Reconstituted Okazaki Fragment Processing Indicates Two Pathways of Primer Removal*

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Eukaryotic Okazaki fragments are initiated by an RNA/DNA primer and extended by DNA polymerase δ (pol δ) and the replication clamp proliferating cell nuclear antigen (PCNA). Joining of the fragments by DNA ligase I to generate the continuous double-stranded DNA requires complete removal of the RNA/DNA primer. Pol δ extends the upstream Okazaki fragment and displaces the downstream RNA/DNA primer into a flap removed by nuclease cleavage. One proposed pathway for flap removal involves pol δ displacement of long flaps, coating of those flaps by replication protein A (RPA), and sequential cleavage of the flap by Dna2 nuclease followed by flap endonuclease 1 (FEN1). A second pathway involves reiterative single nucleotide or short oligonucleotide displacement by pol δ and cleavage by FEN1. We measured the length of FEN1 cleavage products on flaps strand-displaced by pol δ in an oligonucleotide system reconstituted with Saccharomyces cerevisiae proteins. Results showed that in the presence of PCNA and FEN1, pol δ displacement synthesis favors formation and cleavage of primarily short flaps, up to eight nucleotides in length; still, a portion of flaps grows to 20–30 nucleotides. The proportion of long flaps can be altered by mutations in the relevant proteins, sequence changes in the DNA, and reaction conditions. These results suggest that FEN1 is sufficient to remove a majority of Okazaki fragment primers. However, some flaps become long and require the two-nuclease pathway. It appears that both pathways, operating in parallel, are required for processing of all flaps.

Eukaryotic DNA replication proceeds discontinuously via Okazaki fragment synthesis and maturation on the lagging strand. DNA polymerase α primes the lagging strand, synthesizing about 10 nucleotides (nt) of RNA followed by an additional 10–20 nt of DNA. The primer is extended by a complex of DNA polymerase δ (pol δ), the sliding clamp proliferating cell nuclear antigen (PCNA), and the clamp loader replication factor C (RFC). Pol δ continues extension of the upstream fragment until it runs into the downstream fragment, displacing its 5′-end region into a flap. The flap can be cleaved by a nuclease to form a nick, which will be sealed by DNA ligase I to generate the continuous double-stranded DNA.

Cleavage of the flap can be completed by flap endonuclease 1 (FEN1), a 5′ structure-specific endonuclease and the product of the RAD27 gene in Saccharomyces cerevisiae. One proposed model suggests FEN1 cleavage of primarily short flaps displaced by pol δ (3–5). As pol δ displaces the downstream primer into a flap, FEN1 can track from the 5′-end (6, 7) to the base of the flap for cleavage (6, 8, 9) that generates a nick for ligation. Through Okazaki fragment processing reconstitution studies in vitro, Ayyagari et al. (3) showed that short flaps, 10 nt in length, are processed efficiently by FEN1 to generate ligatable replication intermediates. More recent experimentation revealed that in the presence of pol δ and FEN1, strand-displacement and cleavage generated mainly mononucleotide products. In contrast, strand-displacement by mutant forms of pol δ, deficient in its proofreading 3′-5′ exonuclease activity, and cleavage by FEN1 generated mono- through hexanucleotide products. It was proposed that the 3′-5′ exonuclease activity of pol δ, coordinated with FEN1, prevents extensive strand-displacement because pol δ 3′-5′ exonuclease mutants exhibit greater strand-displacement activity than wild-type pol δ (4, 5). This idea is supported by the synthetic lethal interaction between 3′-5′ exonuclease mutants of pol δ (pol3-exo) and either rad27 deletion mutants or those that are compromised for interaction with its cofactor PCNA (rad27-p) (10–12). In addition, the rare pol3-exo rad27-p double mutants that are not lethal require intact recombination and double-strand break repair machinery (12). Altogether, these results suggest that with wild-type proteins, the system is generally designed to strand-displace and cleave short flaps, while failure to cleave at the short stage, either because strand-displacement is enhanced or nuclease cleavage inhibited, leads to the possibility that long flaps occasionally form (3–5).

If a flap escapes cleavage by FEN1, it can become long and follow a secondary pathway. A model put forward by Bae et al. (13) asserts that flaps greater than 27 nt in length are coated by the single-stranded-binding protein replication protein A (RPA). RPA inhibits cleavage by FEN1 but stimulates cleavage by Dna2 nuclease/helicase (13, 14). Dna2, like FEN1, must track from the 5′-end of the flap to cleave (15, 16). Because Dna2 is unable to cleave at the base of a flap (14), it will cleave within the single-stranded region of the flap generating a short flap, too.
small to be coated by RPA (13). The short flap can then be cleaved by FEN1 to generate the nick for ligation (13, 15). Through time course assays in vitro, Bae et al. (13) demonstrated that Dna2 cleavage products appear before FEN1 cleavage products, supporting their hypothesis of the sequential action of Dna2 and then FEN1 to remove the primer.

