

Okazaki Fragment Maturation in Yeast

II. COOPERATION BETWEEN THE POLYMERASE AND 3′–5′-EXONUCLEASE ACTIVITIES OF POL δ IN THE CREATION OF A LIGATABLE NICK*

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To address the different functions of Pol δ and FEN1 (Rad27) in Okazaki fragment maturation, exonuclease-deficient polymerase Pol δ -01 and Pol δ -5DV (corresponding to alleles *pol3-01*-(D321A, E323A) and *pol3-5DV*-(D520V), respectively) were purified and characterized in this process. In the presence of the replication clamp PCNA, both wild-type and *exo*⁻ Pol δ carried out strand displacement synthesis with similar rates; however, initiation of strand displacement synthesis was much more efficient with Pol δ -*exo*⁻. When Pol δ -*exo*⁻ encountered a downstream primer, it paused with 3–5 nucleotides of the primer displaced, whereas the wild type carried out precise gap filling. Consequently, in the absence of FEN1, Pol δ exonuclease activity was essential for closure of simple gaps by DNA ligase. Compared with wild type, Okazaki fragment maturation with Pol δ -*exo*⁻ proceeded with an increased duration of nick translation prior to ligation. Maturation was efficient in the absence of Dna2 and required Dna2 only when FEN1 activity was compromised. In agreement with these results, the proposed generation of double strand breaks in *pol3-exo*⁻ *rad27* mutants was suppressed by the overexpression of DNA2. Further genetic studies showed that *pol3-exo*⁻ *rad27* double mutants were sensitive to alkylation damage consistent with an *in vivo* defect in gap filling by exonuclease-deficient Pol δ .

Efficient and faithful maturation of Okazaki fragments during DNA replication in eukaryotes depends on a coordinated degradation of the RNA primer strand by one or more nucleases along with gap-filling DNA synthesis by a replicative DNA polymerase followed by ligation of the remaining nick. Previous models based on a combination of biochemical and genetic studies have indicated a role for the flap 5′-endonuclease FEN1 and the nuclease/helicase Dna2 in carrying out degradation including the removal of a displaced flap and a role for DNA polymerase δ (Pol δ)¹ to carry out DNA synthesis (1). However,

biochemical experiments in the accompanying paper (3) indicate that the main degradative force is provided by FEN1. The activity of Dna2 becomes crucial only in cases where strand displacement proceeds to the extent that proteins inhibitory to FEN1 can bind to the displaced 5′-strand (2, 3).

Many DNA polymerases have an intrinsic 3′–5′ exonuclease activity, which corrects polymerase errors and prevents mutations. Recently, we provided genetic evidence for the action of the 3′–5′-exonuclease of Pol δ in the process of Okazaki fragment maturation *in vivo* (4, 5). This was indicated by synthetic lethality of *rad27* (FEN1) mutants with several *exo*deficient mutants in Pol δ and by a dramatic increase in duplication mutations in viable *pol3-exo*⁻ *rad27* double mutants. We have suggested that the 3′–5′-exonuclease could be specifically involved in preventing the excessive formation of 5′-flaps by strand displacement synthesis.

Okazaki fragment maturation is mediated by the concerted strand displacement of Pol δ and degradation of the displaced strand by the nuclease activity of FEN1, a process called nick translation followed by sealing of the nick by DNA ligase I (6). However, beside FEN1 and Dna2, at least one more nuclease activity may function during nick translation. It is likely that strand displacement achieved by the 5′–3′ polymerization activity of Pol δ is counteracted by the 3′–5′ exonuclease activity intrinsic to the polymerase. *Exo*deficient mutants of T4 or T7 DNA polymerase, or *Escherichia coli* DNA polymerase II carry out more efficient strand displacement than the wild-type enzymes (7–9). *In vivo*, a loss of the 3′–5′-exonuclease activity of Pol δ results in a large increase in DNA duplications, which can be thought to originate from increased 5′-flap formation (5). Therefore, the 3′–5′-exonuclease activity of Pol δ may supplement the function of FEN1 and Dna2 in creating or maintaining a ligatable nick.

In this paper, we describe studies that indicate that the exonuclease activity of Pol δ beside that of replication error correction has an important role in limiting inappropriate strand displacement synthesis and therefore is an important determinant in creating ligatable nicks.

EXPERIMENTAL PROCEDURES

Materials and Strains—The strains used in this study, wild type, *rad27-p*, *rad27-null*, *pol3-01*, *pol3-5DV*, double mutant *pol3-5DV rad27-p*, and triple mutant *pol3-5DV rad27-p rad51-null*, are isogenic to CG379 (*MATa ade5-1 his7-2 leu2-3,112 trp1-289 ura3-52*) and have been described previously (5). Plasmids pGAL18 (2 mM ori *URA3 GAL1-10*-HA tag) and pGAL-DNA2 (pGAL18 but *GAL1-10-HA-DNA2*) were used for plasmid loss experiments (10). Pol δ -5DV was purified

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¹ The abbreviations used are: Pol δ , DNA polymerase δ ; Pol δ -01, Pol δ with Pol3-01 mutant subunit; Pol δ -5DV, Pol δ with Pol3-5DV mutant subunit; RFC, replication factor C; RPA, replication protein A; PCNA, proliferating cell nuclear antigen; SS, single-stranded; MMS,

methylmethane sulfonate; DSB, double-stranded breaks; WT, wild type; kb, kilobase.

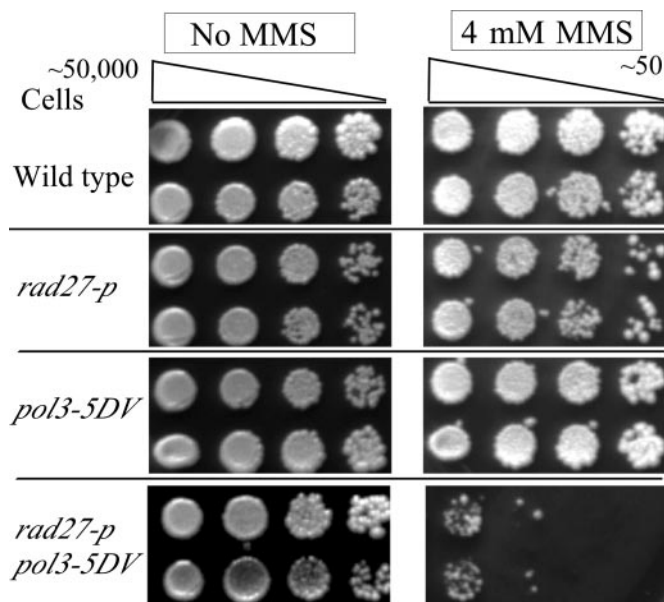


FIG. 1. Sensitivity of *rad27-p pol3-5DV* double mutants to MMS. Serial dilutions of single and double mutant strains were plated on YPD plates and on MMS-containing YPD plates and grown at 30 °C (for details see “Experimental Procedures”).

