On the Roles of *Saccharomyces cerevisiae* Dna2p and Flap Endonuclease 1 in Okazaki Fragment Processing*

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Short DNA segments designated Okazaki fragments are intermediates in eukaryotic DNA replication. Each contains an initiator RNA/DNA primer (iRNA/DNA), which is converted into a 5’-flap and then removed prior to fragment joining. In one model for this process, the flap endonuclease 1 (FEN1) removes the iRNA. In the other, the single-stranded binding protein, replication protein A (RPA), coats the flap, inhibits FEN1, but stimulates cleavage by the Dna2p helicase/nuclease. RPA dissociates from the resultant short flap, allowing FEN1 cleavage. To determine the most likely process, we analyzed cleavage of short and long 5’-flaps. FEN1 cleaves 10-nucleotide fixed or equilibrating flaps in an efficient reaction, insensitive to even high levels of RPA or Dna2p. On 30-nucleotide fixed or equilibrating flaps, RPA partially inhibits FEN1. CTG flaps can form fold-back structures and were inhibitory to both nucleases, however, addition of a dT12 to the 5’-end of a CTG flap allowed Dna2p cleavage. The presence of high Dna2p activity, under reaction conditions favoring helicase activity, substantially stimulated FEN1 cleavage of tailed-foldback flaps and also 30-nucleotide unstructured flaps. Our results suggest Dna2p is not used for processing of most flaps. However, Dna2p has a role in a pathway for processing structured flaps, in which it aids FEN1 using both its nuclease and helicase activities.

Cellular DNA is replicated by continuous leading-strand synthesis and discontinuous lagging-strand synthesis. In eukaryotic cells, each discontinuous DNA segment, or Okazaki fragment, is 100–150 nucleotides long, and its synthesis is primed by RNA. DNA polymerase α-primase complex (pol α) is required to generate an initiator primer composed of an 8- to 12-nucleotide RNA and 20-nucleotide DNA (iRNA/DNA) (1). The remaining DNA is generated by DNA polymerase δ (pol δ) after a “polymerase switching” reaction (2, 3). In this process, replication factor C (RFC) displaces pol α. RFC also loads the toroidal homotrimer, proliferating cell nuclear antigen (PCNA), which recruits pol δ onto the 3’ terminus of the growing chain (2–5). Because pol α does not possess 3’ → 5’ exonuclease activity, it synthesizes DNA with relatively low fidelity (6). To maintain the integrity of the genome, the entire iRNA/DNA synthesized by pol α on each Okazaki fragment should be removed prior to joining of the remaining DNA segments (7–9).

The iRNA/DNA segments are thought to be excised while in a flap intermediate. The strand displacement activity of pol δ has been proposed to be responsible for the formation of the flap structures (8, 9). Our understanding of the mechanism of flap removal is still evolving. There are three models proposed to date. In the original model, the iRNA was proposed to be removed by the sequential action of RNase H and flap endonuclease 1 (FEN1) (10). Here, the iRNA is cleaved by RNase H before the formation of the flap. Cleavage by RNase H occurs specifically at the 5’-end of the ribonucleotide at the RNA-DNA junction, leaving a single ribonucleotide that is displaced into a flap by pol δ (10–13). FEN1, a structure-specific endonuclease, recognizes the specific flap intermediate containing both a several-nucleotide long 5’-flap and an one-nucleotide 3’-tail (14). Stimulated by an interaction with PCNA, FEN1 cleaves this intermediate at the position one nucleotide into the downstream duplex DNA (14, 15). The cleavage produces a nick that is ligated into a contiguous lagging strand.

FEN1 has been shown to play an important role in Okazaki fragment processing in vivo in yeast (16). However, RNase H is not an essential gene in *Saccharomyces cerevisiae* (17). Moreover, RNase H has not been a necessary component in these reconstituted reactions, and flaps are processed well in the absence of RNase H (18). Thus, other models have been proposed.

The second model involves the Dna2 helicase/nuclease. Dna2p was found to be essential in budding yeast (19, 20), and the enzyme is conserved among all organisms, including humans (21–26). In a temperature-sensitive dna2 mutant, the extent of DNA synthesis was reduced, and the majority of products were shorter than full-length (20, 27). In another mutant, there appeared to be a defect late in S phase, although bulk DNA synthesis appeared to occur, as measured by flow cytometry (28). Similar findings were made with *Schizosaccharomyces pombe* dna2 mutants (22, 24). Dna2p was found to copurify with FEN1, and all alleles tested were found to be synthetically lethal with the null mutant of FEN1 in *S. cerevisiae* (rad27Δ) (29). Overproduction of Rad27p suppressed the temperature-sensitive growth of dna2 mutants and even partially suppressed dna2Δ (29, 30). This led to the conclusion that Dna2p may have a role in Okazaki fragment processing and that its nuclease activity might be important. Additional studies in *S. pombe* demonstrated genetic interactions with other lagging strand replication proteins, i.e. subunits of pol δ and DNA...
ligase (24). Defects that inactivate nuclease activity are lethal, whereas full helicase activity is not required on media containing galactose that supports slow growth, instead of glucose or glucose supplemented with sorbitol (27, 28, 31–34). These characteristics, especially the ability of overproduction of Rad27p to suppress deletion of DNA2 and the characteristics of the nuclease activity (29, 30, 32, 33), suggested a role for Dna2p in flap removal. Later studies revealed that eukaryotic single-stranded binding protein, replication protein A (RPA) also interacts with Dna2p genetically and physically on flap substrates (35). An RPA-coated flap intermediate inhibits FEN1 cleavage but stimulates Dna2p activity (35, 36), although another group has reported that RPA stimulates FEN1 up to 3-fold in yeast (37).

