, the absence of induced mutagenesis in *pol32* mutants could not be considered an argument against the “one strand – one DNA polymerase” model.

Finally, recent careful comparison of the *in vitro* activities of yeast Pol ϵ and Pol δ has shown that Pol ϵ is a less efficient enzyme than Pol δ [3]. Although not directly contradictory to the current replication fork model, this fact does not agree well with the proposed role of Pol ϵ as a major leading strand replicase.

7. The integrated new model

The model we are proposing was inspired by the original Sugino’s lab model. It, however, takes into account recent studies of the properties of the replicative Pols, as well as earlier data that the original model does not easily explain. Our model is based on the view that all three enzymes, Pol α , Pol δ , and Pol ϵ , contribute to the chromosomal DNA replication, for which there is compelling evidence [4,6,19,50]. Similar to the currently accepted model, Pol α synthesizes short RNA-DNA fragments at origins and on the lagging strand, and Pol δ extends these fragments on the lagging strand (Fig. 4). We discussed the extensive evidence for this assignment in the previous sections. The principal novel feature of our model is in the mechanism of leading strand synthesis. We postulate that Pol ϵ is responsible for the initiation of leading strand synthesis (Fig. 4a), as well as elongation of the leading strand in the vicinity of the origin (Fig. 4b). It dissociates from the primer terminus with an increasing probability as the distance from the origin increases, and Pol δ takes over the leading strand synthesis. As a result, the majority of the genome replication involves copying of both DNA strands by Pol δ (Fig. 4c). This DNA polymerase arrangement was previously described as an “alternative” replication fork that could be assembled if functional Pol ϵ is not available or upon replication restart after the standard fork stalls [4]. We propose that this “alternative” fork is a rule rather than exception, whereas the strict assignment of Pol ϵ and Pol δ to the leading and lagging strands, respectively, is only observed in the vicinity of the origin. We summarize the arguments in favor of the leading strand synthesis scenario shown in Fig. 4 below.

There is substantial evidence for the involvement of Pol ϵ in early chromosomal replication at chromosomal origins, mediated by interactions with GINS [84,89–92]. This is formally consistent with the possibility that, once initiated at the origin, the Pol ϵ -dependent leading strand synthesis continues until the entire replicon is copied. However, evidence has been accumulated that the leading strand DNA synthesis is not truly continuous. Multiple *in vivo* studies reported that the products of both leading and lagging strand replication first appear as short fragments, suggesting discontinuous synthesis of both strands [93,94]. As we discuss below, multiple circumstances could lead to the untimely pausing or termination of the leading strand synthesis. This suggests that re-initiation of DNA synthesis must occur on the leading DNA strand at sites other than the replication origins. While the initiation of DNA synthesis at origins is tightly regulated and is allowed only once per cell cycle, the replication restart

away from the origin has to be regulated in a cell cycle-independent manner. Accordingly, while the cell cycle-regulated Pol ϵ activity might be an excellent candidate for the initiation of the leading strand synthesis at origins, it might not be able to re-bind to the primer terminus at other genome sites. This idea is supported, although indirectly, by the fact that Pol ϵ does not participate in the replication initiated at the cell cycle-independent SV40 origin [95]. In contrast, Pol δ , the major lagging strand replicase, is clearly capable of rebinding to the primer terminus multiple times during a cell cycle. Thus, it is possible that a restart of replication on the leading DNA strand requires the activities of Pol α -primase and Pol δ rather than Pol ϵ . The idea of the limited involvement of Pol ϵ in the replication of regions distant from the origins is supported by the observation that less Pol ϵ is detected at such sites by chromatin immunoprecipitation [89].