from overproduction strain YH712 (*MATa ade5-1 his7-2 leu2-3,112::lys2D5'-LEU2 lys2::InsHS-D trp1-289 ura3-52 pol3-5DV pep4::KanMX*) carrying pBL336-5DV (2 mM ori *TRP1 GAL1-pol3-5DV* (D520V)) and pBL340 (2 mM ori *URA3 GAL1-POL31 GAL10-POL32*) (5, 11). Because plasmid pBL336-5DV could not be recovered in *E. coli* after standard subcloning procedures, the 5DV mutation was introduced into this plasmid by gap repair through transformation of pBL336 from which a 1.4-kb *BglIII-NdeI* fragment surrounding the *POL3*-amino acid 520 region had been removed into strain YH712. The resulting plasmid allele was verified by PCR amplification and sequencing of the entire plasmid *POL3* gene. Pol δ -01 was similarly purified from strain PY168 (*Mata ura3-52 trp1-289 leu2-3,112 prb1-1122 prc1-407 pep4-3 pol3-01*) carrying pBL336-01 (2 mM ori *TRP1 GAL1-pol3-01* (D321A,E323A)) and pBL340 (2 mM ori *URA3 GAL1-POL31 GAL10-POL32*). Strain growth, extract preparation, and purification of the mutant forms of Pol δ were as described previously for wild type (11). As a final step, the purified enzymes were passed over a Superose 6 gel filtration column to remove trace levels of a low molecular weight nuclease contamination. All other enzymes and DNA substrates were as described by Ayyagari *et al.* (3).

The 88-mer double hairpin oligonucleotide (5'-C₉AAAACCAACCCCACT₅GTTGGGTTGGTTTTGGGA₅CTTCTCCTTCTCTCCT₅GGAGAGA-AAGGAGAAG-3') was extended by a single dTTP residue with carrier-free [α -³²P]dTTP exonuclease-deficient DNA polymerase I and Klenow fragment and purified by phenol extraction and Sephadex G50 gel filtration.

DNA Polymerase and Exonuclease Assays—DNA polymerase assays were carried out on DNase I-activated salmon sperm DNA (11). The 50- μ l nuclease assay contained 20 mM Tris-HCl, pH 7.8, 8 mM MgAc₂, 0.2 mg/ml bovine serum albumin, 4% glycerol, 1 mM dithiothreitol, 100 fmol of 3'-end-labeled pUC19 DNA, and enzyme. The DNA substrate was prepared by linearizing pUC19 DNA with *EcoRI* and filling in with dATP and [³H]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 the addition of 100 μ l of 25 mM EDTA, 25 mM sodium pyrophosphate, and 50 μ g/ml carrier DNA followed by 125 μ l of 10% trichloroacetic acid. After 10 min on ice, the tubes were spun in a Microfuge for 10 min. 200 μ l of the supernatant was added to a water miscible scintillation fluid and counted in a liquid scintillation counter. All other replication and Okazaki fragment maturation assays were essentially as described by Ayyagari *et al.* (3).

Replication Assays—Standard 30- μ l assays contained 20 mM Tris-HCl 7.8, 1 mM dithiothreitol, 100 μ g/ml bovine serum albumin, 8 mM MgAc₂, 1 mM ATP, 100 μ M of each dNTPs, 100 mM NaCl, 100 fmol of primed template, 400 fmol (for oligos) or 10 pmol (for SKII DNA) of RPA, and 150 fmol of all other enzymes (RFC, Pol δ wild-type or Pol δ -5DV, FEN1, Dna2, and DNA ligase) unless indicated otherwise. In

general, the DNA was preincubated with RPA, PCNA, and RFC for 1 min at 30 °C, and the reaction was started by adding the other proteins in a mixture. Incubations were at 30 °C. Radiolabel was either incorporated in the primers by extension with a single radiolabeled [α -³²P]dNTP (300 Ci/mmol) as appropriate (see above) or added as [α -³²P]dATP during the replication assay. In the latter case, the concentration of non-radioactive dATP was lowered to 20 μ M. Reaction products were analyzed by electrophoresis on a 8% polyacrylamide 7 M urea gel on a 1% alkaline-agarose gel or a 1% agarose gel in the presence of 0.5 μ g/ml ethidium bromide (12). The gels were dried and analyzed on a PhosphorImager. Quantitation was carried out using ImageQuant software. The images in the figures were contrast-enhanced for visualization purposes.

Yeast Genetic Methods—Yeast genetic methods were as described previously (4, 5). For the study of methylmethane sulfonate (MMS) sensitivity, the double mutants *pol3-5DV rad27-p* were obtained from a double mutant strain carrying pLC80 (*RAD27-TRP1*) as fresh plasmid loss isolates. Colonies of 12 independent isolates were suspended in H₂O at a density of $\sim 10^7$ cells/ml. Serial 10-fold dilutions were made in microtiter plates, and small (1 μ l) drops of cells were placed on the YPD and YPD + 4 mM MMS media.

To study the rescue of synthetic lethality by overexpression of *DNA2*, plasmid pGAL18-*DNA2* or vector pGAL18 was introduced into the triple mutant strain *pol3-5DV rad27-p rad51-null* harboring plasmid pLC80 (*RAD27-TRP1*). Such triple mutants require the presence of plasmid pLC80 for growth (5). Cells were grown on galactose medium lacking uracil (Gal-Ura) to allow the loss of plasmid pLC80 under conditions of overexpressing *DNA2*. Several independent isolates were picked and diluted, and ~ 200 cells were plated on Gal-Ura medium. Three days later, the cells were replica-plated on Gal-Ura and Gal-Ura-Trp. The percent of colonies that were unable to grow on Gal-Ura-Trp was determined.