These observations led to proposal of the Dna2p/RPA/FEN1 model (35), in which the iRNA/DNA is displaced by pol δ into a long flap that becomes coated by RPA. The RPA prevents FEN1 from accessing the flap, but favors Dna2p cleavage. Dna2p shortens the flap so that it can no longer bind RPA. FEN1 can then remove the remaining flap to allow ligation (35). Supporting this series of events, the strand displacement activity of pol δ was shown to be robust at flap generation, but RPA binding limited the flap length to about 30 nucleotides (38). An RPA-coated flap of this length would be the ideal substrate for Dna2p cleavage.

A much less complex model can also lead to an intermediate in which upstream and downstream segments are separated by a nick suitable for ligation. In this case, FEN1 cleavage is assumed to be very efficient. As the flap is produced and before it can bind RPA, it is removed by one or more FEN1-directed cleavages (8). This latter model is consistent with previous results showing that reconstituted Okazaki fragment-processing reactions did not require Dna2p for fragment joining (10, 18, 39–41). A recent study by Burgers and colleagues (42) has shown that FEN1 and pol δ perform “nick translation” efficiently, in a similar manner as DNA polymerase I in prokaryotes. This process supports nearly 100% ligation during Okazaki fragment maturation (42). Addition of Dna2p did not alter this efficiency. Dna2p only aided the reaction when FEN1 activity was low and a long fixed flap was tested. The patch displaced by pol δ in this system was estimated to be 8–12 nucleotides, indicating that the flap intermediates are recognized and cut by FEN1 as soon as they are formed. Thus, a different model, the FEN1-only model, was proposed for the Okazaki fragment-processing pathway (42, 43). In this model, FEN1 is an ever-present component of the processing system, cleaving most of the flap intermediates. However, when FEN1 activity is impaired or low, such as after occasional formation of long flaps coated by RPA, a pathway involving Dna2p may be needed. FEN1 recognizes a free 5’-end of single-stranded DNA and traverses the flap to the duplex junction, the position of cleavage (36). Bound protein, some chemical modifications, and some folding structures of the flap will block cleavage by FEN1, allowing the flap to lengthen (15, 36, 44, 45).

All three models have supporting evidence. We propose that the most common pathway depends on the usual length of flap produced. A short flap will lead to the possibility of the FEN1-only or the RNase H/FEN1 pathways, but a long flap may require the Dna2p/RPA/FEN1 pathway. One approach to discerning the most common pathway has been to determine the length of the pol δ strand-displacement patch. However, this has suggested production of both long and short flaps (38, 42). Here we employ a different approach to evaluate these models by testing the intrinsic enzyme specificity and preferences on different model substrates that could be potentially created by pol δ.

This approach suggests that FEN1 is the predominant enzyme in processing most flaps. We also used substrate structure and cleavage specificity to analyze whether Dna2p could be beneficial when FEN1 activity is compromised or lowered. Results suggest that Dna2p could aid with cleavage of a subset of flaps containing foldbacks or repeat sequences. In this reaction, the helicase activity of Dna2p is critical, and the role of Dna2p during the maturation pathway is to process structured flaps.

**EXPERIMENTAL PROCEDURES**

**Materials**—Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). Radionucleotides (γ−32P)ATP (6000 Ci/mmol) and [α−32P]dCTP (6000 Ci/mmol) were from PerkinElmer Life Sciences (Boston, MA). The T4 polynucleotide kinase (labeling grade), the Klenow fragment of DNA polymerase I, and ATP were from Roche Molecular Biochemicals. RNase-free solutions and reagents were from Ambion, Inc. (Austin, TX). All other reagents were the best available commercial grade.

**Enzyme Expression and Purification**—S. cerevisiae Dna2p was cloned into the s9 baculovirus expression vector (Invitrogen, San Diego, CA). The amplification/expression and purification conditions were the same as described previously (31), except that High Five cells were utilized for the final expression step of the protein. S. cerevisiae FEN1 was expressed in Escherichia coli, and it was subsequently purified with the same conditions described elsewhere (14). S. cerevisiae RPA was expressed and purified according to Sibenaller et al. (46), but the Mono-Q column was omitted.

**Oligonucleotide Substrates**—Oligomer sequences are listed in Table 1, and they were annealed as described in the figure legends to form various structures, such as short fixed double-flap, long fixed double-flap, short equilibrating, long equilibrating, foldback, and CTG repeat substrates. For both short and long double-flap substrates, different lengths of the downstream flaps were formed by annealing different downstream primers to a template or annealing one downstream primer to multiple templates. Both foldback and repeat substrates were generated by including different foldback structures or CTG repeats on the downstream flaps. The upstream primer utilized in these fixed flap structures was the same oligomer containing one unannealed 3’-tail. The equilibrating substrates were generated by annealing the same downstream primer to different templates and upstream primers that contain 10-nucleotide or 30-nucleotide overlapping sequences to the downstream flaps. Annealing and labeling conditions were the same as described previously (47), except that the annealing ratio is 1:2:4 (downstream:template:upstream). All radiolabeled primers were purified by gel isolation from 15% or 18% polyacrylamide, 7 M urea denaturing gels.

The 10-nucleotide markers were obtained by Cleaved Exonuclease I digestion of the Okazaki fragment. The 30-nucleotide markers from Invitrogen with radionucleotides (γ−32P)ATP (6000 Ci/mmol, PerkinElmer Life Science) and heat-denatured prior to loading of the gel. The nucleotide ladders were obtained by digesting the 5’-labeled substrate oligonucleotide in a time course with snake venom phosphodiesterase (Warington, Frechold, NJ).

