The 3′→5′ exonuclease of DNA polymerase δ can substitute for the 5′ flap endonuclease Rad27/Fen1 in processing Okazaki fragments and preventing genome instability

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Many DNA polymerases (Pol) have an intrinsic 3′→5′ exonuclease (Exo) activity which corrects polymerase errors and prevents mutations. We describe a role of the 3′→5′ Exo of Pol δ as a supplement or *backup* for the Rad27/Fen1 5′ flap endonuclease. A yeast *rad27* null allele was lethal in combination with Pol δ mutations in Exo I, Exo II, and Exo III motifs that inactivate its exonuclease, but it was viable with mutations in other parts of Pol δ. The *rad27-p* allele, which has little phenotypic effect by itself, was also lethal in combination with mutations in the Pol δ Exo I and Exo II motifs. However, *rad27-p* Pol δ Exo III double mutants were viable. They exhibited strong synergistic increases in *CAN1* duplication mutations, intrachromosomal and interchromosomal recombination, and required the wild-type double-strand break repair genes *RAD50*, *RAD51*, and *RAD52* for viability. Observed effects were similar to those of the *rad27*-null mutant deficient in the removal of 5′ flaps in the lagging strand. These results suggest that the 3′→5′ Exo activity of Pol δ is redundant with Rad27/Fen1 for creating ligatable nicks between adjacent Okazaki fragments, possibly by reducing the amount of strand-displacement in the lagging strand.

DNA polymerases (Pol) play essential roles in DNA replication, repair, and recombination. Several of the prokaryotic and eukaryotic DNA polymerases have an intrinsic 3′→5′ exonuclease (Exo) activity. Structural studies (1–3) indicate that the Exo catalytic site is located in an N-terminal domain that is separated from the polymerase domain. The movement of the 3′ end of the nascent DNA strand from the polymerase to the Exo site requires denaturation of 3–4 terminal base pairs. If the 3′ end of the nascent DNA strand is delayed in the polymerase site, for example when Pol generates misincorporation or misalignment, terminal nucleotides are likely to be partitioned to Exo site and hydrolyzed, resulting in proofreading of an error (reviewed in refs. 4–6). The structure of the Exo active site is highly conserved. Two divalent metal ions are present, and they are coordinated by the amino acids of three signature Exo motifs conserved throughout all families of DNA polymerases (ref. 7 and Fig. 1).

The proofreading function of Pol Exo is consistent with the observation that deficiency in a polymerase-associated exonuclease can lead to a strong mutator phenotype and a high error rate during *in vitro* replication (reviewed in refs. 1 and 8). Replication errors that escape Pol Exo proofreading can be corrected by postreplication mismatch repair. Cells that are deficient in both proofreading and mismatch repair are hypermutable and in some cases experience catastrophic rates of recessive lethal mutations (9–11). The biological importance of Pol Exo is emphasized by the observation of high incidence of tumors in mutant mice homozygous for a Pol δ Exo-deficient allele (B. Preston and R. Goldsby, personal communication).

A function for the 3′→5′ exonuclease of Pol δ distinct from correcting replication errors was proposed to explain why yeast double mutants carrying *pol3-01* and *rad27* (homolog of human *FEN1*, encoding for 5′ flap endonuclease) are inviable (12, 13). Synthetic lethality was observed not only with a *rad27*-Δ allele, but also with the subtle *rad27-p* defect (12). This mutation results from two amino acid changes F346A/F347A that eliminate the interaction between Rad27 and PCNA and has few phenotypic effects on its own. Unlike the pattern of synthetic lethality of *pol3-01* in combination with mutations in mismatch repair genes (9, 14), *pol3-01 rad27* was lethal not only in haploid, but also in diploid strains, even in *pol3-01/POL3*$^+$ heterozygotes. This is inconsistent with a catastrophic rate of recessive lethal mutations leading to synthetic lethality.

There were several possible explanations for the synthetic lethality of *pol3-01* with the *rad27* defect. For example, it could have resulted not from the Pol δ exonuclease defect, but from some other change in function of this polymerase. Because both *pol3-01* and *rad27* defects interact with checkpoint mechanisms (15, 16), synthetic lethality could also be the consequence of cell cycle arrest caused by simultaneous activation of different branches of checkpoint control.