RESULTS

Two exonuclease-deficient forms of Pol δ were initially investigated in this study. Pol δ -01 carries the classical *pol3-01* mutations (D321A,E323A) in the EXO-I motif of the exonuclease domain, which cause a strong mutator phenotype in yeast because of a defect in proofreading of replication errors (13, 14). In part, this mutator phenotype can be attributed to the constitutive activation of mutagenic DNA repair in the mutant (15). The mutant is inviable with a FEN1 deletion (*rad27 Δ*) as well as with a milder defect in FEN1 (*rad27-p*, F346A/F347A), which cripples the interaction with PCNA (4, 16). On the other hand, the *pol3-5DV* mutation (D520V), which localizes to the EXO-III motif, is a less strong mutator and shows synthetic lethality with *rad27 Δ* but not with the *rad27-p* allele (5). The viable *pol3-5DV rad27-p* double mutant has an elevated rate of large duplications that are diagnostic of increased flap formation rather than increased replication errors (5).

Deficiency in Pol δ Exonuclease Reduces Efficiency of Alkylation Damage Repair—If the exonuclease-deficient Pol δ has an increased strand displacement capacity *in vivo*, it could lead to problems during other cellular processes where flaps are created and processed, *e.g.* the major pathway of base excision repair in yeast proceeding via FEN1 and Pol δ holoenzyme (for review see Ref. 17). Therefore, we sought to determine whether the repair of alkylation damage, which to a large extent is channeled through the FEN1-dependent base excision repair pathway, is impaired in the Pol δ exonuclease-deficient mutant. For this purpose, we used a viable *pol3-5DV rad27-p* double mutant. Whereas neither of the single mutants is sensitive to MMS treatment, the double mutant is exquisitely sensitive (Fig. 1). This finding suggests that either both exonuclease activities can act on the same DNA structure or a set of rapidly interconverting structures such as 5'- and 3'-flaps or that the exonuclease deficiency of Pol δ generates aberrant DNA structures that can only be processed by a fully functional FEN1. This result also indicates that the 3'-5'-exonuclease activity of Pol δ has a biological function distinct from that of proofreading of replication errors, presumably by preventing

FIG. 2. **Strand displacement synthesis by exonuclease-deficient Pol δ .** A, DNA polymerase and 3'-5'-exonuclease activities of wild-type Pol δ (*wt*), Pol δ -01 (*O1*), and Pol δ -5DV (*5DV*). B, strand displacement synthesis was carried out under standard conditions with 20 mM NaCl and the indicated forms of Pol δ at 37 °C for the indicated times (RPA, PCNA, and RFC were not included in this assay). Electrophoresis was on a 8% polyacrylamide, 7 M urea gel (for details see "Experimental Procedures").

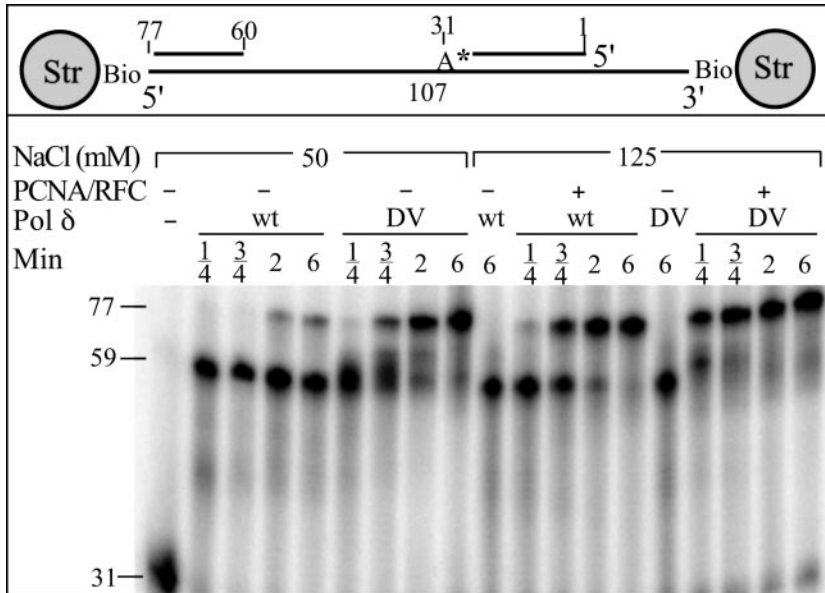
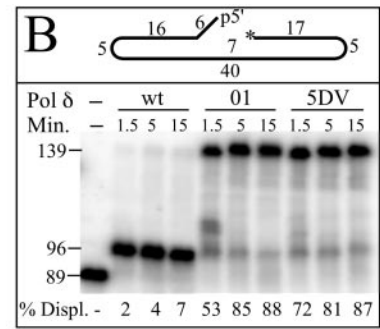
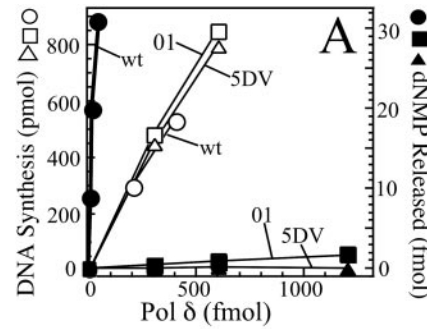


FIG. 3. **PCNA stimulated strand displacement synthesis.** Standard replication reactions on the streptavidin-bound 107-nucleotide 3'- and 5'-biotinylated template (5'-AGTGGGTTGGTTTTGGG-T₃₀CTCCCTTCTCTCTCCCTCTCCC-TTCCT₃₁-3') with a 3'-end labeled primer C12 (5'-AGGGAAGGGAGAGGGA-GGAGAAGAAGGGAG-3') and a downstream primer dc10 (5'-CCCAAACCAA-CCCAC-3') contained RPA and NaCl, PCNA and RFC, and either wild-type or 5DV Pol δ as shown in the figure for the indicated times at 30 °C. The reactions were preincubated with RPA and PCNA and RFC, if present, for 1 min at 30 °C and then started by the addition of Pol δ . The products were separated on a 7 M urea/12% polyacrylamide gel. The length of extension products with relation to the downstream primer is indicated above the figure.

excessive strand displacement (see below).