**Enzyme Assays**—The reaction mixture was performed in 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 0.25 mM bovine serum albumin, 30 mM NaCl, and various ratios of MgCl2 and ATP. Enzyme stocks were diluted in 50 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 10% glycerol, 0.1 mM MgCl2, and 0.02% Nonidet P-40. The “maximum nuclease condition” assays include excess MgCl2 over ATP (2 mM MgCl2 and 1 mM ATP), whereas the “maximum helicase condition” assays contain excess ATP over MgCl2 (1 mM MgCl2 and 2 mM ATP). Most assays were performed under these conditions, except for those in Fig. 1 (where 0.5 mM MgCl2 with and without 2 mM ATP was utilized), Fig. 2 (where MgCl2 was included but not ATP), and Fig. 5 (where a simple nuclease assay with or without ATP was employed). Each reaction contained 300 nM substrate in a 20-μl reaction mix with different amounts of the enzymes indicated under the figure legends. All the assays were incubated at 37 °C for 10 min and stopped by the addition of 20 µl of 2× termination dye (95% formamide (w/v) with bromphenol blue and xylene cyanole) except for maximum helicase assays and assays testing helicase activity of Dna2p (Figs. 1 and 5) in which 5-min preincubation with the enzyme, ATP, and substrates were employed prior to reaction. The maximum helicase reactions were initiated with MgCl2 at the end of the preincubation time. The denatured reactions were resolved on 15% polyacrylamide, 7 M urea denaturing gels. Each gel was quantitated using a PhosphorImager (Molec...
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### Table I

**Oligonucleotide sequences (5'-3')**

| Downstream primers<sup>a</sup><sup>b</sup> | D<sub>1</sub>, 34-mer | CCAAGCCACCGTCACCGACACCTCG
| D<sub>2</sub>, 40-mer | AGGTCCTGACATCCACCGTCAACCGACACCTCG
| D<sub>3</sub>, 55-mer | AGGTCCTGACATGGGATCTCGACTACCCCGTCAACCGACACCTCG
| D<sub>4</sub>, 61-mer | AGGTCCTGACATGGGATCTCGACTACCCCGTCAACCGACACCTCG
| D<sub>5</sub>, 69-mer | AGGTCCTGACATTATAGACACCCGAGTACGAGCTTGTTTCTCCACCGTCAACCGACACCTCG
| D<sub>6</sub>, 85-mer | AGGTCCTGAGCCCTGCTCATTATAGACACCGTCAACCGACACCTCG

| Upstream primers | U<sub>1</sub>, 26-mer | CGACCGTGCCAATTTCAATTTCAATTTACATGCTCAG
| U<sub>2</sub>, 35-mer | CGACCGTGCCAATTTCAATTTCAATTTACATGCTCAG
| U<sub>3</sub>, 55-mer | CGACCGTGCCAATTTCAATTTCAATTTACATGCTCAG

| Templates | T<sub>1</sub>, 54-mer | GCACCGTGCGCCAGCCTAAATTTCAATAGGTCTCGACTAACTCTAGTCGTTGTTCCA
| T<sub>2</sub>, 40-mer | AGGTCTCGACTACCACCCGTCCACCCGACGCCACCTCCTG
| T<sub>3</sub>, 55-mer | AGGTCTCGACTACCACCCGTCCACCCGACGCCACCTCCTG

<sup>a</sup> The bolded nucleotides are the flanking regions.

<sup>b</sup> The underlined nucleotides are the CTG repeats.

### RESULTS

Dna2p is a multifunctional enzyme with 5' → 3' helicase, 5'-nuclease, 3'-nuclease, and ATPase activities (29, 31, 33). In *vitro*, these activities can be differentially regulated by altering the ratio of MgCl<sub>2</sub> and ATP. The helicase activity is optimal when nuclease activity is reduced, and the nuclease activity is maximal when helicase activity is suppressed (31). We developed “maximum nuclease” (MgCl<sub>2</sub> > ATP) and “maximum helicase” (ATP > MgCl<sub>2</sub>) with a 5-min preincubation of the enzyme to move closer to the flap junction, generating 18-nucleotide to 24-nucleotide products (lanes 1, 10, 11–20, and 21–30, respectively). In a simple nuclease titration assay without ATP, Dna2p cleaved the 6-nucleotide substrate poorly (lanes 1–5). Both 12-nucleotide and 27-nucleotide substrates were cleaved well by Dna2p (lanes 11–15 and 21–25), but 27-nucleotide substrates were slightly preferred (compare lanes 15 and 25). Dna2p cleavage produced 2–18 nucleotide products from 5'-labeled substrates. There is a slight sequence dependence of cleavage by Dna2p, evident from different intensities of the different sized cleavage products (because a substrate containing unique sequences was tested instead of a poly-T tract). Dna2p was not able to cleave at the base of the flap. Therefore, between the two nucleases, only FEN1 can generate the nicked product to support the final ligation step (in the absence of other proteins).

### Under maximum helicase conditions (ATP > MgCl<sub>2</sub>)

The 5'-nuclease activity is reported to be stimulated up to 10-fold by RNA in low salt conditions (33). We were unable to observe this stimulation with our Dna2p (data not shown). Our results are consistent with another report that Dna2p acts poorly in the presence of RNA in a fully reconstituted reaction (42). A recent study with the Dna2p homologue from *Pyrococcus horikoshii* concluded that RNA inhibits both nuclease and helicase activities (49). RNA effects on Dna2p may be dependent on assay conditions or preparations of the enzyme. A detailed analysis of these parameters will be required to resolve this discrepancy.

We initially assessed the differences between the 5' and 3'-nuclease activities. We found that the 3'-nuclease activity is greatly reduced by addition of either RPA or ATP, whereas the 5'-nuclease activity is not affected by ATP and is stimulated by RPA (data not shown), suggesting that the 3'-nuclease is not biologically relevant. In fact, 5'-directional helicase movement is expected to drive Dna2p off of a 3'-flap. These considerations together with the proposed role of only the 5'-nuclease activity in Okazaki fragment processing (35) convinced us to focus further investigation on the 5' activity.