Additional evidence of a role for both Dna2 and FEN1 in Okazaki fragment maturation includes physical and genetic interactions between the two proteins. Dna2 and FEN1 have been shown to interact by immunoprecipitation (17). Also, studies of mutants in S. cerevisiae revealed a synthetic lethal interaction between dna2-1 and rad27 and a partial rescue of the temperature-sensitive phenotype of dna2-1 mutants when Rad27 is overexpressed (17, 18). Similarly, the temperature-sensitive growth phenotype of rad27 deletion strains is rescued by overexpression of Dna2 (17). Finally, the lethality of recombination-defective pol3-exo rad27-p double mutant strains is also suppressed by Dna2 overexpression (4).

Because of the biochemical and genetic connection between FEN1 and Dna2, we investigated whether properties of the reconstituted system suggest a role for the proposed pathway involving Dna2 nuclease. Even if only a small fraction of flaps escape FEN1 cleavage to become long, the actual number could be significant in vivo because there are millions of Okazaki fragments processed in mammalian cells. Therefore, the proportions of short and long products become an important factor to consider in understanding primer removal.

To address the question of whether the action of FEN1 alone is sufficient to remove the Okazaki fragment primer, we reconstituted Okazaki fragment processing in vitro, with proteins from S. cerevisiae. We asked which is the predominant pathway for primer removal, concentrating on various factors that might affect the proportions of short and long products.

**EXPERIMENTAL PROCEDURES**

Materials—Oligonucleotides were synthesized by either Midland Certified Reagents Company (Midland, TX) or Integrated DNA Technologies (Coralville, IA). Radionucleotides [gamma-^32^P]ATP and [alpha-^32^P]dCTP were obtained from PerkinElmer Life Sciences. Polynucleotide kinase, the Klenow fragment of Escherichia coli DNA polymerase I, and streptavidin were purchased from Roche Applied Science (Indianapolis, IN). All other reagents were the best commercially available.

**Enzyme Expression and Purification—**S. cerevisiae wild-type pol Delta (pol Delta-wt) (19) and 3′-5′ exonuclease deficient pol Delta (pol Delta 3-01) (4, 19) were overexpressed in S. cerevisiae and purified as previously described. S. cerevisiae RFC was overexpressed and purified from E. coli as previously described (20). S. cerevisiae wild-type Rad27 (21) (referred to as FEN1) and Rad27-G67S (22) (referred to as G67S FEN1) were cloned into T7 expression vector pET-24b (Novagen/EMD Biosciences, WI), expressed in E. coli strain BL21(DE3) codon plus (Stratagene), and purified as previously described, resulting in recombinant protein with a C-terminal His_6 tag.

Wild-type S. cerevisiae PCNA was expressed in E. coli strain BL21(DE3) (Novagen/EMD Biosciences, WI) by T7 expression vector pET-24b (Novagen/EMD Biosciences, WI) to generate recombinant PCNA with a C-terminal His_6 tag. Protein purification was performed using the procedure described by Liu and Bambara (23) with the following modifications. The flow through from the Mono S column was collected and loaded onto a 1-ml Mono Q column (Amersham Biosciences). Protein was eluted with a gradient of Hi buffer (30 mM HEPES and 0.5% isoinositol) with 30–2000 mM KCl.

**Oligonucleotide Substrates—**Using oligonucleotide primers, substrates were designed to simulate Okazaki fragment processing intermediates. The primer sequences are listed in Table 1.

| Primer | Length | Sequence |
|--------|--------|----------|
| U1     | 20     | GCCACCCGACGCGCACCTCC |
| T1     | 110    | GCCGTGCTGCGGTGGATACGGCCAGTGCCGACCGTGCCAGCCTAAATTTCAATCCACCC |
| T2     | 110    | AGAGGGTGCCGCTGCGGTGGATACGACGGCCAGTGCCGACCGTGCCAGCCTAAATTTCAATCCACCC |

* Underline indicates RNA segment.
* Both templates T1 and T2 are biotinylated at both the 5′ and 3′-ends.
at 95 °C for 5 min, transferring to 70 °C, and cooling to room temperature.

There are two primary sets of substrates used for the following experiments. All substrates contain a 20-nt upstream primer and a 60-nt downstream primer annealed to the 3′- and 5′-ends, respectively, of a 110-nt template. However, the two sets differ in the nucleotide content of the downstream region. One set contains a nucleotide sequence balanced in AT/GC content in the downstream region and will be referred to as the standard substrate. The second set of substrates contains a GC-rich region at the 5′-end of the downstream primer and will be referred to as the GC-rich substrate. The specific substrate used in any experiment will be indicated in the figure legend and depicted above the figure.

**Enzyme Assays**—Biotinylated substrates (5 fmol per reaction) were preincubated with streptavidin (500 fmol each reaction) on ice for 20 min prior to initiating the reaction. In a total reaction volume of 20 μl, substrate was then incubated with 22.7 fmol pol δ-wt or pol δ 3-01 (indicated in figure legend), 50 fmol of PCNA, 50 fmol of RFC, and 20 fmol of FEN1 (or titration amount indicated in figure legends) for 10 min at 30 °C. Reaction buffer contained 50 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 1 mM ATP, 50 μM dNTPs, and 75 mM NaCl (unless otherwise indicated). Reactions were stopped by addition of 10 μl 2× termination dye (90% formamide (v/v), 10 mM EDTA with 0.01% bromphenol blue and xylene cyanol) and heating for 5 min at 95 °C. Reaction products were loaded onto a 15%/7 M urea denaturing polyacrylamide gel and separated by electrophoresis. The gel was scanned by a Molecular Dynamics PhosphorImager and results analyzed with Image Quant version 1.2 software.