Characterization of Exonuclease-deficient Pol δ —Preliminary studies with partially purified preparations had already indicated that both mutant forms of Pol δ indeed showed exonucleolytic defects (5, 13). To allow a more thorough characterization and obtain pure enzyme useful for mechanistic studies, the mutant forms were purified to homogeneity from yeast overexpression systems (see "Experimental Procedures"). DNA polymerase activity was unaffected by the exo⁻ mutations. However, the exonuclease activity of Pol δ -01 was ~0.5% wild type, whereas that of Pol δ -DV was undetectable (<0.1%) (Fig. 2A). Because Pol δ -DV is completely exonuclease-deficient, further studies were primarily carried out with that enzyme rather than with Pol δ -01, which still shows residual 3'-5'-exonuclease activity. However, when tested in strand displacement, nick translation, and Okazaki fragment maturation assays described below, Pol δ -01 showed activities comparable with those of Pol δ -DV (data not shown).

Exonuclease-deficient Pol δ Has Increased Strand Displacement Activity—For several DNA polymerases, it has been noted that inactivation of the exonuclease activity increases the strand displacement activity of the enzyme (7–9). These include phage T4 and T7 DNA polymerases and *E. coli* DNA polymerases I and II. The same enhancement of strand displacement was observed upon inactivation of the exonuclease activity of Pol δ . Strand displacement synthesis by the three different forms of Pol δ was carried out on a self-priming double hairpin template, which made a rapid determination of factors determining strand displacement synthesis possible (Fig. 2B). Under all conditions tested, both Pol δ -01 and Pol δ -DV were much more active in strand displacement synthesis than wild-

type Pol δ . However, even strand displacement synthesis by the exonuclease-deficient forms of Pol δ was not very efficient. It required conditions that destabilized double-stranded DNA, *i.e.* elevated temperatures and low salt concentrations, and was stimulated by elevated dNTP concentrations (data not shown).

To be able to assess the contribution of the replication clamp PCNA to strand displacement synthesis, we used a model oligonucleotide system with terminal biotin-streptavidin anchors to prevent PCNA from sliding off the DNA (18–20). In this system, displacement synthesis of a downstream primer by wild-type Pol δ was previously shown to depend not only on the presence of PCNA and the clamp loader RFC but also on the presence of the streptavidin blocks (3). In the absence of PCNA, strand displacement synthesis by wild-type Pol δ on the model oligonucleotide substrate was very poor but detectable when the salt concentration was at 50 mM NaCl (Fig. 3). In contrast, strand displacement synthesis by Pol δ -DV was 10–20-fold more efficient. The inclusion of 125 mM NaCl in the assay completely inhibited strand displacement synthesis by both DNA polymerases. However, in the presence of PCNA, efficient strand displacement synthesis was observed at 125 mM NaCl with both the wild-type and mutant Pol δ , although Pol δ -DV was still ~4-fold more efficient.

Even in the presence of PCNA, strand displacement synthesis is preceded by extensive pausing of the polymerase at the downstream primer. With Pol δ -WT, two prominent pause sites were observed, one at the 0 position corresponding to precise gap filling and one at the +1 position corresponding to displacement of a single nucleotide by the polymerase (Fig. 4). In sharp contrast, Pol δ -DV formed prominent pause sites at +3 to +5 positions for all downstream primers tested whether they were

FIG. 5. Pol δ -5DV readily initiates strand displacement synthesis. Alkaline-agarose electrophoretic analysis of strand displacement synthesis is shown. Standard assays contained 100 mM NaCl. The reactions were preincubated with RPA, PCNA, and RFC for 1 min at 30 °C and then started by the addition of either wild type or 5DV Pol δ , and incubation was continued for the indicated times at 30 °C. (for details see "Experimental Procedures"). The arrow indicates fully replicated Bluescript SKII DNA (2.9 kb).

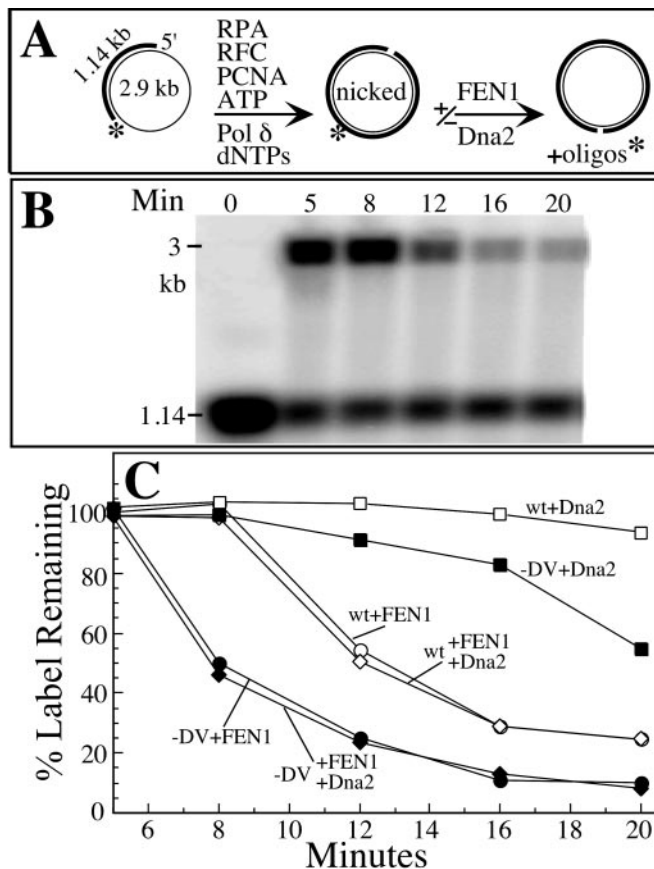
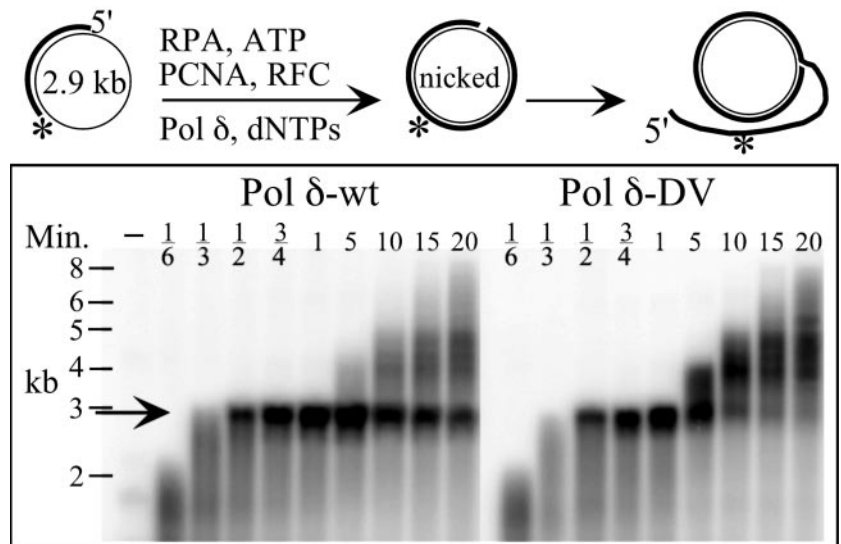