Alternatively, synthetic lethality could be the result of synergy between Pol δ Exo and 5′ flap endonuclease Rad27/Fen1 participating as redundant functions in an important DNA metabolic process such as DNA replication. Based on *in vitro* studies, Rad27/Fen1 is required for processing Okazaki fragments into ligatable nicks because of its capability to remove 5′ ribonucleotide and 5′ flaps caused by strand displacement by DNA polymerase. Flap removal is essential for maturation of shorter Okazaki fragments into long continuous DNA strand (17, 18). Yeast *rad27*-Δ null mutants are prone to an unusual category of mutations, extended duplications of up to 108 bp flanked by short direct repeats (19). It was suggested that the duplications result from incorporation of unremoved 5′ flaps into replicated DNA. Increased recombination and chromosome loss in the *rad27*-Δ mutant indicated that unremoved 5′ flaps can lead to double-strand breaks (DSB). In support of this, the *rad27*-Δ mutant required the *RAD52* group of recombinational DSB repair genes for viability (19, 20). We speculated that both the 5′ flap endonuclease Rad27/Fen1 and the 3′→5′ exonuclease of Pol δ could act together to process adjacent Okazaki

Abbreviations: Pol, DNA polymerase; Exo, exonuclease; DSB, double-strand break; Ts, temperature-sensitive.

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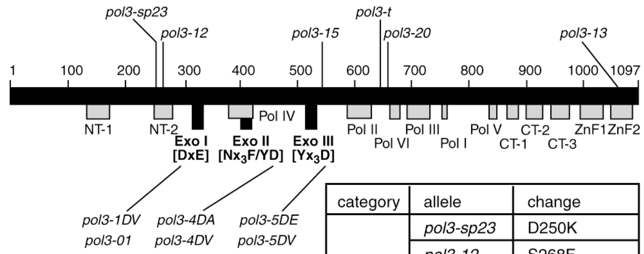

category	allele	change
Ts	*pol3-sp23*	D250K
	pol3-12	S268F
	pol3-15	R542C
	pol3-t	D643N
	pol3-20	T655I
	pol3-13	C1074S
Exo I	*pol3-01*	D321A, E323A
	pol3-1DV	D321V
Exo II	*pol3-4DA*	D407A
	pol3-4DV	D407V
Exo III	*pol3-5DE*	D520E
	pol3-5DV	D520V

Fig. 1. Mutations in the yeast *POL3* gene. The 1097-aa yeast *POL3* ORF is shown schematically as a black bar. Names and positions of conserved regions are designated as in ref. 53. Gray boxes, conserved regions, other than nuclease; black boxes, exonuclease conserved regions. Consensus sequences of the 3′→5′ exonuclease signature motifs (7) are shown in brackets. Ts mutations are shown above and Exo mutations below the *POL3* gene. Amino acid changes in different mutant alleles are shown in the table.

fragments to create ligatable nicks and that toxic intermediates could occur in replication forks when mutant *pol3-01* enzyme is included in the replication complex. We proposed that the accumulation of DSBs was the cause of inviability in the double mutant.

In this study we show that the synthetic lethality with a *rad27* defect is a general feature of nuclease-deficient Pol δ Exo-motif mutants and is not observed with other Pol δ mutations. A specific prediction of the hypothesis of functional redundancy was that the combination of partial Pol δ Exo and Rad27/Fen1 defects could cause genetic changes typical of mutants lacking the Rad27/Fen1 function. Some Pol δ Exo mutations were viable in combination with a partial Rad27 defect, and the viability required a functional DSB recombinational repair system. Surprisingly, the double mutants did not increase the rate of point mutations that might result from DNA polymerase errors, but instead caused synergistic increases in the rates of duplications and mitotic recombination. This suggests that the Pol δ 3′→5′ exonuclease is important not only for correcting replication errors, but also is redundant with Rad27/Fen1 in processing Okazaki fragments and preventing genome instability.

Materials and Methods

Yeast Strains and Plasmids. The wild type, *rad27-p*, *rad27*-Δ, and *pol3-01* isogenic strains used in this study have been described (12, 13, 21). Each strain was *MAT*α *ade5-1 his7-2 leu2-3,112 trp1-289 ura3-52*. Strains also carried mutant *lys2* alleles used as mutation or recombination reporters. Interchromosomal recombination strains ALE100 and ALE101 were described in refs. 12 and 22. Strains ALE1000 and ALE1001 (a gift from K. Lobachev, National Institute of Environmental Health Sciences, Research Triangle Park, NC) with an intrachromosomal recombination reporter carried the 5′ truncated *lys2* sequence, and the *LEU2* gene has been integrated into chromosome II as a direct repeat with the *lys2*::*HS-D* allele.