The exact mechanism of the switching from Pol ϵ to Pol δ could depend on the particular circumstances that led to the termination of DNA synthesis by Pol ϵ . Two possible scenarios are shown in Fig. 4. Pol ϵ could potentially pause due to the incorporation of an incorrect nucleotide, which generates a primer terminus that is difficult to extend. The pausing could subsequently lead to the dissociation of Pol ϵ . The abandoned primer terminus could then be bound by Pol δ that would correct the mismatch through its proofreading activity and continue the leading strand synthesis (Fig. 4). The existence of this mechanism is supported by our new experimental data described below. This way of polymerase switching could be more frequent in cells carrying inaccurate variants of Pol ϵ due to the higher rate of mismatch generation.

Alternatively, Pol ϵ could stall due to the presence of a lesion in the template (Fig. 4, insert). Cellular DNA is continuously damaged by endogenously generated agents, such as oxygen radicals, and lesions are routinely encountered by the replication fork. According to one of the current models of TLS [96], replication stalling at a lesion site results in a quick restart of replication downstream of the lesion, which leaves a gap between the site of the lesion and the site of the restart. TLS polymerases then bypass the lesion and, possibly, fill the remaining gap. As discussed above, Pol δ may be a more likely candidate for accomplishing the replication restart than Pol ϵ , and, thus would be in a position to continue the leading strand synthesis. Note that, in this scenario, TLS polymerases assist the leading strand replication by accomplishing the bypass of the lesion that triggered the polymerase switch (Fig. 4, insert). Obviously the TLS polymerases participate in the bypass of lesions on the lagging strand template as well, although the mechanism of the replication restart might not differ significantly from the normal Okazaki fragment initiation process.

In addition, a recent study suggested that a collision of the *E. coli* replisome with RNA polymerase could be a source of discontinuities in the leading strand synthesis [97]. If similar events take place in eukaryotic cells, this could potentially lead to the dissociation of Pol ϵ and the Pol δ -mediated restart downstream of the RNA polymerase. Finally, as mentioned previously, Pol ϵ is substantially less efficient and somewhat less processive than Pol δ while replicating circular ssDNA *in vitro* [3]. Pausing and/or dissociation of Pol ϵ for reasons other than template damage, the generation of mismatched primer termini or the collision with RNA polymerase could also lead to switching to the more mobile and robust Pol δ .

This model allows us to explain a number of observations that could not easily be reconciled with the strict assignment of Pol δ and Pol ϵ to the lagging and leading DNA strands, respectively. First, it would explain the fact that mutations affecting Pol δ typically have a much stronger effect on strain growth and the genome stability than analogous defects in Pol ϵ (Table 1; see previous section). Second, the model in Fig. 4 satisfactorily explains the competition of the exonucleases of Pol δ and Pol ϵ for the same pool of replication errors suggesting that the two Pols can correct each other's errors [60]. In our unpublished experiments, we further characterized this competition. We combined mutations affecting the

fidelity of polymerization by one of the two Pols, Pol δ and Pol ϵ , with mutations inactivating the exonuclease of the other Pol. The strain with the polymerization fidelity defect of Pol δ and the exonuclease defect of Pol ϵ , showed an additive increase in the mutation rate (Table 1). This implies that Pol ϵ does not proofread errors generated by Pol δ . In a reciprocal experiment, when a mutation affecting the polymerization fidelity of Pol ϵ was combined with an exonuclease defect of Pol δ , a synergistic increase in the mutation rate was seen for several reporter genes. This is consistent with the idea that mistakes made by the inaccurate variant of Pol ϵ are corrected by the proofreading activity of Pol δ . Accordingly, the model in Fig. 4 predicts that Pol δ takes over the primer terminus generated by Pol ϵ during the leading strand synthesis, but Pol ϵ does not access the primer termini generated by Pol δ . We propose that the reporters that detect the synergistic interaction of the Pol ϵ nucleotide selectivity and Pol δ proofreading defects are in the regions, where the switching from Pol ϵ to Pol δ is most probable.

