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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Yong Hwan Jin‡§, Rao Ayyagari§¶, Michael A. Resnick‡, Dmitry A. Gordenin‡,¶ and Peter M. J. Burgers§**

From the ¶Laboratory of Molecular Genetics, NIEHS, National Institutes of Health, North Carolina 27709, ¶¶Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, Missouri 63110, and §§Lindenwood University, St. Charles, Missouri 63301

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 exonuclease-deficient Pol δ carried out strand displacement synthesis with similar rates; however, initiation of strand displacement synthesis was much more efficient with Pol δ-01. When Pol δ-01 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 alkyla
tion 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 5 (Pol 5)\(^1\) 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 nucleic acid 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 nucleic acid 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 C3579 (MATa ade5–1 his7–2 leu2–3,112 trp1–289 ura3–52) and have been described previously (5). Plasmids pGAL18 (2 m M 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 from methylene methane sulfonate; DSB, double-stranded breaks; WT, wild type; kb, kilobase.

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** To whom correspondence should be addressed. Tel.: 314-362-3872; Fax: 314-362-7183; E-mail: burgers@biochem.wustl.edu.
§ 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, mutagen.

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from overproduction strain YH712 (MATa ade5-1 his7-2 leu2-3,112:lys2D5::LEU2 lys2::Ina5:His-D trp1-289 ura3-52 pol3-5DV pep4::KanMX) carrying pBL336–5DV (2 mb ori TRP1 GAL1-pol3-5DV (D520V)) and pBL340 (2 mb 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 BglII-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 5-01 was similarly purified from strain FY168 (Matm ura3-52 trp1-289 leu2-3,112 prb1-1122 prc1-407 pep4–3 pol3–01) carrying pBL336–01 (2 mb ori TRP1 GAL1-pol3–01 (D321A,E323A)) and pBL340 (2 mb ori URA3 GAL1-POL31 GAL10-POL32). Strain growth, extract preparation, and purification of the mutant forms of Pol 5 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'-GCTGAGGGATTGGTTGGTTTTGGGA 8CTTCTCCTTTCTCTCCT 5'-GGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA...
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 parent 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...
fully hybridized (lane 3) or with a 5'-flap (lane 6) or whether they were in a GC-rich region (lane 3) or in a poly(dA) stretch (lane 8) and even when a RNA-DNA primer was used (lane 10). This distribution of pause sites remained constant during the course of the reaction and was similar at 30 and 22 °C (data not shown). In comparison, sequenase formed one major pause site at the precise nick position (lane 4).

The model oligonucleotide system conveniently measures whether a polymerase carries out strand displacement or not but does not distinguish between initiation and elongation events. Elongation of strand displacement can more easily be measured as rolling circle DNA replication on circular templates such as the 2.9-kb Bluescript SKII template we used in this study. The products of rolling circle replication were separated by electrophoresis on a denaturing agarose gel, and rates were calculated from the size distribution of the displaced strand (Fig. 5). Surprisingly, we noted that the difference between wild-type and exodeficient Pol δ is not in the rate of strand displacement synthesis but rather at an initiation step.

With Pol δ-WT, replication of the primed DNA proceeded continuously around the circle at a rate of 40–60 nucleotides/sec at 30 °C until the circle was fully replicated after 30–45 s. However, after 5 min of incubation, only 22% of the replicated strands were longer than unit length, indicating that the majority of replication complexes had not initiated strand displacement synthesis. After 10 min of incubation, 50% of the complexes had initiated strand displacement synthesis. With Pol δ-DV, the same rate of DNA replication of the SS template was observed. However, in contrast with wild type, 63% Pol δ-DV complexes had initiated strand displacement synthesis after 5 min of incubation and 86% Pol δ-DV complexes had initiated strand displacement synthesis after 10 min. The rate of displacement synthesis by the two enzymes was actually not significantly different (1.5 ± 0.5 nucleotides/sec for wild type and 2 ± 0.5 nucleotides/sec for the exodeficient enzyme).