### Substrate Specificity of Dna2p—Even though Dna2p has been suggested to cleave long flaps in Okazaki fragment processing (35, 42), cleavage specificity of Dna2p on substrates containing different flap lengths was not been performed. Therefore, we examined the cleavage specificity of Dna2p on substrates that contain 6, 12, and 27 nucleotide flaps (Fig. 1, lanes 1–10, 11–20, and 21–30, respectively). In a simple nuclease titration assay without ATP, Dna2p cleaved the 6-nucleotide substrate poorly (lanes 1–5). Both 12-nucleotide and 27-nucleotide substrates were cleaved well by Dna2p (lanes 11–15 and 21–25), but 27-nucleotide substrates were slightly preferred (compare lanes 15 and 25). Dna2p cleavage produced 2–18 nucleotide products from 5'-labeled substrates. There is a slight sequence dependence of cleavage by Dna2p, evident from different intensities of the different sized cleavage products (because a substrate containing unique sequences was tested instead of a poly-T tract). Dna2p was not able to cleave at the base of the flap. Therefore, between the two nucleases, only FEN1 can generate the nicked product to support the final ligation step (in the absence of other proteins). Under maximum helicase conditions (ATP > MgCl<sub>2</sub>), the Dna2 nuclease activity on the 6-nucleotide substrates was completely suppressed (lanes 6–10). Under these conditions, the cleavage of 27-nucleotide substrates by Dna2p was 2-fold higher than that of 12-nucleotide substrates (compare lanes 20 and 30). Moreover, the helicase activity of Dna2p allows the enzyme to move closer to the flap junction, generating 18-nucleotide to 24-nucleotide products (lanes 28–30). Cleavage at that position requires the simultaneous action of both helicase and nuclease. Interestingly, this helicase-coupled nuclease activity is only observed with the 27-nucleotide substrate (lanes 26–30). In addition, PCNA inhibits Dna2p cleavage on 6-nucleotide substrates but has no effect on Dna2p cleavage on 27-nucleotide substrates (data not shown). These observations led us to conclude that substrates with flaps 27 or more nucleotides in length are preferred by Dna2p, a conclusion also implied by other studies (32, 33, 35).
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The Effect of RPA on Dna2p and FEN1 Cleavages—Because Dna2p prefers long flap substrates, and RPA binds long flaps and stimulates Dna2p, RPA was proposed to coordinate the two nucleases in processing the flaps (35). However, RPA has two binding modes, an 8-nucleotide binding mode (unstable) and a 30-nucleotide binding mode (stable) (50, 51). Whether or not the unstable 8-nucleotide binding mode is able to support this coordination between the two nucleases was unknown. To examine potential coordination, we measured the effect of RPA on the two nucleases with both short (10-nucleotide) and long (30-nucleotide) flap substrates (Fig. 2).

An RPA titration with Dna2p (lanes 3–8 and 17–22) or FEN1 (lanes 9–14 and 23–28) was employed in a simple nuclease assay (in the presence of 0.5 mM MgCl₂, without ATP). Consistently, cleavage by Dna2p only occurred within the flap, whereas FEN1 was capable of generating a nicked DNA for subsequent ligation. As reported before (35), RPA stimulated Dna2p cleavage of long flap substrates in a dose-dependent manner (lanes 17–22), and this stimulation was up to 5-fold (compare lanes 17 and 22). RPA inhibited FEN1up to 18-fold on this long flap substrate (lanes 23–28 and compare lanes 23 and 28). This inhibition relies on RPA coating of the flap and blocking the tracking mechanism of FEN1. FEN1 cleavage was unaffected by the presence of RPA on a 10-nucleotide substrate even though RPA could bind a 10-nucleotide flap via its 8-nucleotide binding mode (lanes 9–14). The rate of FEN1 cleavage on this shorter substrate was also not changed by RPA as determined in a time-course assay (data not shown). Dna2p, on the other hand, was slightly inhibited by RPA on a short flap substrate (lanes 3–8), further supporting the conclusion that a long flap is preferred. The stimulation of Dna2p by RPA requires stable RPA binding of flaps, which suggested the formation of a stable ternary complex among DNA, Dna2p, and RPA as the intermediate prior to cleavage. This complex has been detected in a gel-shift assay (35). Altogether, we conclude that, RPA coating of long flap substrates inhibits FEN1 but stimulates Dna2p and that FEN1 efficiently processes short flap substrates in the presence and absence of RPA.

Partially Reconstituted Reactions with Short Flap Substrates—To elucidate how the two nucleases process flap intermediates in the context of all three proteins, we employed a partially reconstituted reaction favoring maximum nuclease activity of Dna2p. Also, different enzyme ratios, including low enzyme activities (15 fmol of Dna2p and 0.05 fmol of FEN1 in lanes 1–7), high FEN1 activity (15 fmol of Dna2p and 2 fmol of FEN1 in lanes 8–14), or high Dna2p activity (100 fmol of Dna2p and 0.05 fmol of FEN1 in lanes 15–21), were tested in Fig. 3 and 4. We first used short flap substrates (Fig. 3A). In low enzyme reactions (Fig. 3A, lanes 1–7), FEN1 is the dominant nuclease. Dna2p fails to compete with FEN1 on this substrate, even when Dna2p is raised to high level of activity (15 fmol versus 100 fmol of Dna2p in a 20-μl reaction containing 5 fmol of substrates) (Fig. 3A, compare lanes 1–7 and 15–21).

A flap displaced by pol δ can be simulated by an equilibrating substrate. The 10-nucleotide equilibrating substrate that we employed has a 10-nucleotide overlapping region between the 3'-end of the upstream primer and the 5'-end of the downstream primer. As with the fixed flaps (Fig. 3A), FEN1 is much more effective on this substrate than Dna2p under all conditions tested (Fig. 3B). However, lower cleavage activity of equilibrating substrates was observed with both nucleases. The reason for this is that, at any point in the reaction, only a portion of the substrate molecules will possess the right configuration for Dna2p or FEN1 cleavage during the equilibrating process. A similar experiment with the equilibrating substrates was performed under maximum helicase conditions. The helicase function did not confer the ability on Dna2p to compete with FEN1 (data not shown). This analysis suggested that, on a short flap substrate, FEN1 is the overwhelmingly dominant nuclease. These properties are consistent with the FEN1-only mechanism in which FEN1 captures and cleaves the flap intermediates during nick translation as soon as they are formed (42).