For the time course reactions examining FEN1 cleavage on 3′-labeled substrates, a volume of 120 μl of master reaction mix containing 30 fmol of biotinylated substrate (preincubated with 3000 fmol of streptavidin on ice for 20 min), 136 fmol of pol δ-wt or pol δ 3-01 (indicated in figure legend), 300 fmol of PCNA, 300 fmol of RFC, and 120 fmol of FEN1 in reaction buffer (described above) was incubated at 30 °C. At each time point (0.5, 1, 2.5, and 10 min), 20 μl of the master reaction mix was removed and added to a tube containing 10 μl of 2× termination dye. Reactions were heated for 5 min at 95 °C. Reaction products were loaded onto a 15%/7 M urea denaturing polyacrylamide gel and separated by electrophoresis. The gel was scanned by a PhosphorImager and results analyzed with Image Quant.

**Cleavage Product Distribution Analysis**—First, bands were identified on the gels and correlated with flap cleavage lengths. Then, using Image Quant software, the gel lanes were scanned and peaks of signal intensities were detected. Bands on the gels corresponding to different flap cleavage lengths were matched with the peaks detected by Image Quant. The intensity of peaks from the FEN1 alone cleavage was subtracted from the intensity of peaks in the presence of pol δ, PCNA/RFC, and FEN1. A graph of flap length versus peak intensity was generated, and a moving average trendline (period of 3) was added to smooth and indicate the trend of the data.

**RESULTS**

Our goal was to test whether long flaps (greater than about 8 nt) are generated in an Okazaki fragment processing reconstitution following strand-displacement by pol δ. The presence of long flaps would suggest that in addition to being subject to FEN1 cleavage, some could be long enough to become substrates for Dna2. First, we examined pol δ strand-displacement and FEN1 flap cleavage with wild-type enzymes. Then, we determined how mutations that impair pol δ or FEN1 influence the length of flaps and their cleavage.

**Long Flaps Are Created and Cleaved by FEN1 Following Displacement by Both pol δ-wt and pol δ 3-01**—This first experiment used either pol δ-wt or pol δ 3-01, deficient in 3′-5′ exonuclease activity, in reconstitutions with the standard substrate, which has nearly equal AT/GC content at the 5′-end of the downstream primer. This substrate, like all the substrates used in the following experiments, resembles a lagging strand synthesis intermediate with an upstream and downstream primer annealed to the 3′- and 5′-ends, respectively, of an oligonucleotide template such that there is a gap between the two primers. Substrates are depicted above the figures. When investigating synthesis by pol δ, the substrate is radiolabeled at the 5′-end of the upstream primer, allowing observation of extension by the polymerase. Full-length extension of the 20-nt upstream primer results in a product 110-nt in length, as indicated in all figures displaying synthesis products. In the presence of PCNA/RFC and FEN1, both the pol δ-wt and pol δ 3-01 displayed some level of full-length synthesis (Fig. 1A, lane 5 and lane 9, respectively). The same was also true for the GC-rich substrate (Fig. 1B, lane 5 and lane 9, respectively). This evidence of strand-displacement indicates that flaps for cleavage by FEN1 were generated.

Initial FEN1 cleavage products were evaluated by using a substrate radiolabeled at the 5′-end of the downstream primer, to allow visualization of the first product cleaved from each downstream primer. The maximum length FEN1 cleavage product for a reconstitution with pol δ-wt was about 30 nt versus about 41 nt with pol δ 3-01 (Fig. 1A, lanes 14 and 16, respectively). The length of flap released correlated with the extent of strand-displacement. Greater strand-displacement resulted in longer flap displacement and consequently longer flap cleavage products. Displacement synthesis by pol δ 3-01 in this system generates longer cleavage products than pol δ-wt on the standard substrates. In addition to the cleavage products dependent on pol δ strand-displacement synthesis, there is a large amount of cleavage exhibited by FEN1 in the absence of polymerase (Fig. 1A, lane 11). This non-synthesis-dependent FEN1 activity is high with the standard substrate and creates an undesirable background in the reactions with displacement synthesis.

To suppress this background, a substrate was designed that is GC-rich at the 5′-end of the downstream primer. Because GC residues base pair more strongly than AT residues, we predicted that the GC-rich sequence would stabilize the annealed 5′-end of the downstream primer. This stabilization limited the amount of non-synthesis-dependent FEN1 cleavage (Fig. 1A, lane 11 versus Fig. 1B, lane 11, respectively). In the presence of pol δ-wt, FEN1 cleaved flaps up to about 14-nt long (Fig. 1B,
while in the presence of the pol δ 3-01, flaps up to 27-nt long were cleaved (Fig. 1B, lane 16). The overall length of pol δ-displaced and FEN1-cleaved flaps decreased on the GC-rich substrate. However, for both the standard and GC-rich substrates, pol δ 3-01 displacement generates longer cleavage products than the pol δ-wt (Fig. 1, A and B, lane 16 versus lane 14). This result is consistent with previous biochemical results demonstrating that the exonuclease deficient pol δ 3-01 generates longer strand-displacement products than the wild-type polymerase (4, 5).