The plasmids used to make two-step gene replacements of genomic *POL3* by *pol3-t* (p171; ref. 21) and by *pol3-01* (YIpAM26; ref. 9) mutations have been described. Other *pol3* mutations were created either in the plasmid YIpKhr5 or in p170. The YIpKhr5 plasmid with the *POL3* N-terminal region was a gift from A. Sugino and Y. Pavlov (Osaka University, Osaka). *pol3* mutations in the plasmids were made with the Quick Change Site Directed Mutagenesis Kit (Stratagene). Mutated plasmids were sequenced to verify that there were no additional mutations. The LC80B–ARS-CEN-*TRP1* plasmid with wild-type *RAD27* was described in ref. 12. Construction of deletions was as described for *rad50*–pNKY83 (23), *rad51*–pΔRAD51 (24), *rad52*–pΔ52Blast (25), and *pep4*::*KanMX* (26). Sequences of mutant plasmids and oligonucleotides are available on request.

Table 1. Mutation rates of *pol3* mutants

POL3 allele	*lys2*::*InsLD*	*his7-2*	*CAN1*
WT*			
POL3^{+}	1.0	1.0	1.0
Ts			
pol3-t	120	1.2	5.9
pol3-12	67	1.1	2.9
pol3-13	69	2.7	4.8
pol3-15	8.7	0.9	1.4
pol3-20	23	1.1	2.2
pol3-sp23	250	3.6	15
Exo			
pol3-01	0.6†	74	110
pol3-1DV	3.3	37	86
pol3-4DA	1.3	90	55
pol3-4DV	1.0	68	76
pol3-5DE	3.5	39	79
pol3-5DV	2.1	7.4	20

Rates are relative to wild type: *lys2*::*InsLD* reversion, 0.1×10^{-8}; *his7-2* reversion, 1.9×10^{-8}; *CAN1* forward mutation, 29×10^{-8}. Yeast were grown at 25°C.

*Wild type.

†Data from ref. 21.

Table 2. Loss from *pol3 rad27* strains of the *TRP1* marker associated with the plasmid containing a wild-type *RAD27* gene

	rad27 Δ		*rad27-p*	
POL3 allele	*TRP1* loss, %*	Viability	*TRP1* loss, %	Viability
Ts				
pol3-sp23	11–38	+	43–58	+
pol3-12	7.3–33	+	21–43	+
pol3-15	14–31	+	21–51	+
pol3-t	7.2–29	+	19–42	+
pol3-20	15–31	+	33–61	+
pol3-13	19–27	+	55–66	+
Exo				
pol3-01†	0–0.06	−	0–1.0	−
pol3-1DV	0	−	0	−
pol3-4DA	0	−	0	−
pol3-4DV	0	−	0	−
pol3-5DE	0	−	2.6–46‡	+
pol3-5DV	0	−	20–59	+

Yeast were grown at 23°C.

*Presented are the minimal and maximal values of the *TRP1* marker loss obtained in 4–12 independent experiments for viable and 9–12 experiments for nonviable combinations. About 100–300 colonies were counted in each experiment.

†Data from ref. 12.

‡Median value is 11%.

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Table 3. Mutation and recombination rates in *pol3 rad27* double mutants

		CAN1		Recombination	
Genotype	*his7-2*	Total	DUPL*	INTRA	INTER
wild type	1.0	1.0	0 (0/20)	1.0	1.0
rad27-p	1.8	2.7	1.3 (9/19)	1.9	1.9
rad27-Δ	15	24	18 (15/20)§	18	15
pol3-5DE	51	26	ND	1.1	1.1
pol3-5DE rad27-p	62†	100‡	ND	33‡	14‡
pol3-5DV	14	5.9	0 (0/20)	1.3	0.9
pol3-5DV rad27-p	27†	49‡	36 (19/26)§	14‡	3.9‡

Rates are relative to wild type: *his7-2* reversion, 1.2×10^{-8}; *CAN1* forward mutation rate, 51×10^{-8}; INTRA (intrachromosomal recombination), 1400×10^{-8}; INTER (interchromosomal recombination), 9.9×10^{-8}. Yeast were grown at 30°C.

*Rates of extended duplications (DUPL) in *CAN1* relative to the total rate of *can1* mutations in the wild type were calculated based on the fraction of duplications among all *can1* mutants sequenced (numbers of duplications and the total numbers of sequenced *can1* mutants are given in parenthesis as numerator and denominator, respectively). Mutation spectrum is presented in the supplemental data.