FIG. 6. Increased nick translation by Pol δ -5DV and FEN1. *A*, schematic of the assay. *B*, alkaline-agarose electrophoretic analysis of nick translation. Standard assays contained 100 mM NaCl. The reactions were preincubated with RPA, PCNA, and RFC for 1 min at 30 °C and then started by the addition of FEN1 together with Pol δ , and incubation was continued for the indicated times at 30 °C. The label remaining at 1.14 kb is material that did not hybridize to the SS SKII DNA during the original priming reaction. *C*, quantitation of the assay in *B* as well as several others. Dna2 was added instead of FEN1 or together with FEN1 as indicated. The same assays were also carried out with Pol δ -5DV replacing wild type.

zaki fragment maturation. As DNA ligase I does not ligate DNA to RNA ends, degradation of the RNA portion is a prerequisite. Fully ligated products were observed with almost 100% efficiency with both forms of Pol δ when FEN1 was included in the assay, whereas Dna2 functioned poorly with Pol

TABLE I
Comparative activities of Pol δ -WT and Pol δ -DV
Activities are either expressed as relative to wild type = 100% or in absolute numbers. In the maturation assays, the DNA was present at a concentration of 3.3 nM nt, nucleotide.

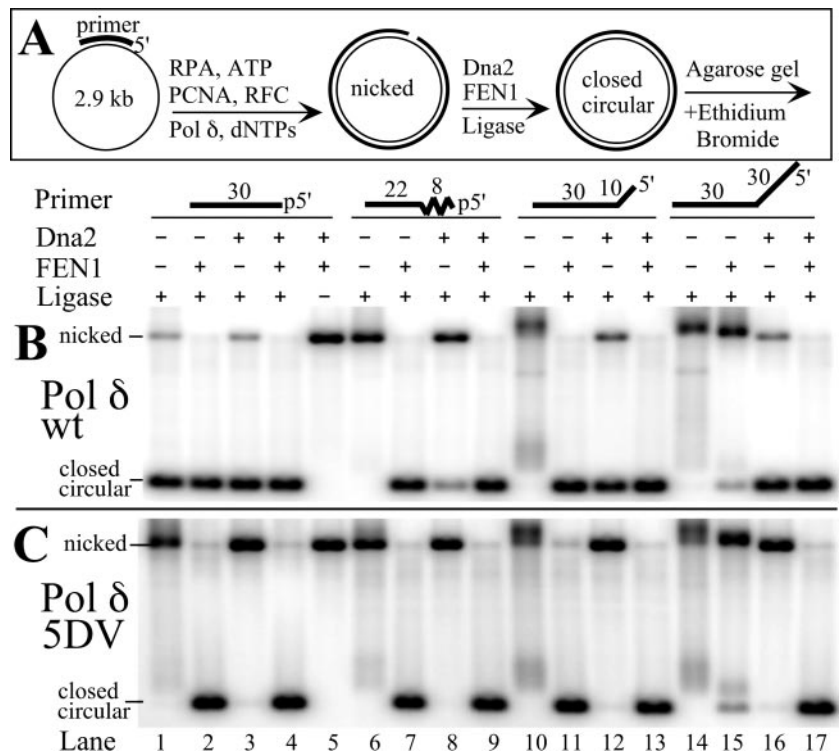
	WT	DV
DNA polymerase activity (relative)	(100)	103
3'-5'-exonuclease activity (relative)	(100)	<0.1
Strand displacement synthesis (relative)		
No PCNA, 50 mM NaCl	1	15
No PCNA, 125 mM NaCl	<1	<1
+ PCNA, 125 mM NaCl	(100)	400
Pausing positions at nick, + PCNA	0, +1	+3 to +5
Strand displacement rate (nt/sec)	1.5	2
Nick translation rate (nt/sec)	1.7	2.5
Production of ligatable nicks (%)		
with ds 5'-p-DNA primer, no FEN1	87	0
with ds 5'-p-DNA primer, + FEN1	97	94
with RNA primer, + FEN1	95	96
with RNA primer, + Dna2	27	0
with 30-nt flap, + FEN1	14	9
with 30-nt flap, + Dna2	75	1
with 30-nt flap, + FEN1 + Dna2	96	96
Maturation time of an Okazaki fragment (sec)		
5 nM ligase	22-25	—
50 nM ligase	15-17	18-20
Nick translation patch past RNA/DNA (nt)		
5 nM ligase	8-12	25-40
50 nM ligase	4-6	7-11

δ -WT and not at all with Pol δ -DV (*lanes 7 and 8*). Maturation of a DNA primer with a 10-nucleotide 5'-flap was similar to that of the RNA primer in which the formation of ligatable products required exclusively FEN1 in order to degrade the 5'-flap and create a ligatable nick (*Fig. 7, B and C, lanes 11*).

As noted previously, the maturation of substrates with long 5'-flaps to which RPA can bind requires the action of Dna2 prior to FEN1, because FEN1 is unable to slide onto flaps to which protein is bound (1-3). The primer with the 30-nucleotide 5'-flap did not permit efficient formation of covalently closed circles with either wild type or exo⁻ DNA polymerase together with FEN1 (*Fig. 7, B and C, lanes 15*). More efficient ligation was observed with the Dna2 nuclease but only for Pol δ -WT (*Fig. 7B, lane 16*). Pol δ -DV together with Dna2 did not yield significant ligation products, and both Dna2 and FEN1 were required for efficient ligation (*Fig. 7C, lanes 16 and 17*).

Overexpression of Dna2 Can Rescue the Lethality in pol3-5DV rad27-p rad51—Based on the evidence above, Dna2 could assist in the removal of large flaps in the cell that otherwise might be expected to lead to double strand breaks (DSBs).