Salt Decreases Non-synthesis-dependent FEN1 Cleavage While Still Allowing for Strand-displacement Synthesis—The studies presented in Fig. 1 were carried out at low salt buffer conditions (0 mM NaCl as described under “Experimental Procedures”). Increasing salt stabilizes duplex DNA. It also inhibits FEN1 interaction with DNA, particularly when PCNA is not present (8, 24). As a result, both the initiation of strand-displacement synthesis by pol δ, and non-synthesis-dependent FEN1 cleavage should be inhibited when increasing amounts of salt are added into the reconstitution. Increasing the salt concentration slightly decreased both the amount of substrate usage by the polymerase and overall amount of synthesis with both the standard (Fig. 2A, lanes 2–6 and 7–11) and GC-rich substrates (Fig. 2B, lanes 2–6 and 7–11). Yet, higher salt still allowed complete strand-displacement synthesis to occur on both the standard and GC-rich substrates (Fig. 2, A and B, lanes 2–11), although notable pausing by the polymerase was observed upon encountering the 5′-end of the downstream primer, a position corresponding to the nick. Significantly, higher salt markedly decreased the amount of non-synthesis dependent FEN1 cleavage (Fig. 2A, lanes 14–17 compared with lane 13). This trend is similar for both the standard (Fig. 2A) and the GC-rich substrates (Fig. 2B, lanes 14–17 compared with lane 13). The remaining experiments were done at 75 mM NaCl, which effectively limits the amount non-synthesis-dependent FEN1 cleavage without significantly affecting polymerase activity.

**FIGURE 1.** Long flap formation and cleavage following pol δ displacement synthesis. A, synthesis (lanes 2–9) by pol δ-wt, denoted wt (lanes 2–5), and pol δ 3-01, denoted 01 (lanes 6–9), was assayed on the standard substrate (U1:T1:D1). Reactions were performed in the presence of PCNA/RFC and FEN1 at 0 mM NaCl, as described under “Experimental Procedures.” Cleavage by FEN1 (lanes 11–16) was assayed on the standard substrate in the presence of PCNA/RFC and either pol δ-wt (lanes 13 and 14) or pol δ 3-01 (lanes 15 and 16) at 0 mM NaCl, as described under “Experimental Procedures.” B, synthesis (lanes 2–9) and cleavage (lanes 11–16) reactions are the same as described in A except the substrate used was the GC-rich substrate (U1:T2:D2). The substrates are depicted above the figure. The asterisk indicates location of radiolabel.
Endonuclease-deficient FEN1 Generates a Larger Proportion of Long Cleavage Products Compared with Wild-type FEN1—The effects of a mutant FEN1 on flap formation were investigated. The G67S FEN1, which was identified in a screen for replication and repair mutants having a tendency toward insertion or deletion of nucleotides within a repeat tract, has a point mutation in the proposed catalytic site. As observed through assays in vitro, the G67S mutant is deficient in double and single flap endonucleolytic cleavage and exonucleolytic cleavage. The maximum amount of cleavage on a single-flap substrate with the wild-type FEN1 was 90% versus 60% with the G67S FEN1. Although deficient in cleavage, the mutant nuclease still retains a measurable level of cleavage activity (22). We compared wild-type FEN1 to the G67S mutant in reconstitution assays with either pol/δ/H9254-wt or pol/δ/H92543-01 and PCNA/RFC on both the standard and GC-rich substrates.

Moreover, the mutant and wild-type FEN1 support comparable amounts of full-length extension by pol δ 3-01 (Fig. 3, A and B, lanes 5–8 and 10–13). On the standard substrate, with pol δ-wt and PCNA/RFC, cleavage products were seen up to 27-nt long with both the wild-type and mutant FEN1 (data not shown). However, the G67S FEN1 generated a larger proportion of long cleavage products relative to short products when compared with the wild-type FEN1 (data not shown). For the purposes of describing cleavage product proportions, we defined short products as those 1–8 nt in length and long products ranging from 9–59 nt. With the GC-rich substrate, we were unable to detect cleavage products longer than 5 nt in the presence of pol δ-wt (data not shown).

In contrast, products up to about 50-nt long were observed with both substrates in assays with pol δ 3-01 (Fig. 3, A and B). On the standard substrate the ratio of long products to short products is 0.33 with the wild-type FEN1 (Fig. 3 A, lane 21) compared with 1.4 with the G67S mutant (Fig. 3A, lane 28).