†No statistically significant increase over rates in the corresponding single *pol3*-Exo III mutant based on overlapping 95% confidence intervals.

‡Statistically significant increase over the rates in single mutants based on nonoverlapping 95% confidence intervals.

§No statistically significant difference between fractions of duplications, $P = 0.88$.

Yeast Genetic Methods. Yeast genetic methods were as described in ref. 12. *pol3*-Ts (temperature-sensitive) mutants were propagated at 23°C. Temperatures used in specific experiments are given in footnotes to Tables 1–4. *CAN1* mutants were obtained from independent single colony isolates. The *CAN1* gene was PCR amplified and sequenced as described in ref. 19.

Purification of Pol δ. The protease-deficient *pep4*::*KanMX* strains were grown as described in (27). The cells (about 25 g wet weight) were resuspended in 10 ml water and frozen as kernels in liquid nitrogen. They were disrupted in a blender with dry ice in lysis buffer as described (28). The lysate was fractionated with polymin P and ammonium sulfate, and the dialyzed crude fraction was subjected to phosphocellulose chromatography and MonoQ FPLC (27).

The peak MonoQ fractions containing Pol δ activity were diluted with an equal volume of buffer A_0 (30 mM Hepes-NaOH, pH 7.4/1 mM EDTA/0.5 mM EGTA/10% glycerol/0.01% Nonidet P-40/5 mM DTT/2 μM pepstatin A/2 μM leupeptin; and NaCl as indicated by a subscript in mM) and injected onto a 0.5-ml MonoS column equilibrated in buffer A_{50}. The column was washed with 1 ml of buffer A_{50} and eluted with a 10-ml linear gradient from 30–500 mM NaCl in buffer A. Pol δ eluted at $\approx A_{350}$. The elution positions of the mutant enzymes were identical to wild type. The total yield of partially purified DNA polymerase, measured as units of polymerase activity on activated DNA, from the *pol3-5DE* and *pol3-5DV* mutants was comparable to that of wild type. However, the yield from the *pol3-01* mutant polymerase was about half that of wild type.

DNA Polymerase and Exonuclease Assays. DNA polymerase assays were carried out on DNase I-activated salmon sperm DNA (29).

The 50-μl nuclease assay contained 20 mM Tris·HCl, pH 7.8, 8 mM $MgAc_2$, 0.2 mg/ml BSA, 4% glycerol, 1 mM DTT, and 100 fmol (as 3′ termini) of 3′-endlabeled pUC19 DNA plus enzyme. This DNA concentration was saturating for both the wild-type and mutant polymerases. The DNA substrate was prepared by linearizing pUC19 DNA with *Eco*RI and filling in with dATP and [^{3}H]dTTP, followed by purification of the DNA. Assays were assembled on ice in microfuge tubes and incubated at 37°C for 15 min. They were stopped by addition of 100 μl 25-mM EDTA, 25 mM sodium pyrophosphate, and 50 μg/ml carrier DNA, followed by 125 μl 10% trichloroacetic acid. After 10 min on ice, the tubes were spun in a microfuge for 10 min. Supernatant (200 μl) was added to a water miscible scintillation fluid and counted in a liquid scintillation counter.

Results

***pol3* Mutations.** A series of isogenic *Saccharomyces cerevisiae* strains was constructed that carry *POL3* alleles with mutations in different parts of the large Pol δ subunit (Fig. 1). In addition to the previously studied *pol3-01* mutation in the Exo I motif, five alleles were created that contained mutations in conserved residues of the Exo I, Exo II, or Exo III motifs (Exo-mutations; Fig. 1). Four of these mutations, *pol3-01* (9, 10), *pol3-1DV*, *pol3-4DA* (30), and *pol3-5DV* (B. Preston, personal communication) were previously characterized as strong mutators.

We also studied six Ts mutations of Pol δ. Five of these cause genome instability and/or repair defects in *S. cerevisiae*: *pol3-t* (13, 21, 22, 31); *pol3-13* (32); *pol3-12*, *pol3-15*, and *pol3-20* (33). The sixth mutation, *pol3-sp23* results in a mutant protein with the same amino acid change as a Ts mutant characterized in the fission yeast *Schizosaccharomyces pombe* (*cdc6-23*; ref. 34). None of the Ts mutants grew at 37°C, whereas all Exo mutants grew normally at all temperatures tested (23°C, 25°C, 30°C, and 37°C).