FIG. 7. Defective gap filling and Okazaki fragment maturation by Pol δ -5DV. **A**, schematic of the assays. **B**, maturation with wild-type Pol δ on SS SKII DNA primed with a 5'-phosphate containing primer (SKdc10, lanes 1–5), a RNA-DNA 5'-phosphate containing primer (SKrc14, lanes 6–9), a 10-nucleotide 5'-flap primer (SKdc11, lanes 10–13), or a 30-nucleotide 5'-flap primer (SKdc12, lanes 14–17). Standard assays contained 100 mM NaCl. The reactions were preincubated with RPA, PCNA, and RFC for 1 min at 30 °C and then started by adding the indicated proteins in a mixture and continued for 4 min at 30 °C. **C**, the same assay as in **B** but with Pol δ -5DV (for details see "Experimental Procedures"). Analysis was on a 1% agarose gel with 0.5 μ g/ml ethidium bromide present. Quantitation of selected lanes is given in Table I.



DSBs have been proposed to form in the double mutant *pol3-5DV rad27-p* because the viability of this mutant depends on an intact DSB repair system (*i.e.* a *pol3-5DV rad27-p rad51* triple mutant is inviable) and can be maintained only in the presence of a plasmid carrying wild-type *RAD27* (pRAD27) (5). To assess whether increased levels of Dna2 would rescue the viability of the triple mutant, we overexpressed *DNA2* from a galactose-inducible promoter and monitored loss rates of the complementing pRAD27 plasmid (monitored by the loss of the *URA3* marker as described under "Experimental Procedures"). In agreement with a previous study (5), cultures carrying empty vector pGAL18 showed a low frequency of loss of plasmid pRAD27 (average = 0.4%; range 0–2% over four independent cultures). In contrast, the average loss of pRAD27 from the triple mutant also containing pGAL-*DNA2* plasmid was 39% (range 10–61% over seven independent cultures) but only if cells were grown on galactose to induce overexpression of *DNA2*. Colonies that had lost pRAD27 on galactose media were replated on either inducing (galactose) or repressing (glucose) medium (Fig. 8). Thus, the viability of the triple mutant depends on the overexpression of *DNA2* because *pol3-5DV rad27-p rad51* (pGAL-*DNA2*) strains were able to grow only on galactose where *DNA2* is overexpressed.

The 3'-5'-Exonuclease of Pol δ Limits the Patch Length of Nick Translation—The kinetics of maturation have been studied with a model RNA-DNA-primed circular DNA substrate (see Fig. 7A) (3). In that study (3), we noted that the maturation time, *i.e.* the time required to convert nicked DNA circles into covalently closed DNA, was substantially decreased when a large molar excess of DNA ligase was present. In the presence of a 15-fold molar excess of DNA ligase, the maturation time with wild-type Pol δ was 15–17 s. Under the same experimental conditions, the maturation time with Pol δ -DV was 18–20 s (Table I). This slightly longer maturation time could be indicative of extended nick translation by Pol δ -DV prior to ligation.

In the accompanying paper (3), we have described an assay to determine the nick translation patch length, *i.e.* how far nick translation proceeds past the RNA-DNA junction prior to ligation. A set of RNA-DNA primers was used, each with a radio-

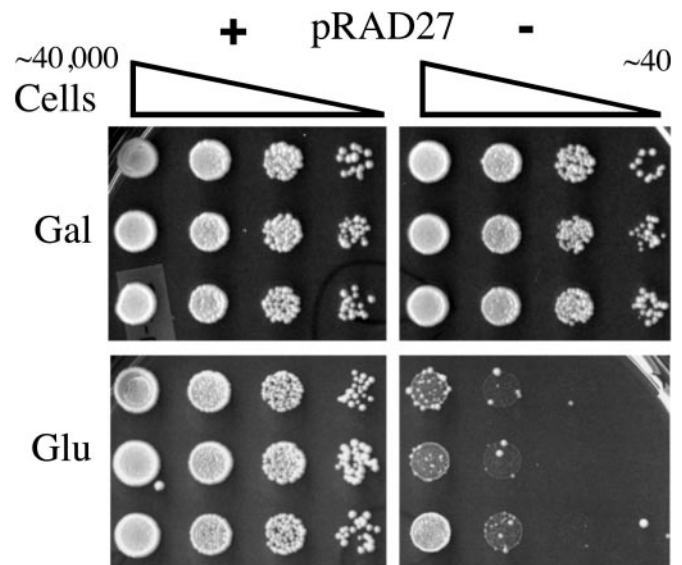


FIG. 8. Rescue of lethality of a *pol3-5DV rad27 rad51* mutant by overexpression of *DNA2*. Strains carried the galactose-inducible overexpression plasmid pGAL-*DNA2* and either the pRAD27-complementing plasmid (left panel) or empty vector (right panel). Growth under inducing (galactose) or repressing (glucose) condition was determined for serial 10-fold dilutions.

active label incorporated at a different position at 6, 12, or 22 nucleotides past the RNA-DNA junction. A loss of label in the covalently closed product indicates that nick translation proceeded past the labeled position prior to ligation. Under standard replication conditions with the relevant enzymes (Pol δ , FEN1, and DNA ligase) in 50% molar excess over DNA substrate, the measured nick translation patch length was 8–12 nucleotides for Pol δ -WT and 25–40 nucleotides for Pol δ -DV (a range of three independent experiments for Pol δ -WT and two for Pol δ -DV). However, one major factor determining the rate of Okazaki fragment maturation is the DNA ligase concentration. Surprisingly, a 50% excess of DNA ligase was far from

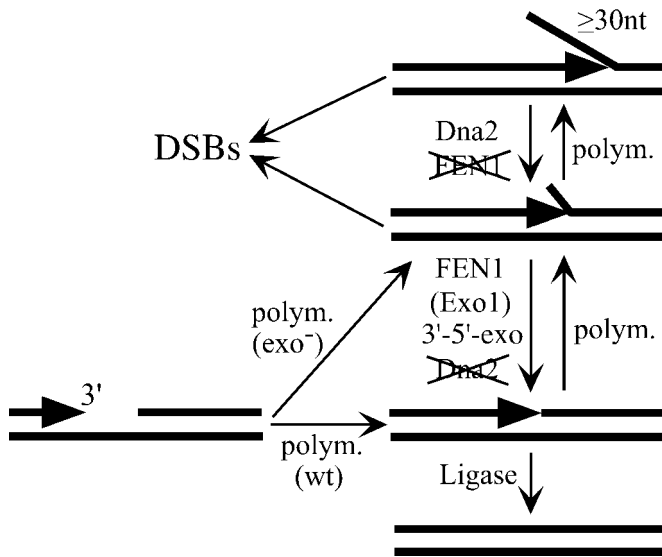


FIG. 9. Model for activities operating during gap-filling synthesis. Cooperation among Dna2, FEN1, and the DNA polymerase and exonuclease activities of Pol δ operating during gap-filling synthesis. In this model, RPA binds to long flaps only, thus preventing cleavage by FEN1 and stimulating cleavage by Dna2. The crossed out enzyme shows no or poor activity on the indicated substrate. Exo1 is shown in parentheses as it is proposed to replace FEN1 in a *rad27*- Δ strain, but it was not used in our studies.

saturating, and a 15-fold molar excess of ligase was required for rapid maturation. Accordingly, with DNA ligase in high excess, the nick translation patch was reduced to 4–6 nucleotides for Pol δ -WT and 7–11 nucleotides for Pol δ -DV (Table I). Increasing the levels of the other enzymes did not substantially alter the nick translation patch length.