FIGURE 2. Salt decreases non-synthesis-dependent FEN1 cleavage. A, synthesis (lanes 2–11) by pol δ-wt, denoted wt (lanes 2–6), and pol δ 3-01, denoted 01 (lanes 7–11), was assayed on the standard substrate (U1:T1:D1). Reactions were performed in the presence of PCNA/RFC and FEN1, as described under “Experimental Procedures,” with increasing amounts of NaCl (0, 25, 50, 75, 100 m). Cleavage by FEN1 (lanes 13–17) was assayed on the standard substrate, as described under “Experimental Procedures,” with increasing amounts of NaCl (0, 25, 50, 75, 100 m). B, synthesis (lanes 2–11) and cleavage (lanes 13–17) reactions are the same as described in A except the substrate used was the GC-rich substrate (U1:T2:D2). The substrates are depicted above the figure. The asterisk indicates location of radiolabel.
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FIGURE 3. Larger proportion of long cleavage products generated with endonuclease deficient FEN1. A, synthesis (lanes 2–13) by pol δ 3-01 was assayed on the standard substrate (U1:T1:D1). Reactions were performed in the presence of PCNA/RFC and increasing amounts (10, 20, 50, 100 fmol) of either wild-type FEN1 or G67S FEN1 as described under “Experimental Procedures.” FEN1 cleavage was assayed by a time-course reaction (0.5, 1, 2, 5, 10 min) on the standard substrate (U1:T1:D1). Reactions were performed in the presence of PCNA/RFC and either pol δ-wt, denoted wt (lanes 6–10), or pol δ 3-01, denoted 01 (lanes 13–17), as described under “Experimental Procedures.” Control reactions (lanes 1–5 and 11 and 12) were all run for 10 min. B, cleavage reactions were the same as described in A except the substrate used was the GC-rich substrate (U1:T2:D2). The substrate is depicted above the figure. The asterisk indicates location of radiolabel.

FIGURE 4. Extent of FEN1 cleavage indicates pol δ displacement 52 nt into downstream primer. A, progressive cleavage by FEN1 into the downstream primer was assayed by a time-course reaction (0.5, 1, 2, 5, 10 min) on the standard substrate (U1:T1:D1). Reactions were performed in the presence of PCNA/RFC and either pol δ-wt, denoted wt (lanes 6–10), or pol δ 3-01, denoted 01 (lanes 13–17), as described under “Experimental Procedures.” Control reactions (lanes 1–5 and 11 and 12) were all run for 10 min. B, cleavage reactions are the same as described in A except the substrate used was the GC-rich substrate (U1:T2:D2). The substrate is depicted above the figure. The asterisk indicates location of radiolabel.

Our results suggest that although FEN1 cleavage of displaced flaps results in products up to about 45-nt long, the majority of displaced and cleaved flaps are short, up to about 8 nt. Because the RNA/DNA primers initiating Okazaki fragments are about 20–30 nt in length (1, 2), we examined whether pol δ displacement and FEN1 cleavage extend far enough into the downstream primer region to remove at least 30 nt corresponding to the approximate length of the entire initiating RNA/DNA primer. To accomplish this, we radiolabeled the substrate at the 3'-end of the downstream primer. This allowed visualization of products remaining after cleavage. The generation of progressively shorter products with time is a result of multiple flap cleavages by FEN1 as strand-displacement proceeds. On the standard substrate, in the presence of pol δ-wt or pol δ 3-01, PCNA/RFC, and FEN1 (Fig. 4A, lanes 10 and 17, respectively), cleavage products ranging in size from 8 to 59 nt were seen. Cleavage products 8-nt long result from cleavage 52 nt into the downstream primer.

to short products is higher with the mutant FEN1 compared with wild-type, 0.8 versus 0.001 (Fig. 3B, lane 28 versus lane 21). We could also compare the total generation of products greater than 8 nt in length as the concentration of FEN1 was increased. These products are beyond the length of non-synthesis-dependent FEN1 cleavage and so were completely derived from strand-displacement synthesis. This comparison would reveal whether excess FEN1 could capture and cleave more or all flaps before they become long. As the concentration of FEN1 was increased from approximately half-stoichiometric to ~5-fold excess, in relation to pol δ 3-01, the amount of long products cleaved by wild-type FEN1 decreased to about half (Fig. 3, A and B, lanes 18–21) on both the standard and GC-rich substrates. The moderate decrease in cleavage of long products probably occurs in part because the initial level of FEN1 is substoichiometric. It also may be a result of structure forming in flaps released by initial FEN1 cleavage that can support further cleavage at high FEN1 concentration. In contrast, the amount of long products cleaved with the G67S FEN1 increased between 30–80% over the increase in FEN1 concentration (Fig. 3, A and B, lanes 25–28). This possibly occurs in part because the mutant FEN1 may not recognize folded single strands as substrates. Alternatively, the mutant nucleases may actually interfere with cleavage at high concentration.