The *pol3-4DA* mutation in the Exo II motif eliminates the 3′→5′ exonuclease activity of Pol δ *in vitro* (30). To confirm the loss of exonuclease activity for mutants in the Exo I and Exo III motifs, Pol δ was purified to ≈30% purity from *pol3-01*, *pol3-5DE*, *pol3-5DV*, and wild-type strains (data not shown). Relative to wild-type Pol δ, the exonuclease activities of *pol3-01*, *pol3-5DE*, and *pol3-5DV* mutants were estimated to be < 0.9%, 1.3%, and 1.7%, respectively (Fig. 2).

***pol3*-Exo and *pol3*-Ts Mutations Cause Different Types of Genetic Instability.** The effects of twelve *pol3* alleles on genetic stability (Table 1) were studied with several reporter systems previously used to characterize the effects of the *pol3-t* (Ts) and *pol3-01* (Exo) mutations: (*i*) *lys2*::*InsLD* is a 31-bp insertion mutation that only reverts by deletions between 7-bp direct repeats, most likely via replication slippage (21, 31); (*ii*) the *his7-2* allele

Table 4. Effect DSB recombinational repair defects on the loss from yeast *pol3*-Exo III and *rad27-p* mutants of the *TRP1* marker associated with the *RAD27* plasmid

Genotype	*TRP1* loss, %*	Viability
pol3-5DE rad27-p	12–48	+
pol3-5DV rad27-p	66–69	+
rad27-p rad51Δ	33–43	+
pol3-5DE rad51Δ	45–78	+
pol3-5DV rad51Δ	53–76	+
pol3-5DE rad27-p rad51Δ	0	−
pol3-5DV rad27-p rad51Δ	0–1.1†	−
rad27-p rad50Δ	8.9–30	+
pol3-5DE rad27-p rad50Δ	0–1.6‡	−
rad27-p rad52Δ	15–24	+
pol3-5DE rad27-p rad52Δ	0–2.1§	−

Yeast were grown at 30°C.

*Presented are the minimal and maximal values of the *TRP1* marker loss obtained in two to eight independent experiments for viable and seven to eight experiments for nonviable combinations.

†There was no plasmid loss in seven of eight isolates.

‡There was no plasmid loss in six of eight isolates.

§There was no plasmid loss in six of seven isolates.

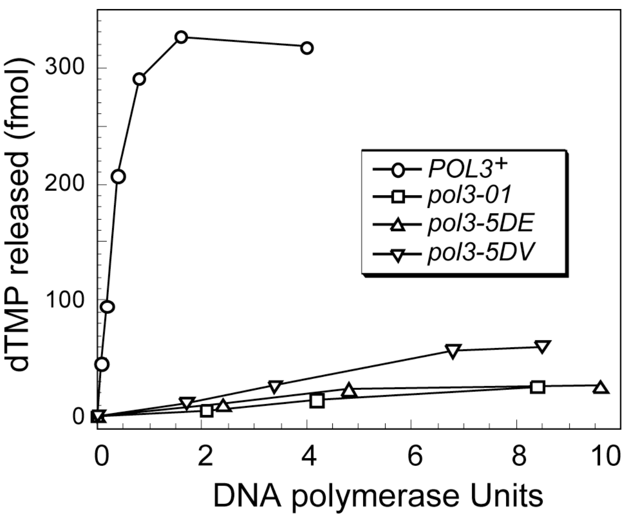

Fig. 2. Exonuclease activity of mutant Pol δ. Assays were as described in *Materials and Methods*.

contains a −1 frameshift in a run of eight A-T base pairs and reverts via +1 or −2 frameshifts (35); and (*iii*) canavanine-resistant forward mutants of the *CAN1* gene can occur via a broad spectrum of point mutations and rearrangements (ref. 15 and references therein).

The exonuclease-deficient Pol δ Exo-motif mutations had different effects on genetic stability than the Pol δ alleles that are Ts (Table 1). The Exo-motif alleles did not cause a statistically significant increase in *lys2*::*InsLD* deletions, but the Ts mutations elevated the rate of these events from 9- to 250-fold. In marked contrast to Ts alleles, Pol δ Exo-motif alleles were stronger mutators in the *his7-2* frameshift reversion or *can1* forward mutation detection systems (7.4- to 110-fold).