Even though Dna2p and RPA have little influence on cleavage of short flaps, the presence of these proteins affects FEN1 cleavage specificity (compare lanes 10 and 14 in Fig. 3, A and B). In high FEN1 reactions, FEN1 alone displays extensive exonucleolytic cleavage (lane 10), but the additional presence of Dna2p, RPA, or both, inhibits this exonucleolytic cleavage (lane 14). This inhibition does not affect the endonucleolytic cleavage efficiency of FEN1 (95% cleavage versus 91% cleavage for fixed-flap substrates). Possibly the physical interaction between Dna2p and FEN1, and between RPA and the substrate, influence the specificity of FEN1. However, the detailed mechanism of how this occurs remains to be investigated.

Partially Reconstituted Reactions with Long Flap Substrates—Because the results from the short flap substrates suggest the FEN1-only model, we wanted to test whether long
flap substrates would support the Dna2p/RPA/FEN1 model as suggested previously (35, 42). We performed a partially reconstituted reaction with Dna2p, FEN1, and RPA on either fixed or equilibrating long flap substrates under maximum nuclease conditions (Fig. 4, A and B, respectively). At low enzyme levels (15 fmol of Dna2p and 0.05 fmol of FEN1 in lanes 1–7, Fig. 4A), we did not see stimulated FEN1 cleavage (i.e., increased nicked products) in the presence of Dna2p (compare lanes 5 and 7). According to the model, RPA inhibits FEN1 but stimulates Dna2p, and cleavage by Dna2p should have relieved the inhibition of FEN1 by RPA (35).

When FEN1 activity was high (15 fmol of Dna2p and 2 fmol of FEN1 in lanes 8–14), it thoroughly out-competed Dna2p (lanes 12–14), as with the short flap substrates (Fig. 3). This indicates that high FEN1 activity is capable of processing the long flap substrates, and this activity can even relieve the inhibition by RPA (compare lanes 3, 5, 10, and 12).

Even though Dna2p prefers long flaps (Fig. 2), it does not compete well with FEN1 as a nuclease. We were not able to observe RPA coordination of the two nucleases under most conditions favoring nuclease function. Even though short flaps were produced by Dna2p, they did not appear to be captured and cleaved well by FEN1 (lane 7). Interestingly, FEN1 competes well with Dna2p for all the flap substrates in the absence of RPA (lanes 6, 13, and 20), verifying that it is the presence of RPA but not the flap length that is inhibitory. The only situation in which we were able to show the coordination between the two nucleases by RPA and increased FEN1 cleavage by Dna2p was when Dna2p activity was high (lanes 15–21). Dna2p was then able to process most of the long flaps to short flaps, presumably no longer tightly bound by RPA. These short flaps then became favorable substrates for FEN1 (compare lanes 19 and 21). The reason why these Dna2p products were used by FEN1, but the ones made under low-Dna2p conditions were not, is obscure.

A similar trend was evident with the 30-nucleotide equilibrating substrates (Fig. 4B), but the percentage of the product formation was less compared to that with fixed substrates. Under maximum nuclease conditions, Dna2p reacts poorly with equilibrating substrates. We also tested the long equilibrating substrates under maximum helicase conditions, but as with 10-nucleotide equilibrating substrates, the helicase activity of Dna2p did not improve cleavage (data not shown). We then attempted to determine whether substrate specificity of the two nucleases would suggest that Dna2p helicase activity aids in processing flaps that become abnormally long, because they form secondary structures that resist FEN1 cleavage.

Dna2p Cleavage on Flaps Containing Foldback Structures—We first analyzed cleavage by Dna2p on foldback substrates in a simple nuclease assay in the presence and absence of ATP and/or RPA, as indicated under “Experimental Procedures” (Fig. 5A). A study of Dna2p cleavage on a 10-bp foldback with 20 thymines at the 5′-end had been reported, and the helicase activity of Dna2p facilitated cleavage on this substrate (52). We then tested the range of foldback stabilities over which this facilitation would occur. Substrates with 6, 10, and 18 bp at the 5′ terminus, representing weak, medium, and strong secondary structures on the flaps (lanes 7–12, 13–18, and 19–24), respectively, were employed. A reaction with FEN1 was included to define the position of cleavage at the base of the flap. High FEN1 was needed, because increasing length foldbacks are progressively more inhibitory to FEN1 cleavage (lanes 2, 8, 14, and 20) (45). Dna2p and FEN1 both effectively cleave a 30-nucleotide flap control (lanes 1–6). Addition of ATP allows Dna2p to cleave further into the flap regions, indicative of helicase activity (compare lanes 3 and 5 and lanes 4 and 6). We expected that Dna2p would be able to cleave foldback structures that were refractory to FEN1. Surprisingly, none of the foldback substrates allowed only Dna2p cleavage while...
continuing to exclude FEN1. The stronger the structure, the weaker cleavage activity was for either nuclease.

There are 18 nucleotides of single-stranded DNA between the base of the flap and the double strand of the foldback in these substrates. Neither nuclease was able to cleave by binding directly to the single-stranded region. This observation implies that Dna2p, like FEN1, requires a free 5'-end to recognize and load onto flap substrates for nuclease function (33, 36). Dna2p cleavage levels on foldback structures without a "b" region are not improved by the presence of ATP (Lanes 6–24). This was anticipated because the helicase activity of Dna2p also requires an unannealed 5'-end for loading (32). The strong structure (18-bp foldback) is disfavored for Dna2p cleavage. Virtually no cleavage is seen even in the presence of RPA (lanes 22 and 24). These results suggest that neither Dna2p nor FEN1 is designed to deal with fully annealed foldback structures (45).