FEN1 Can Cleave, Following pol δ-wt and pol δ 3-01 Displacement, up to 52 nt into Downstream Primer—Our results suggest that although FEN1 cleavage of displaced flaps results in products up to about 45-nt long, the majority of displaced and cleaved flaps are short, up to about 8 nt. Because the RNA/DNA primers initiating Okazaki fragments are about 20–30 nt in length (1, 2), we examined whether pol δ displacement and FEN1 cleavage extend far enough into the downstream primer region to remove at least 30 nt corresponding to the approximate length of the entire initiating RNA/DNA primer. To accomplish this, we radiolabeled the substrate at the 3'-end of the downstream primer. This allowed visualization of products remaining after cleavage. The generation of progressively shorter products with time is a result of multiple flap cleavages by FEN1 as strand-displacement proceeds. On the standard substrate, in the presence of pol δ-wt or pol δ 3-01, PCNA/RFC, and FEN1 (Fig. 4A, lanes 10 and 17, respectively), cleavage products ranging in size from 8 to 59 nt were seen. Cleavage products 8-nt long result from cleavage 52 nt into the downstream primer.
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FIGURE 5. Comparison of synthesis and cleavage on DNA versus RNA-containing substrates. Three types of radiolabeled substrates were used in this experiment, label on the 5'-end of the upstream primer (lanes 1–3 and 14–16), label on the 5'-end of the downstream primer (lanes 4–8 and 17–21), and label on the 3'-end of the downstream primer (lanes 9–13 and 22–26). Synthesis was assayed on the standard substrate (U1; T, D) (lanes 2 and 3) and the RNA-containing substrate (U1; T, D) (lanes 15 and 16) in the presence of PCNA/RFC, FEN1, and either pol δ-wt, denoted wt, or pol δ 3-01, denoted 01, as described under “Experimental Procedures.” Initial cleavage by FEN1 was assayed on the standard substrate (lanes 5–8) and the RNA-containing substrate (lanes 18–21) in the presence of PCNA/RFC and either pol δ-wt or pol δ 3-01, as described under “Experimental Procedures.” The extent of FEN1 cleavage into the downstream primer was assayed on the standard substrate (lanes 10–13) and the RNA-containing substrate (lanes 23–26) in the presence of PCNA/RFC and either pol δ-wt or pol δ 3-01, as described under “Experimental Procedures.” Underlined bands indicate products corresponding to 10, 20, 30, or 40 nt. The substrates are depicted above the figure.

stream primer. The greater part of cleavage products seen in assays with pol δ-wt ranged between 59–37 nt in size corresponding to cleavage occurring up to 23 nt into the downstream primer (Fig. 4A, lanes 6–10 and 13–17). The same holds true for the GC-rich substrate (Fig. 4B, lanes 6–10 and 13–17). Although the cleavage pattern is similar for assays with pol δ-wt or pol δ 3-01, the amount of FEN1 cleavage is greater with pol δ 3-01 (Fig. 4, A and B, lane 17 compared with lane 10), consistent with better displacement synthesis by pol δ 3-01 (4, 5).

Comparison of pol δ-wt and pol δ 3-01 Synthesis and FEN1 Cleavage on Substrates with a DNA- versus RNA-initiated Downstream Primer—Natural Okazaki fragments are initiated with about 10 nt of RNA followed by DNA (1, 2). Because of this, it was important to determine whether the patterns of synthesis and cleavage in our reconstituted system change when a substrate is used that contains an initiator RNA at the 5’-end of the downstream primer. The RNA-containing substrate used in this experiment has the same sequence as the standard substrate, described earlier. However, in this substrate, the 5’-end of the downstream primer is initiated with 12 ribonucleotides followed by 48 nt of DNA, unlike the downstream primer in the standard substrate, which contains 60 nt entirely of DNA. In the presence of pol δ-wt or pol δ 3-01, PCNA/RFC, and FEN1, the overall level of synthesis for the RNA-containing substrate (Fig. 5, lanes 15 and 16) is comparable to the standard substrate without initiator RNA (Fig. 5, lanes 2 and 3). For the RNA-containing substrate, there is a slightly higher amount of larger synthesis products (Fig. 5, lanes 15 and 16) compared with the standard substrate (Fig. 5, lanes 2 and 3). This result indicates that the polymerase synthesizes and strand-displaces further into the downstream primer region on the RNA-containing substrate. Interestingly, in the presence of PCNA/RFC and FEN1, there is strong pausing in pol δ-wt synthesis at the nick position on the standard substrate that is absent on the RNA-containing substrate (Fig. 5, lane 2 compared with lane 15). Yet, on the RNA-containing substrate, synthesis pauses in a region corresponding approximately to the beginning of the DNA (Fig. 5, lane 15), suggesting that polymerase pausing limits strand-displacement to avoid degradation well into the DNA.

The size of initial cleavage products following displacement synthesis by pol δ is similar for both the standard substrate and the RNA-containing substrate (Fig. 5, lanes 7 and 8 compared with lanes 20 and 21). The maximal length of cleavage products, as measured on substrates labeled at the 5’-end of the downstream primer, was 23 nt with pol δ-wt (Fig. 5, lane 7) and 44 nt with pol δ 3-01 (Fig. 5, lane 8) for the standard substrate. Maximal lengths were 20 nt (Fig. 5, lane 20) and 44 nt (Fig. 5, lane 21), respectively, for the RNA-containing substrate. In addition, the amount of displacement and cleavage measured on the 3’-end-labeled substrates was the same for both the standard and RNA-containing substrates. In reconstitution assays with pol δ-wt and pol δ 3-01, cleavage occurred about 52 nt into the downstream primer (Fig. 5, lanes 12 and 13 and 25 and 26).