Synthetic Lethality of *pol3*-Exo with *rad27* Defects. The genetic interaction between *POL3* and *RAD27* was examined by determining the viability of strains carrying different alleles of these two genes (Table 2). The viability of a double mutant was evaluated by the ability to lose a plasmid carrying a wild-type copy of the *RAD27* gene and a *TRP1* marker (12). Double mutants of *rad27* mutations with *pol3*-Ts showed high frequencies of *TRP1* marker loss. In contrast, all of the double mutants carrying *rad27*-Δ and *pol3*-Exo mutations showed little or no loss of the *TRP1* plasmid marker, indicating that the combination of Exo-deficient *pol3* alleles with a deficiency of *RAD27* function is lethal. (Rare *TRP1* marker loss could be due to intraplasmid rearrangement or to reversion of the *rad27* or *pol3*-Exo mutation.) Mutations in the Exo I and Exo II motifs were lethal even when combined with a subtle *rad27-p* defect. However, mutations in the Exo III motif (*pol3-5DE* and *pol3-5DV*) were viable with *rad27-p*. A reduced frequency of cells without the plasmid was observed in several cultures of the *pol3-5DE rad27-p* double mutant, but this is likely due to the double mutant growing at a reduced rate in the absence of the plasmid (data not shown).

It was previously shown (12) that heterozygous *pol3-01*/*POL3*$^+$ diploids were also inviable with either *rad27*-Δ or *rad27-p*. Therefore, the viability of diploid heterozygotes for other mutations in Exo I, Exo II, and Exo III motifs was examined in this study. All *pol3*-Exo/*POL3*$^+$ were synthetic lethal with *rad27*-Δ, and heterozygotes for *pol3-1DV* and *pol3-4DV* were synthetic lethal even with *rad27-p* (data not shown).

Genetic Instability in *pol3*-Exo *rad27-p* Double Mutants. Synthetic lethality in *pol3*-Exo *rad27* could result from an accumulation of DNA lesions in the double mutant cells that might be detectable as genome instability in the viable double mutants, *pol3-5DE* or *-5DV* with *rad27-p*. Double mutants were created in the presence of the plasmid LC80B carrying wild-type *RAD27* and *TRP1*. The rates of mutation and recombination were determined by using fresh Trp$^-$ isolates that lost the plasmid (Table 3). This approach was taken to avoid accumulation of suppressor mutants that might confer growth advantage and possibly alter the genetic instability phenotype. All strains carried the reporters for +1 frameshift mutation (*his7-2*), forward mutation (*CAN1*), and either interchromosomal or intrachromosomal recombination.

Unlike the synergistic interaction between mutations inactivating mismatch repair and defects of either Pol δ or Pol ε Exo (9, 10, 14), there was no synergistic increase in the rate of +1 frameshift reversions of *his7-2* in the *pol3*-Exo III *rad27-p* double mutants. This supports the conclusion that the negative interaction of Pol δ 3′→5′ exonuclease and *rad27* defects resulting in lethality and genetic instability is not due simply to high levels of unrepaired mismatches generated by replication.

The *rad27*-Δ strains exhibit increased mutation and recombination rates (Table 3; ref. 12 and references therein). Similar to ref. 19, we also observed a large increase in duplications flanked by short direct repeats among forward mutations in the *CAN1* gene. The *rad27-p* mutation has little or no effect on the overall rates of recombination and mutation (Table 3; ref. 12). Importantly, it caused a small but detectable increase in duplications (11–96 bp flanked by 5–13-bp direct repeats), indicating that the mutant protein may be only partially defective in flap removal. Single mutations in the Pol δ Exo III motif are mutators for both *his7-2* and *CAN1*. Sequencing 20 *CAN1* mutants in the *pol3-5DV* revealed only point mutations and the spectrum did not differ significantly from the wild type (see supplemental data, which is published on the PNAS web site, www.pnas.org). A 4- to 8-fold increase in the *CAN1* forward mutation rate over a single *pol3*-Exo III mutants was observed in the double mutants. Importantly, the increased mutability of *CAN1* in the *pol3-5DV rad27-p* double mutant is mainly due to extended duplications (7–64 bp flanked by 2–10-bp direct repeats). Thus, the addition of the *pol3-5DV* mutation to the *rad27-p* caused about 28-fold increase in the rate of duplications (Table 3, second and last lines).