The model shown in Fig. 4 is also consistent with the earlier genetics data demonstrating the primary roles for Pol δ and Pol ϵ in the copying of the opposite DNA strands [61,62,69,79]. All these experiments monitored DNA replication at a reporter gene located close to a replication origin. Interestingly, and in agreement with our model, we were able to detect some proofreading activity of Pol δ on the strand that is preferentially proofread by Pol ϵ even at the reporter placed close to the origin (~4.4 kb; [61]). In general, however, the genetic studies confirm the idea that the fork arrangement proposed by the Sugino lab, exists at the vicinity of the origin. Future studies will help establish if this arrangement could also be seen further away from the origin or, as shown in Fig. 4, Pol δ takes over the synthesis of both strands as the fork progresses. We observed previously that the leading and lagging DNA strands have unequal susceptibility to base analog-induced replication errors, and the strand bias is maintained over the entire replicon studied [98]. One explanation for the bias is the involvement of different Pols in the copying of the two strands [4,98]. In the case of 8-oxoguanine-generated errors, however, the bias was shown to result from differential MMR efficiency on the leading and lagging strands [80]. The example of *E. coli*, where the two strands are copied by the same Pol III holoenzyme with unequal fidelity [54], indicates that there could be inherent differences in the error rate on the leading and lagging strands. Apparently, this bias could reflect the fundamental differences in the mechanism of replication of the two strands rather than the identity of Pol that does the copying.

The last comment we would like to make relates to the possible role of Pol ζ , the fourth eukaryotic B family polymerase, in the chromosomal DNA replication. Fig. 4 illustrates that TLS polymerase activity is an integral part of the genome replication. TLS polymerases, including Pol ζ , as well as the Y family DNA polymerases, help to overcome replication barriers created by lesions [26,96]. Our model proposes that, in addition to other genome sites, the TLS activity could be specifically observed in areas where the switching from Pol ϵ to Pol δ occurs on the leading DNA strand. In addition, our earlier and more recent studies suggested that Pol ζ may be recruited to restart DNA synthesis at stalled or slowly progressing replication forks regardless of the presence of DNA damage. A Pol ζ -dependent increase in spontaneous mutagenesis is observed in strains with defects in normal replicative DNA polymerases [36, 65,99]. Genetic studies suggested that this mutagenesis results from error-prone copying of undamaged DNA (M. R. Northam, H. A. Robinson and P. V. Shcherbakova, unpublished observations). Moreover, some fraction of the errors introduced by Pol ζ is corrected by MMR, similar to the regular DNA replication errors [65]. It is, therefore, possible that Pol ζ assists the main trio during the genome replication. The role of Pol ζ in the copying of undamaged DNA likely reflects its ability to extend terminally mismatched primers and other aberrant substrates that are poorly extended by the replicative DNA polymerases [26]. However, Pol ζ is dispensable for normal growth and replication in yeast. We propose that Pol ζ is the fourth Pol at the fork, whose involvement is of a limited extent and in a highly controlled fashion.

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Figure 1. Three-polymerase model of replication fork in eukaryotes

A. The model proposed in 1990 by Sugino group (adapted from [2]).

(i) Pol α synthesizes short DNA segments (straight lines) primed by RNA (solid circles) at the replication origin (open arrowheads). (ii) Pol ϵ synthesizes the leading strand (dashed line); Pol α synthesizes short RNA-DNA stretches on the lagging strand, which are subsequently extended by Pol δ . (iii) After removal of RNA primers, Pol δ completes the lagging strand synthesis (dotted lines).

B. The currently accepted model (adapted from [85]).

The model illustrates primary roles for Pol ϵ (green oval) in leading DNA strand replication (dashed line) and Pol δ (red oval) in lagging strand replication (dotted line). Other proteins

shown include the Pol α -primase (blue oval) synthesizing RNA-DNA hybrids (solid circles and straight lines), the MCM helicase (pink), the eukaryotic single-stranded-DNA-binding protein, replication protein A (RPA; gray ovals), the sliding clamp proliferating cell nuclear antigen (PCNA; gray ring) and the Fen1–DNA ligase complex (khaki-yellow).