**Exonuclease-deficient Pol δ Carries Out More Rapid Nick Translation with FEN1**—Rates of nick translation could be measured with the same circular template primer used for strand displacement synthesis, because the 1.14-kb-long primer is labeled at the 3'-position. Therefore, label will be lost from the DNA after nick translation has proceeded for over 1.14-kb (Fig. 6A). An example of this analysis is shown in Fig. 6B for Pol δ-WT + FEN1. The time point at which half of the label had been lost was taken to calculate the average rate of nick translation. This time was corrected for the period (~45 s) required for complete replication of the SS DNA circle. As with strand displacement synthesis, not all complexes with Pol δ-WT proceeded with nick translation. Complex disassembly probably accounts for the ~20% label that remains even after 20 min of incubation. Nick translation with Pol δ-DV proceeded both to a higher extent and at a higher rate. In the presence of FEN1, the rate of nick translation was 2.5 nucleotides/sec with Pol δ-DV compared with 1.7 nucleotides/sec with Pol δ-WT (Fig. 6C and Table I). Those rates were not altered by the addition of Dna2. Dna2 alone poorly supported nick translation with Pol δ-WT and the rate with Pol δ-DV was also very low (~0.9 nucleotides/sec). Nick translation over extended stretches of DNA as carried out in this experiment, although probably not of physiological relevance, allowed us to determine an elongation rate for this process because the initiation step dominates the kinetics of nick translation through short stretches of DNA.

**Gap Filling with Exonuclease-deficient Pol δ Does Not Produce Ligatable Nicks**—In the presence of PCNA, Pol δ-DV appears to produce exclusively products with 3'-5' nucleotide-long 5'-flaps when encountering a downstream double-stranded region during gap-filling synthesis (Fig. 4). Ayyagari et al. (3) used a primed circular SS DNA substrate to assay the accuracy of gap-filling synthesis and Okazaki fragment maturation. DNA ligase-catalyzed ligation of the nick resulting from precise gap filling or proper Okazaki fragment maturation produces covalently closed circular DNA, which has a unique migration position when electrophoresed through an agarose gel in the presence of ethidium bromide (Fig. 7A). Indeed, when SS SKII DNA was primed with a 5'-phosphorylated primer, replication by Pol δ holoenzyme in the presence of DNA ligase I produced predominantly (87%) covalently closed DNA (Fig. 7B, lane 1). In sharp contrast, replication by Pol δ-DV produced no detectable covalently closed DNA (Fig. 7C, lane 1), suggesting that the enzyme did not produce ligatable nicks. However, when FEN1 was also added to the assay, proper processing of unannealed strand produced ligatable nicks with full efficiency (Fig. 7C, lane 2). Dna2 was completely inefficient (Fig. 7C, lane 3).

We used a RNA-DNA primer as a model substrate for Oka-
and then started by the addition of FEN1 together with Pol

Nick translation. Standard assays contained 100 mM NaCl. The react-
schematic of the assay. B

100% efficiency with both forms of Pol

requisite. Fully ligated products were observed with almost

DNA to RNA ends, degradation of the RNA portion is a pre-
zaki fragment maturation. As DNA ligase I does not ligate
DNA during the original priming reaction.

remaining at 1.14 kb is material that did not hybridize to the SS SKII
DNA. Lane 14, Pol δ-DV; lanes 15–16, Pol δ-DV together with FEN1 as indicated. The same assays were also carried out

with RNA primer, +PCNA
with RNA primer, +FEN1
with RNA primer, +Dna2
with 30-nt flap, +FEN1
with 30-nt flap, +Dna2
with 30-nt flap, +FEN1 + Dna2
Maturation time of an Okazaki fragment (sec)

Table 1

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.

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

Pol 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-nucleo-
tide 5’-flap did not permit efficient formation of covalently
closed circles with either wild type or exo+ DNA polymerase

To further evaluate their activity, the primer was incubated with

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).
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 radioactive 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...
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, 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\(^{-}\)-Rad27\(^{-}\)-Rad51\(^{-}\)) (Fig. 7). 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 \(\delta\) during DNA Repair**—Another severe problem that may occur in pol3-exo\(^{-}\) strains is suggested from our observation that gap filling by Pol \(\delta\)-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 \(\delta\)-5DV produced no detectable ligated products, whereas 87% ligated products were observed with Pol \(\delta\)-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-exo\(^{-}\) 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 \(\delta\), 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 \(\delta\) is required for repair synthesis. The most probable candidate would be RAD27, the homologue of RAD27, which also interacts with PCNA (32). Alternatively, another DNA polymerase such as Pol \(\delta\) might function generally during gap filling or specifically in the pol3-exo\(^{-}\) strains (33).

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**REFERENCES**