DISCUSSION

It is a common view that the maturation of Okazaki fragments results from the interaction among many proteins in the cell. In this study, we investigated the cooperation among four biochemical activities presumably involved in Okazaki maturation *in vivo*. This allowed us to establish the major reaction pathways leading to the creation of a ligatable nick. Okazaki fragments that are not ligated could lead to DSBs. Considering the large number of Okazaki fragments (~100,000/yeast genome), even a small percentage (<0.1%) of ligation failures might lead to a number of DSBs that would exceed the capacity of the DSB repair system (~30 DSBs/yeast cell) and therefore cause lethality (21, 22). An even higher reliability of maturation is required for larger genomes such as in humans where Okazaki fragments are expected to be 100–1000-fold more numerous and where the number of DSBs tolerated is similar.

Although FEN1 has been established as a key activity for Okazaki fragment maturation, additional functions can contribute to the highly efficient maturation required for successful genome duplication. The schematic diagram presented in Fig. 9 describes how the maturation can be accomplished, the role of FEN1 and Dna2 in processing various intermediates, and the impact of mutants. The following discussion summarizes the *in vitro* and *in vivo* observations that support the roles of the various components in maturation.

Pol δ 3'-5'-Exonuclease Is Required for Creating a Ligatable Nick under Conditions of FEN1 Deficiency—This conclusion follows from the gap-filling experiments presented in Fig. 7 and is in agreement with the MMS sensitivity observed in a *pol3-exo⁻ rad27* double mutant (Fig. 1) and previous genetic studies (5). The current study established several distinguishing properties of exonuclease-deficient Pol δ that may aid in explaining

the defects of *pol3-exo⁻* mutants that are not related to an increase in spontaneous mutation rates. A comparison of the activities of wild type and exonuclease-deficient Pol δ is given in Table I. The exonuclease-deficient Pol δ is more efficient than wild type in strand displacement synthesis, but this increased efficiency can primarily be attributed to an initiation step (Fig. 5). Once strand displacement synthesis has initiated, elongation rates measured by rolling circle DNA replication are similar for both types of enzymes. Increased strand displacement obviously would be problematic in a cell that already is crippled for FEN1 such as in a *rad27* complete or partial mutant.

Fig. 9 represents a model of how the exonuclease activity of Pol δ can reduce the initiation of strand displacement synthesis. This model is based on the assumption that when the polymerase is in the strand displacement mode, there is a high probability of the 3'-end of the DNA partitioning into the exonuclease site, leading to degradation of the newly synthesized DNA back to the position of the nick. Our observation—that pausing by Pol δ -WT when a downstream double-stranded region is encountered is at the position of the nick, whereas pausing by Pol δ -*exo⁻* is at a +3 to +4 position—is consistent with this idea (Fig. 4). This finding suggests a model in which the wild-type polymerase is actually idling at a nick, *i.e.* going through futile cycles of incorporation with limited strand displacement followed by exonucleolytic degradation back to the nick position. Idling by the wild-type polymerase would explain why simple gap-filling synthesis followed by ligation is quite successful for this enzyme, whereas it is a total failure for the exonuclease-deficient enzyme.

Similar considerations can be put forward to explain why maturation with just Dna2, *i.e.* without FEN1, gives substantial yields of ligated products with wild-type Pol δ but absolutely none with Pol δ -DV (Fig. 7, compare *B* with *C*, lanes 3, 8, 12, and 16). Dna2 specifically cuts long 5'-flaps, leaving small 5'-flaps of 5–10 nucleotides in length, and the removal of these short flaps by Dna2 is inefficient (23). When Dna2 action is coupled to strand displacement synthesis by Pol δ , it is likely that its cutting specificity remains similar, *i.e.* at any given time the site on the DNA where polymerization and degradation occur contains a small 5'-flap rather than a nick (see "Discussion") (3). Therefore, in the absence of FEN1, a ligatable nick could only be produced through degradation of the 3'-strand by the 3'-5'-exonuclease activity of Pol δ in order to allow the displaced 5'-strand to rehybridize to the template and produce a proper nick for ligation. The observation of a complete failure to produce ligatable nicks by the combined action of the exonuclease-deficient Pol δ and Dna2 strongly supports this interpretation.

Cooperation between 5'-Nucleases—In as much as *rad27*- Δ mutants are viable, an additional 5'-nuclease may participate in creating ligatable nicks in cooperation with Pol δ . A related exonuclease, Exo1, is the most likely candidate, because *rad27 exo1* double mutants are lethal and overexpression of *EXO1* suppresses some of the *rad27*- Δ -associated defects (24, 25). At the same time, *EXO1* is unable to fully substitute for *RAD27* in the absence of the Pol δ exonuclease activity, indicating a limited role for Exo1. Of course, Dna2 is also a 5'-nuclease implicated into the maturation of Okazaki fragments (1, 26–28). Unlike *rad27*-null or *exo1*-null, null mutants in *DNA2* are lethal and so are mutations that inactivate the nuclease but not the helicase activity of Dna2 (29, 30). Our biochemical results indicate that the essential role of Dna2 is to clip long (>30 nucleotides) flaps, which can neither be cleaved by FEN1 nor realigned by the exonuclease activity of Pol δ on the neighboring 3'-strand. In our biochemical studies, neither wild type nor exonuclease-deficient Pol δ created detectable amounts of large

flaps in the presence of FEN1. A low frequency (<5%) of large flaps would not be detected in our *in vitro* analysis, but such a low frequency of large flaps occurring *in vivo*, particularly in *pol3-exo⁻* mutants, could eventually lead to the accumulation of a lethal number of DSBs. The overexpression of *DNA2* can reduce the need for DSB repair following Okazaki maturation and render viability to a triple mutant *pol3-5DV rad27-p rad51* (Fig. 8). Possibly, normal Dna2 levels enable processing of only a limited number of long flaps, whereas overexpression levels of Dna2 help Exo1 and/or the crippled FEN1-p of the *rad27-p* mutant to process flaps generated by increased strand displacement synthesis in the *pol3-exo⁻* background.