Figure 2. A schematic diagram of conserved blocks of the four eukaryotic B-family DNA polymerases in comparison with the phage RB69 DNA polymerase

The drawing is based on the amino acid sequences of the catalytic subunits of yeast Pols and RB69 Pol and is roughly to scale. The inactivated C-terminal domains of Pol2, uracil-recognizing domains and exonuclease domains of Pol1 and Rev3 are marked by “X” symbols. Zn-finger 2* denotes the distinct version of this module in Pol2 that is highly similar to the Zn-finger of archaeal PolD [7].

Figure 3. Models of Pol δ -mediated induced mutagenesis

Pol32 mediates the recruitment of the mutasome including Rev1 and Pol ζ to the lagging strand (A) or both strands (B).

Pol holoenzymes are drawn in blue (Pol α), red (Pol δ), yellow (Pol ϵ), purple (Pol ζ) and green (Rev1). A solid arrow acknowledges the proven interaction of Rev1 with Pol32. A broken arrow with a question mark indicates the hypothetical interaction of mutasome with Pol ϵ . A black square represents damaged DNA.

Figure 4. Model of replication fork

a) Pol ϵ and Pol α are recruited to the origins (ARS, standing for Autonomous Replication Sequence according to the yeast nomenclature) by the replication initiation machinery and start leading DNA strand synthesis.

b) Pol δ is recruited to the lagging DNA strand, and replication of both strands proceeds further (only one fork moving to the left is shown for simplicity).

c) At a random site away from the origin, Pol ϵ encounters an obstacle and dissociates. Its recruitment back is not possible due to the absence of origin-specific factors. Pol δ rapidly takes over the leading DNA strand synthesis.

c, *inset*) Obstacles that can be overcome neither by Pol ϵ , nor by Pol δ , result in the recruitment of TLS polymerases. In the example shown, the replication block results in a replication restart downstream of the block, which requires Pol α and Pol δ . The remaining gaps are filled postreplicatively, as postulated by one of the current TLS models [98]. Replication block is shown as damaged DNA for simplicity. In reality, an elevated level of dissociation of the main replicative DNA polymerases due to genetic or physiological perturbations can result in the recruitment of TLS Pols to the fork.

Table 1

Severity of the mutator effects of mutations affecting the fidelity of yeast Pol α , δ , ϵ and ζ^* .

Allele	Pol defect	Alone	With defect of Pol ϵ Exo	With defect of Pol δ Exo	With defect of MMR
<i>pol1-L868M</i>	base selection	very weak	weak, additivity	strong, synergy	very strong, synergy
<i>pol2-M644G</i>	base selection	weak	very strong, synergy	very strong, synergy	very strong, synergy
<i>pol3-L612M</i>	base selection	weak	weak, additivity	catastrophic **, synergy	very strong, synergy
<i>rev3-L979F</i>	base selection	very weak	weak, additivity	weak, additivity	weak, additivity
<i>pol2-4</i>	proofreading	moderate	n/a	catastrophic **, synergy y	very strong, synergy
<i>pol3-01</i>	proofreading	very strong	catastrophic **, synergy	n/a	catastrophic **, synergy nergy
<i>pol3-5DV***</i>	proofreading	strong	catastrophic **, synergy rgy	n/a	catastrophic **, synergy nergy

*The compilation is based on [55,56,58,60, 67,69,71,73, 77,79,86,100] and unpublished observations from Y. Pavlov's lab.

** mutation catastrophe - haploids die because of accumulation of lethal lesions and mutations rates could be measured in diploids only

*** original name for this allele[58]. In newer literature, it is often referred to as *pol3-D520V*, because it leads to D520 $\rightarrow$ V amino acid change[101].

Table 2

Nomenclature (yeast)

Gene	Holoenzyme	Catalytic subunit
<i>POL1</i>	Pol α	Pol1
<i>POL2</i>	Pol ϵ	Pol2
<i>POL3</i>	Pol δ	Pol3
<i>REV3</i>	Pol ζ	Rev3