When a fragment of 12 thymines was introduced into the 18-bp foldback structure (lanes 25–30), cleavage by FEN1 did not improve (compare lanes 20 and 26). On the other hand, Dna2p was able to process this strong secondary structure at low efficiency when ATP and/or RPA were included (lanes 29 and 30). This is significant in that it indicates that the helicase function helps processing foldbacks containing the "b" region that allow Dna2p loading (compare lanes 11, 17, 23, and 29). However, the low cleavage efficiency, in this case 22 and 16% (lanes 29 and 30, respectively), is consistent with the previously reported weak helicase activity of Dna2p (32).
Dna2p Cleavage on Flaps Containing CTG repeats—Because Dna2p cleaves some types of foldbacks, we also analyzed cleavage on CTG repeat substrates (Fig. 5B). Such sequences appear naturally, and their self-complementarities allow them to fold back. A simple nuclease assay with or without ATP was performed as in Fig. 5A. A high FEN1 reaction (0.75 fmol of FEN1) was included as the control for the cleavage at the base of the flap (lanes 2, 8, 14, 20, and 26). 100 fmol of RPA and 100 fmol of Dna2p were utilized in this assay. Lanes 1, 7, 13, 19, and 25 are the substrate-only controls. Lanes 1–6 contain a 5′-P32-radiolabeled 30-nucleotide flap control (D5:T2:U1). 6-, 10-, 18-, and 12T-18-bp foldback substrates were tested in lanes 7–12, 13–18, 19–24, and 25–30, respectively (D6:T2:U1, D10:T2:U1, D18:T2:U1, and D12T:T2:U1), and the schematic representation of the structure is depicted on the top of the gel. The symbol “a” represents the number of base pairs on the foldbacks, and “b” is the number of the unannealed nucleotides 5′ to the base pair region on the folded flap. There are 18 nucleotides between the foldback structure and the base of the flap, as indicated in the figure. A 10-nucleotide marker series and nicked products are indicated on the left and right sides of the gel. The 56-nucleotide, 44-nucleotide, 56-nucleotide, 86-nucleotide, and 98-nucleotide bands are the starting substrates. FEN1 cleavage at the base of the flap generates the 26-nucleotide product. Dna2p cleavages are mostly between the 5′-end and base of the flap. B, the assay conditions and experimental protocols are similar to part A of this figure, except for the substrates tested. Lanes 1–6, 7–12, 13–18, and 19–24 are the 27-nucleotide flap control (D4:T2:U1), 5 CTG-containing flap (D5:T2:U1), 10 CTG-containing flap (D10:T2:U1), and 12T-10 CTG-containing flap (D10:T2:U1) substrates, respectively. The symbol “a” represents the structure of the flap on each substrate. A 10-nucleotide marker and nicked products are indicated on the left and right sides of the gel. The 56-nucleotide, 44-nucleotide, 56-nucleotide, and 68-nucleotide bands on each set of reactions are the starting substrates. The 29-nucleotide product derives from cleavage at the base of flap by FEN1.

To further define the role of Dna2p helicase in Okazaki fragment processing, we tested the model substrate with a stable foldback (18 bp) and (CTG)10 in the presence of FEN1, RPA, and high Dna2p (Fig. 6). We performed these partially reconstituted reactions under both maximum helicase and maximum nuclease conditions. If the

FIG. 5. Helicase activity of Dna2p allows the cleavage of flaps containing strong secondary structures. A, a simple nuclease reaction (1 mM MgCl$_2$) was performed in the absence and presence of RPA and/or 2 mM ATP (indicated on top of the gel). The assay conditions were as described under “Experimental Procedures.” A high FEN1 reaction (0.75 fmol of FEN1) was included as the control for the cleavage at the base of the flap (lanes 2, 8, 14, 20, and 26). 100 fmol of RPA and 100 fmol of Dna2p were utilized in this assay. Lanes 1, 7, 13, 19, and 25 are the substrate-only controls. Lanes 1–6 contain a 3′-P32-radiolabeled 30-nucleotide flap control (D5:T2:U1). 6-, 10-, 18-, and 12T-18-bp foldback substrates were tested in lanes 7–12, 13–18, 19–24, and 25–30, respectively (D6:T2:U1, D10:T2:U1, D18:T2:U1, and D12T:T2:U1), and the schematic representation of the structure is depicted on the top of the gel. The symbol “a” represents the number of base pairs on the foldbacks, and “b” is the number of the unannealed nucleotides 5′ to the base pair region on the folded flap. There are 18 nucleotides between the foldback structure and the base of the flap, as indicated in the figure. A 10-nucleotide marker series and nicked products are indicated on the left and right sides of the gel. The 56-nucleotide, 44-nucleotide, 56-nucleotide, 86-nucleotide, and 98-nucleotide bands are the starting substrates. FEN1 cleavage at the base of the flap generates the 26-nucleotide product. Dna2p cleavages are mostly between the 5′-end and base of the flap. B, the assay conditions and experimental protocols are similar to part A of this figure, except for the substrates tested. Lanes 1–6, 7–12, 13–18, and 19–24 are the 27-nucleotide flap control (D4:T2:U1), 5 CTG-containing flap (D5:T2:U1), 10 CTG-containing flap (D10:T2:U1), and 12T-10 CTG-containing flap (D10:T2:U1) substrates, respectively. The symbol “a” represents the structure of the flap on each substrate. A 10-nucleotide marker and nicked products are indicated on the left and right sides of the gel. The 56-nucleotide, 44-nucleotide, 56-nucleotide, and 68-nucleotide bands on each set of reactions are the starting substrates. The 29-nucleotide product derives from cleavage at the base of flap by FEN1.
helicase activity of Dna2p were critical in processing abnormal flaps containing structures during Okazaki fragment maturation, we only expected to see RPA coordination and increased FEN1 products in the presence of both Dna2p and RPA under the maximum helicase conditions on these substrates.

When an 18-bp foldback substrate was utilized in a reconstituted maximum helicase condition assay (Fig. 6A), presence of the 12T fragment in the structure allowed cleavage within the foldback region by Dna2p (lanes 15–21). This generated short flap substrates favored and cleaved by FEN1 (compare lanes 18 and 21).