Cleavage Product Distribution Shows Two Populations—We considered that quantitation of the distribution of products might reveal additional information about their creation. After scanning a gel lane to determine the pixel intensities of the
cleavage bands, we grafted the band intensity versus the size of the flap cleaved. The graph in Fig. 6 shows that the majority of flaps are displaced and cleaved at the short stage up to about 8 nt. A second much smaller population of flaps is displaced and cleaved from about 8 nt up to about 30 nt, in the presence of pol δ-wt, or about 45 nt in the presence of pol δ 3-01. The short products are distributed in a sharp peak, while the distribution of the long products is broad and flat. These general characteristics of the two populations of cleavage products are similar for both the standard and RNA substrates in reconstitution with pol δ-wt and pol δ 3-01, at approximately equal amounts of FEN1 and pol δ (Fig. 6). The same product distribution also holds true when FEN1 is in excess of pol δ-wt or pol δ 3-01 for the standard substrate and when FEN1 is in excess of pol δ 3-01 for the GC-rich substrate (data not shown). There were no cleavage products greater than 5 nt detectable in assays with GC-rich substrate and pol δ-wt (data not shown).

**DISCUSSION**

To assess the mechanisms for eukaryotic Okazaki fragment primer removal, we investigated the lengths of flaps displaced by pol δ and cleaved by FEN1 using an Okazaki fragment processing model system in vitro. Previous biochemical evidence demonstrated that a large majority of flaps strand-displaced by pol δ, and cleaved by FEN1, are short (ranging from 1 to 4 nt) (5). Current observations revealed detectable amounts of longer flap cleavage products, ranging up to 30 nt.

The proportion of flaps that become long is not a fixed value. It can be altered by changing the properties of the enzymes and substrate. To this end, flap cleavage length was measured with both pol δ-wt and the 3’-5’ exonuclease deficient pol δ 3-01. The exonuclease deficient pol δ 3-01 allowed for “amplification” and better visualization of longer cleavage products that would otherwise be undetectable because they form in small amounts with wild-type polymerase. A likely explanation for this effect is that pol δ 3-01 has been reported to be more active at strand-displacement synthesis (4, 5). However, other properties of the polymerase may have been altered by the mutation that disrupts coordination of the polymerase and FEN1.

Likewise, use of the endonuclease-deficient G67S FEN1 augmented the fractions of longer flap cleavage products (Fig. 3). Because the G67S FEN1 cleaves at a lower efficiency than the wild-type (22), we expected that flaps displaced by the polymerase were not cleaved as readily by the G67S FEN1 and more became long.

Long flap formation was also increased with substoichiometric levels of FEN1 relative to pol δ (Fig. 3). Even though FEN1 molecules are in excess over primer termini, lowering FEN1 concentration is expected to deplete some polymerases of a FEN1 partner, allowing pol δ molecules to extend without a corresponding FEN1. In that case, pol δ displacement of a flap might not be met with immediate FEN1 cleavage, leading to displacement and cleavage of long flaps. Significantly, in some experiments FEN1 concentration was in ~5-fold excess over the pol δ concentration (Fig. 3). Even then, FEN1 cleavage generated flaps 8–27 nt in length. This is an important control that verifies that our standard reactions, with about equal pol δ and FEN1, contained enough FEN1 to provide partners for every polymerase. Moreover, excess FEN1 cannot correct the system to eliminate the creation of long flaps. It is probable that pol δ is coordinated with a single FEN1 to cleave displaced flaps. Its occasional failure to do so cannot be corrected by another nearby FEN1.

Another factor influencing strand-displacement and flap cleavage is the nucleotide composition of the substrate. Increasing the GC content decreased the overall length of flap cleavage products (Fig. 1). Compared with the standard substrate, which has a more balanced AT/GC content and showed cleavage products up to 27-nt long in the presence of pol δ-wt, the GC-rich substrate did not generate detectable products longer than 5 nt. We conclude that the amount of displacement synthesis by pol δ is sequence-dependent. Sequences with more stable base pairing inhibit displacement, while the presence of weaker paired sequences promotes displacement. The slower displacement would then allow FEN1 ample time to cleave virtually all flaps before their length could increase. In this way, the GC-rich substrate limits flap length, acting similarly to a polymerase mutation that limits strand-displacement.

In contrast, the presence of RNA initiating the downstream primer does not have a notable affect on the size of flap cleavage products, although it is slightly stimulatory to pol δ synthesis, compared with the standard substrate (Fig. 5). The similarity in cleavage product distribution and synthesis indicates that the non-RNA-containing substrates are an adequate substitute for the RNA-containing substrates for reconstitutions of RNA-initiated Okazaki fragment processing in vitro.

Overall, these results suggest two important conclusions. First, there is indeed a distribution of long products made under the reaction conditions that we feel emulate natural Okazaki fragment processing. These products are not abundant and appear at the limits of detection in our biochemical assays. However, millions of Okazaki fragments have to be processed each time the genome of a eukaryotic cell is replicated. Even a
low level of production of these long products would require the two-nuclease pathway involving RPA, Dna2, and then FEN1. The need for Dna2 in this pathway is a reasonable explanation for the lethality of dna2-null mutants in S. cerevisiae (25, 26). This conclusion is also consistent with the recent report that mutations in the gene for the 5' helicase Pif1 can suppress the dna2 null mutation. Pif1 may augment the ability of pol δ to generate flaps, feeding the two-nuclease pathway (27).