Inappropriate Strand Displacement by Exonuclease-deficient Pol δ during DNA Repair—Another severe problem that may occur in *pol3-exo⁻* strains is suggested from our observation that gap filling by Pol δ -5DV is not precise and pausing of the mutant enzyme occurs when the enzyme enters the duplex region and displaces a 3–5-nucleotide 5'-flap (Fig. 4). In our model system in which simple gap maturation was measured (gap filling followed by ligation), Pol δ -5DV produced no detectable ligated products, whereas 87% ligated products were observed with Pol δ -WT (Fig. 7, lanes 1). However, in the presence of FEN1, this defect of the mutant polymerase was completely corrected (lanes 2). These observations may explain why *pol3-5DV rad27-p* mutants are extremely sensitive to MMS damage (Fig. 1). Simple gap filling as a final step in PCNA-dependent base excision repair requires both FEN1 and Pol δ , and in a *pol3-exo⁻ rad27-p* double mutant, both degradative mechanisms that provide a ligatable nick would be crippled (17, 31). Gap filling also occurs in the final step of nucleotide excision repair. Similarly, this process would be expected to be defective in a *pol3-exo⁻* strain. However, as *pol3-exo⁻* strains are not particularly sensitive to ultraviolet irradiation, another 5'-nuclease might function during gap filling if Pol δ is required for repair synthesis. The most probable candidate would be *RAD2*, the homologue of *RAD27*, which also interacts with PCNA (32). Alternatively, another DNA polymerase such as Pol δ might function generally during gap filling or specifically in the *pol3-exo⁻* strains (33).

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REFERENCES

- Bae, S. H., Bae, K. H., Kim, J. A., and Seo, Y. S. (2001) *Nature* **412**, 456–461
- Murante, R. S., Rust, L., and Bambara, R. A. (1995) *J. Biol. Chem.* **270**, 30377–30383
- Ayyagari, R., Gomes, X. V., Gordenin, D. A., and Burgers, P. M. (2002) *J. Biol. Chem.* **278**, 1618–1625
- Gary, R., Park, M. S., Nolan, J. P., Cornelius, H. L., Kozyreva, O. G., Tran, H. T., Lobachev, K. S., Resnick, M. A., and Gordenin, D. A. (1999) *Mol. Cell. Biol.* **19**, 5373–5382
- Jin, Y. H., Obert, R., Burgers, P. M., Kunkel, T. A., Resnick, M. A., and Gordenin, D. A. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98**, 5126–5127
- Rigby, P. W., Dieckmann, M., Rhodes, C., and Berg, P. (1977) *J. Mol. Biol.* **113**, 237–251
- Engler, M. J., Lechner, R. L., and Richardson, C. C. (1983) *J. Biol. Chem.* **258**, 11165–11173
- Canceill, D., Viguera, E., and Ehrlich, S. D. (1999) *J. Biol. Chem.* **274**, 27481–27490
- Bhagwat, M., and Nossal, N. G. (2001) *J. Biol. Chem.* **276**, 28516–28524
- Budd, M. E., and Campbell, J. L. (1997) *Mol. Cell. Biol.* **17**, 2136–2142
- Gorgens, P. M., and Gerik, K. J. (1998) *J. Biol. Chem.* **273**, 19756–19762
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Simon, M., Giot, L., and Faye, G. (1991) *EMBO J.* **10**, 2165–2170
- Morrison, A., Johnson, A. L., Johnston, L. H., and Sugino, A. (1993) *EMBO J.* **12**, 1467–1473
- Datta, A., Schmeits, J. L., Amin, N. S., Lau, P. J., Myung, K., and Kolodner, R. D. (2000) *Mol. Cell* **6**, 593–603
- Gomes, X. V., and Burgers, P. M. J. (2000) *EMBO J.* **19**, 3811–3821
- Matsumoto, Y. (2001) *Prog. Nucleic Acid Res. Mol. Biol.* **68**, 129–138
- Kaboord, B. F., and Benkovic, S. J. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 10881–10885
- Burgers, P. M. J., and Yoder, B. L. (1993) *J. Biol. Chem.* **268**, 19923–19936
- Podust, L. M., Podust, V. N., Floth, C., and Hubscher, U. (1994) *Nucleic Acids Res.* **22**, 2970–2975
- Resnick, M. A., and Martin, P. (1976) *Mol. Gen. Genet.* **143**, 119–129
- Resnick, M. A. (1978) *J. Theor. Biol.* **71**, 339–346
- Bae, S. H., and Seo, Y. S. (2000) *J. Biol. Chem.* **275**, 38022–38031
- Tishkoff, D. X., Boerger, A. L., Bertrand, P., Filosi, N., Gaida, G. M., Kane, M. F., and Kolodner, R. D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 7487–7492
- Parenteau, J., and Wellinger, R. J. (1999) *Mol. Cell. Biol.* **19**, 4143–4152
- Budd, M. E., Choe, W.-C., and Campbell, J. (1995) *J. Biol. Chem.* **270**, 26766–26769
- Formosa, T., and Nittis, T. (1999) *Genetics* **151**, 1459–1470
- Kang, H. Y., Choi, E., Bae, S. H., Lee, K. H., Gim, B. S., Kim, H. D., Park, C., MacNeill, S. A., and Seo, Y. S. (2000) *Genetics* **155**, 1055–1067
- Lee, K. H., Kim, D. W., Bae, S. H., Kim, J. A., Ryu, G. H., Kwon, Y. N., Kim, K. A., Koo, H. S., and Seo, Y. S. (2000) *Nucleic Acids Res.* **28**, 2873–2881
- Budd, M. E., Choe, W., and Campbell, J. L. (2000) *J. Biol. Chem.* **275**, 16518–16529
- Blank, A., Kim, B., and Loeb, L. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 9047–9051
- Gary, R., Ludwig, D. L., Cornelius, H. L., MacInnes, M. A., and Park, M. S. (1997) *J. Biol. Chem.* **272**, 24522–24529
- Budd, M. E., and Campbell, J. L. (1995) *Mol. Cell. Biol.* **15**, 2173–2179