The presence of Dna2p stimulated nicked product formation up to 5-fold (compare lanes 19 and 21). Dna2p was not able to stimulate nicked product formation on the substrate without the T-tailed free 5’-end (compare lanes 12 and 14). In the same assay but under maximum nuclease conditions, there was no Dna2p-dependent increase of FEN1 cleavage (data not shown). A 30-nucleotide unstructured flap control (lanes 1–7) also shows Dna2p-dependent stimulation of FEN1 cleavage. This is similar to the stimulation seen with this substrate under maximum nuclease conditions in Fig. 4A (lanes 15–21), except that the stimulation of FEN1 is greater (20-fold in lanes 5 and 7 of Fig. 6A and 2-fold in lanes 19 and 21 of Fig. 4A). This could stem from efficient loading of Dna2p under maximum helicase conditions onto a fixed long flap substrate thereby facilitating cleavage.

We also employed (CTG)_{10} substrates in a partially reconstituted maximum helicase assay (Fig. 6B). With a 27-nucleotide unstructured flap control (lanes 1–7), we continued to observe increased FEN1 cleavage in the presence of Dna2p (compare lanes 5 and 7). The 6-fold stimulation was also greater than the 2-fold stimulation in the maximum nuclease assay in Fig. 4A (lanes 15–21). (CTG)_{10} substrates were cleaved poorly by both nucleases (lanes 8–14), whereas 12T-(CTG)_{10} substrates enabled Dna2p to utilize both helicase and nucleases to process the flaps (lanes 15–21). FEN1 cleaved the 12T-(CTG)_{10} substrate with slightly higher activity (compare lanes 10 and 17). The presence of Dna2p greatly stimulated FEN1 cleavage (compare lanes 19 and 21), up to 10-fold. A similar experiment under maximum nuclease conditions did not show this strong Dna2p-dependent stimulation of FEN1 (data not shown), indicating that the helicase function of Dna2p is critical for the stimulation.

**DISCUSSION**

We have examined the substrate specificity of Dna2p alone (Fig. 1) and in partially reconstituted Okazaki fragment processing reactions in an attempt to define the role of Dna2p in DNA replication. Analyzing the creation and cleavage of flaps during synthesis by DNA polymerase δ and FEN1, Burgers and colleagues (42, 43) concluded that FEN1 can effectively cleave nascent flaps before they become long enough for stable binding of RPA. Our analysis of cleavage of 10-nucleotide fixed flaps supports this conclusion. FEN1 is very effective at cleaving short flaps. The presence of RPA, which can bind the short flaps with low affinity (50, 51), has no effect on FEN1 cleavage efficiency (Fig. 3A). The presence of Dna2p at either low or high activity levels is slightly inhibitory to the FEN1 cleavage reaction on short flaps.

The nature of flaps generated by strand-displacement synthesis is that they have overlapping single-stranded regions that competitively bind to the template. This allows for an equilibration process in which many structures can form. Only one of these structures is the favored double-flap cleavage substrate of FEN1 (14). Because of this, FEN1 cleavage activity is lower on equilibrating flaps. We considered the possibility that Dna2p aids FEN1 in cleavage of equilibrating flap structures, but this was not the case. With the short equilibrating
substrate, the presence of RPA was slightly inhibitory to FEN1 cleavage. This could be explained by the fact that RPA preferentially binds to the 3'-flaps (53) and may favor the formation of 3'-flaps during the equilibrating process. The addition of Dna2p further enhanced this inhibition (Fig. 3B).

Together these observations suggest that FEN1 has evolved to rapidly cleave flaps as they are formed. This idea is consistent with the ability of FEN1 to cleave as an RNase within the initiator RNA of a flap (12). The presence of such an activity suggests that FEN1 acts even before any of the DNA beyond the RNA primer is displaced. This reaction may occur multiple times to completely remove the initiator primer. The facts that Dna2p is a deoxyribonuclease but not a ribonuclease, and cannot cleave near the flap base suggest that it acts only on extensively displaced flaps (32, 33, 35). We have not performed a rigorous kinetic analysis of the relative efficiencies of cleavage by FEN1 and Dna2p. Nevertheless, FEN1 appears as the distinctly superior nuclease for flap processing based on three properties. First, the same rate of depletion of starting substrate requires a higher concentration of Dna2p. Second, when both enzymes are present in one reaction with either similar activity or high Dna2p activity, only FEN1 products are observed, indicating that FEN1 is able to consume all the substrates before Dna2p does (lanes 6 and 20 in Figs. 3A, 3B, 4A, and 4B). Finally, the cleavage specificity of FEN1 immediately produces the desired nicked products, whereas Dna2p produces an array of intermediate length products.

Dna2p had been reported to cleave long single-stranded DNA (32, 33) (Fig. 1), and its activity was highest when the DNA strands were coated by RPA (33, 35, 52). On flap substrates, stable coating by RPA inhibited FEN1 but stimulated Dna2p cleavage (35, 36) (Fig. 2), which is the key observation leading to the Dna2p/RPA/FEN1 model (35). Similarly we observed that the efficient FEN1-directed cleavage of a 30-nucleotide flap substrate was compromised by RPA. Our results are consistent with the interpretation that Dna2p shortens the flaps so that RPA binding would be destabilized (35). This creates a better substrate for loading and cleavage by FEN1. However, under maximum nuclease conditions, which simulate the relative concentrations of Mg\(^{2+}\) and ATP in cells, stimulation of FEN1 was not significant until the Dna2p activity was raised to higher levels (Fig. 4A). Similar results were obtained with a 30-nucleotide equilibrating flap substrate (Fig. 4B).

It has been speculated that Dna2p may only be employed when structures form in flaps that inhibit FEN1 and allow the flaps to become long (42). Analysis of fully double-stranded foldback structures in flaps revealed that with increasing length, these become refractory to Dna2p cleavage under every tested condition. However, if the foldback region has a single-stranded 5'-extension and the Dna2p operates under maximum helicase conditions, substantial flap cleavage can occur (Figs. 5A and 6A).