Second, the distribution of flaps into one pathway versus the other appears to be based on a simple balance of displacement and FEN1 cleavage activities. Augmentation of strand-displacement either with a mutation in the polymerase, a change of nucleotide composition of the template, or impairment of FEN1 cleavage shifts the balance in an explainable manner. This suggests that in vivo there is simply some probability that any flap will escape immediate cleavage by FEN1.

Most of our experiments examine the fate of the first FEN1 cleavage product. We also examined the extent of pol δ displacement and multiple FEN1 cleavages on the downstream primer (Fig. 4). Results showed cleavage products corresponding to displacement of flaps 52 nt into the downstream region. Although lengths of 52 nt are well beyond the length of Okazaki fragment RNA/DNA primers (20–30 nt) (1, 2), the majority of cleavage for both the standard and GC-rich substrates, occurring to a distance of ~23 nt, is consistent with removal of primers and not excessive strand-displacement and cleavage. From this analysis of cleavage, it is evident that FEN1 can cleave a sufficient distance into the downstream region to remove the RNA/DNA primer. However, because the majority of cleavage products are only up to about 8-nt long, most primers must be removed in several cleavage events. Presumably, pol δ displaces flaps and FEN1 makes a series of short-spaced, successive cuts. We hypothesize that in vivo a similar mechanism holds true. Previous work has shown that once FEN1 cleaves in a region of DNA, DNA ligase I readily utilizes the nicked product for completion of the processing reaction (3).

There are two populations of cleavage products, a peak of short products up to about 8 nt and much wider, more level distribution of products ranging from 8 nt up to 30 or more nt (Fig. 6). This two-component distribution suggests that a small population of flaps, having escaped immediate FEN1 cleavage, would reel out to form long flaps. It is likely that the two populations of displacement products are processed differently. We hypothesize that ordinarily pol δ and FEN1 are coordinated such that FEN1 captures the flaps as pol δ is displacing them, keeping the flaps short. Possibly a communication between polymerase and nuclease influences the cleavage rate. Once the flap exceeds a particular length this coordination may be lost. We have previously demonstrated that FEN1 needs to track from the 5’-end of a flap (6, 7). For very short flaps, the tracking rate may be irrelevant. At a particular flap length the tracking process would race against the strand-displacement process, with cleavage occurring only when the FEN1 reaches the rapidly moving base of the flap. If the tracking and displacement rates are nearly the same and differ slightly from flap to flap, the effect would be a very wide, flat distribution of cleavage lengths as observed. Once the flap reaches 20–30 nt, RPA binds, and FEN1 tracking is inhibited (13, 14). At that point we envision that the tracking rate of Dna2, previously shown to be a helicase (15, 28) and tracking enzyme (15, 16), could determine the rate of flap cleavage.

Clearly, the process of flap removal is more complex than the “nick translation” originally envisioned in bacteria (29) because it is accomplished by flap cleavage. In E. coli and other bacteria, FEN1 is a domain of a larger protein having a polymerase capable of strand-displacement, allowing for tight coordination of synthesis and cleavage (30). However, even in E. coli there is some evidence that the downstream primer is often cleaved to produce an oligomer (31). Moreover, the bacterial versions of FEN1 display flap cleavage specificity (32).

The eukaryotic pol δ and FEN1 are independent proteins, presumably designed to work together. The preferred substrate of FEN1 in both bacteria (33, 34) and eukaryotes (6, 9, 21) is a double-flap in which the upstream primer overlaps one nucleotide with the base of the downstream primer. This means that FEN1 is most active during the displacement reaction. In eukaryotes it is conceivable that FEN1 cleaves at the 5’-ends of some Okazaki fragments before arrival of the polymerase and the terminus of the extending upstream primer. In our system, we observed a significant amount of this displacement-independent FEN1 cleavage (Fig. 1). This is expected since FEN1 has some activity on the 5’-ends of apparently fully annealed primers (6, 8). Susceptibility to cleavage was attributed to natural thermal “breathing” of the 5’-end region to create a transient flap. In current work, increasing the salt concentration stabi-
lized the DNA helix such that the amount of non-synthesis dependent FEN1 cleavage was reduced (Fig. 2). The cleavage products dependent on pol δ strand-displacement were more evident, although the overall amount of synthesis decreased slightly.

The results presented here support the existence of parallel pathways for removal of eukaryotic Okazaki fragment primers (Fig. 7). A majority of displaced flaps are cleaved while short, before reaching 8 nt in length, though some flaps do escape FEN1 cleavage, growing to 30 nt, which is long enough for stable RPA binding (35). It is likely that FEN1 is sufficient to remove the Okazaki fragment primer through successive cleavage of primarily short flaps. Alternatively, in a subset of long displaced flaps, RPA coating of a flap could mediate the two-nuclease pathway involving sequential action of Dna2 and then FEN1 for primer removal. Examining the influence of RPA and Dna2 on the displacement and cleavage flap length will be important to expand our understanding of the mechanisms of primer removal and Okazaki fragment maturation.

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