The single mutations *rad27-p*, *pol3-5DE*, and *pol3-5DV* had little if any effect on recombination. However, the double mutants *pol3-5DV rad27-p* and *pol3-5DE rad27-p* exhibited elevated rates of interchromosomal and intrachromosomal recombination.

Viability of *pol3*-Exo III *rad27-p* Double Mutants Requires a DSB Recombinational Repair System. One explanation for increased recombination in the viable double mutants carrying *rad27-p* and either *pol3-5DE* or *pol3-5DV* is that these cells have a higher than normal level of DSBs. To test this, mutations in DSB repair genes were introduced into these double mutants and their viability was determined (Table 4). Based on *in vitro* studies, *RAD50*, *RAD51*, and *RAD52* play different roles in DSB repair (36). Each of these genes was deleted in single or double *pol3*-Exo III and *rad27-p* mutants carrying the *RAD27-TRP1* plasmid LC80B.

The double mutants carrying *rad27-p* or *pol3*-Exo III and a DSB-repair defect were able to lose the *RAD27-TRP1* plasmid. The lower frequency of cells lacking the *RAD27-TRP1* plasmid in strains with *rad27-p* and a DSB repair defect is in agreement with the reduced growth rate of such double mutants (ref. 12 and data not shown). However, there was no loss or a drastic reduction in the frequency of loss of the *RAD27-TRP1* plasmid in the triple mutants carrying *rad27-p pol3*-Exo III and any of the DSB repair defects. Therefore, the viability of *pol3*-Exo III *rad27-p* double mutants depends on the presence of a functional DSB recombinational repair system.

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Discussion

Our study demonstrates that a general feature of 3′→5′ exonuclease deficient is synthetic lethality with a *rad27* defect. Synthetic lethality cannot be explained by a catastrophic error rate resulting from the lack of Pol δ proofreading (see the Introduction). The nature of the biological function of the Pol δ Exo activity, which is distinct from correcting replication errors, can be deduced from the phenotype of the *rad27-p pol3*-Exo III double mutant.

The 3′→5′ Exonuclease Activity of Pol δ Can Participate in Processing of Okazaki Fragments. We propose that the 3′→5′ exonuclease activity of Pol δ can act in place of Rad27/Fen1 to process Okazaki replication intermediates. The strongest support for this is the large increase in duplication mutations caused by the *pol3-5DV* mutation when combined with the *rad27-p* partial defect of the 5′ flap endonuclease (Table 3). It is generally accepted (see the Introduction) that the Rad27/Fen1 5′ flap endonuclease is important for prevention and/or removal of displaced 5′ flaps and creating ligatable nicks at the border between Okazaki fragments in the lagging strand. Duplications in *rad27*-Δ mutants are considered to result from the decreased flap removal. The partial *rad27-p* defect that disrupts the interaction between Rad27/Fen1 and PCNA also increased the rate of duplications, but to a smaller extent than found with the *rad27*-Δ mutant. This is in agreement with *in vitro* results indicating that PCNA enhances Fen1 cleavage efficiency via stabilization of the endonuclease on the flap but is not required for flap binding and cleavage (ref. 37 and references therein). The absolute increase in duplication rate caused by addition of *pol3-5DV* to the *rad27-p* is not less than the rate of duplications in the *rad27*-Δ strains. This supports our proposal that, along with the Rad27/Fen1, the Pol δ Exo can process (or prevent) 5′ flap intermediates.

In the presence of *rad27-p* both *pol3*-Exo III mutations led to increased recombination (Table 3). The increase in the recombination rate in most cases was comparable with the hyperrec phenotype of the *rad27*-Δ. Because both *rad27-p pol3*-Exo III mutants required the DSB recombinational repair genes for viability, we suggest that the most likely cause of hyperrecombination is the appearance of DSBs. Similar levels of hyperrecombination and requirements for DSB repair genes were found for the *rad27*-Δ single mutants (19, 20). The DSBs that are proposed to result from retained flaps could be due to unligated Okazaki fragments continuing into the next round of replication. Alternatively, DSBs could be formed in association with a stalled replication fork (38).

DSBs are highly toxic and 20–30 DSBs can kill a repair proficient yeast cell, whereas it takes only one DSB to inactivate a repair deficient *rad52* cell (39, 40). Thus, it is possible that the synthetic lethality observed in certain *pol3*-Exo *rad27* double mutants (Table 2 and refs. 12 and 13) results from an accumulation of DSBs to a level that exceeds the capacity of the DSB-repair system and/or from unrepairable DSBs. Partially defective Rad27/Fen1 (*rad27-p*) is unable to prevent DSBs even when *pol3*-Exo is heterozygous (*pol3*-Exo/$POL3^{+}$), suggesting that unremoved flaps leading to DSBs may arise via concerted action of the mutant proteins in some replication forks.

An Alternative Way to Reduce 5′ Flap Formation. Presented in Fig. 3 is a model explaining how a deficiency in 3′→5′ exonuclease activity could lead to increased formation of 5′ flaps. We propose that Exo-deficient Pol δ has increased strand displacement capacity similar to Exo-deficient mutants of replicative DNA polymerases such as T7 Pol (41–43) and T4 Pol (43–46).

Shown in Fig. 3 is the action of Pol δ, Rad27/Fen1, and DNA ligase at the junction between Okazaki fragments from which the

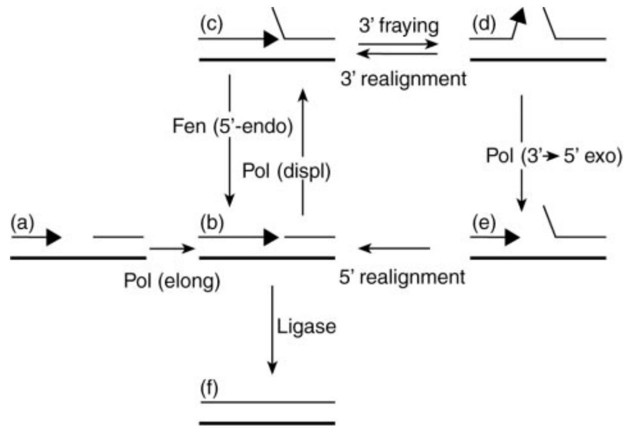

Fig. 3. Possible roles of the 3′→5′ exonuclease of Pol δ and the Rad27/Fen1 5′ flap endonuclease in creating a ligatable nick after strand displacement. The arrowhead shows the growing 3′ end of the nascent (*Top*) strand. Enzymatic reactions and conformational changes of DNA: Pol (elong), Pol δ elongation; Ligase, DNA ligase; Pol (displ), Pol δ displacement; Fen (5′-endo), Rad27/Fen1 5′ flap endonuclease; 3′ fraying, fraying at the 3′ end of the nascent strand; 5′ realignment, realignment of displaced 5′ end; Pol (3′→5′ exo), Pol δ 3′→5′ exonuclease.

RNA primer has already been removed by one of the 5′ nucleases, such as RNase H (47), Exo1 (48), or Dna2 (49). Strand displacement by DNA Pol (b→c) can create a 5′ flap intermediate that cannot be ligated. The 5′ flap can be removed by Fen1 (c→b). Alternatively, a ligatable nick could be created as a result of 3′ degradation by the 3′→5′ activity of DNA Pol (c→d→e→b). This could occur if strand separation (fraying) at the 3′ end (c→d) is followed by removal of terminal 3′ nucleotide(s) (d→e) similar to proofreading reaction (see the Introduction). Realignment of a 5′ end into the gap (e→b) could then create a ligatable nick.

The primary roles for Pol Exo activity is generally considered to be the removal of polymerization errors and prevention of mutations. Here we show that the Exo activity of Pol δ can also prevent extended duplications and recombination, and possibly DSBs, by suppressing excessive strand displacement and 5′ flap formation. The role of Pol δ Exo in suppressing genome instability and DSBs in yeast becomes evident only if the Rad27/Fen1 function is reduced. The Exo deficiency of yeast Pol δ on its own did not cause increased duplications. However, in partially defective Rad27/Fen1 (*rad27-p*) the rate of duplications caused by Exo defect is very high. *pol3*-Exo mutations on their own did not cause an increase in recombination or a requirement for the DSB recombinational repair genes including *RAD52*. These results suggests that in yeast the Rad27/Fen1 protein efficiently removes 5′ flaps in Pol δ Exo-deficient yeast mutants, although it might be working under stress. Delayed cell cycle progression in the *pol3-01* Exo-mutant could be due to increased flap formation activating S-phase checkpoint (15). In higher eukaryotes, the relative roles of Pol δ Exo and Rad27/Fen1 in reducing 5′ flap formation and preventing genome instability remain to be determined. The contribution of Pol δ Exo may vary depending on the activity of Fen1, whose expression (50, 51) and nuclear localization (52) can be altered by proliferation status and DNA damage.

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