We did not observe RPA facilitation of Dna2p cleavage on the 12T-foldback substrates, even though it had been previously observed (52). The most likely explanation is that our substrate had a very stable 18-bp complementarity. This structure may not have responded to the DNA unwinding activity of RPA but instead required the helicase activity of Dna2p.

CTG repeats are one of the commonly found repetitive sequences in human genomes, and they have attracted attention because of their links with genetic conditions termed human triplet repeat disorders (54). They can form a variety of structures, including foldbacks (55). Triplet repeat flaps behaved in a similar manner as the foldback substrates in our assays (Fig. 5B). A (CTG\(_{10}\)) flap was strongly inhibitory to FEN1 cleavage. FEN1 could be stimulated by Dna2p but only if a T-tail was appended onto the 5'-end of the triplet repeat flap (Fig. 6B).

These results emphasize that the helicase function of Dna2p is necessary for stimulation of FEN1 cleavage on both structured and unstructured long flaps. This contention is consistent with genetic data showing that Dna2p with a helicase defect can only grow on media containing galactose that supports slow growth, instead of glucose, or glucose media supplemented with sorbitol (28, 34, 52). Although the helicase function is not essential, these observations are an indication of its importance in DNA replication. The inefficient cleavage of strong foldback substrates, even under the most favorable conditions for Dna2p function, and the requirement for a free 5'-end on the structured flap implies that another helicase or factor may be needed besides Dna2p to optimize the processing of these structured substrates. Significantly, the Bloom syndrome helicase interacts with Dna2p both genetically and physically (23) and may supplement Dna2p helicase functions. In addition, a helicase-deficient strain, dna2-2, is synthetically lethal with a null helicase mutant strain, rrm\(_A\), lacking a helicase that acts in ribosomal DNA replication (56). These
results suggest that systems employed to date in vitro lack components that would augment the capacity of Dna2p to promote FEN1 cleavage of structured flaps.

Our work suggests the following model for the role of Dna2p in DNA replication (Fig. 7). During Okazaki fragment processing, short flaps are the likely intermediates. Pol δ strand displaces the rRNA/DNA into an 8- to 12-nucleotide flap (42). This flap will be processed by FEN1 and supports the subsequent ligation by DNA ligase I and PCNA (14, 15, 57). FEN1 can also cleave into the initiator DNA region synthesized by pol α and is more active in that region if there are any mismatched nucleotides (58). However, under certain conditions, i.e. synthesis across a repeat region, the flap may escape from FEN1 cleavage and become long. In this case, Dna2p and/or another helicase or factor may be needed to resolve the intermediate into a short flap that will support subsequent events to complete the pathway.

The Dna2p/RPA/FEN1 model (35) is an interpretation of the ordered cleavage of long flaps coated by RPA, first by Dna2p, which can interact with the RPA-coated strand, and then by FEN1, which cleaves the remaining bare strand. These specificities hold true for all long flaps irrespective of the presence of the secondary structures, inviting the conclusion that two nucleases are used in the primary pathway for Okazaki fragment processing. However, our results suggest that unstructured long flaps are virtually never generated because of efficient FEN1 cleavage of their short precursors. Instead, only structured flaps survive to become long enough to experience the RPA coating that forces the ordered cleavage by the two nucleases.

Genetic studies in yeast have revealed only a 2- to 3-fold increase in both dinucleotide instability and trinucleotide fragility (CAG-155 tract) in dna2-1 mutants (30, 59). Assuming that repeat sequence expansion occurs via the mechanism of unresolved flap intermediates, these results suggest that Dna2p is not involved in the processing of most Okazaki fragments. The fact that FEN1 null mutants exhibit much higher -fold increase in genomic instability indicates that short flaps are the major intermediates in vivo, and they are processed by FEN1. A failure of FEN1 would then be expected to have a more profound impact on sequence stability. This interpretation is consistent with our biochemical analysis and the fully reconstituted reactions reported earlier (42). The -fold increase of genomic instability measured in dna2-1 mutant should be an indirect indication of the proportion of flaps that escape FEN1 cleavage and become long enough to require the Dna2p/RPA/FEN1 pathway. This -fold increase implies that long flaps are formed within a small population even of structured flap intermediates, and the role of Dna2p in replication is to rescue these occasional flaps by utilizing its helicase and nuclease activities.

Even though we favor the FEN1-only model as the primary pathway, our conclusion does not rule out the possibility of an Rnase H/FEN1 pathway. Rnase H may act much earlier in the process before the strand-displacement synthesis of pol δ, and it is not a rate-limiting step. Most likely, it co-exists with the FEN1-only pathway to make rRNA/DNA removal more efficient.

Our data suggest that the essential role of Dna2p is not in replication, but it is rather an auxiliary pathway for FEN1, because Dna2p rescues the maturation process when FEN1 level is low in reconstituted reactions (42). If Dna2p plays an occasional role in replication, why do Dna2p and FEN1 interact with each other genetically and physically? What makes Dna2p essential? The synthetic lethality suggests that Dna2p and FEN1 are working on the same type of structure in vivo and that structure is suggested by our results. The two proteins might be in parallel pathways or perform parallel functions in the same pathway, because our results do not necessarily support a sequential function. For example in Fig. 4, shortened flaps made by Dna2p did not appear to be captured by FEN1 in some conditions. Perhaps besides processing structured flaps with FEN1, Dna2p carries out another role of regulating FEN1 exonuclease activity. We observed that the presence of Dna2p suppresses exonuclease activity of FEN1, preventing extensive degradation of an annealed fragment (Figs. 3 and 4). In addition, the role of Dna2p may be in some type of gap repair. Other studies suggest that Dna2p participates in telomere replication, stalled replication fork rescue, recombination, double-strand break repair, and destabilization of minisatellites in the genome (30, 56, 60, 61). We have observed that Dna2p nuclease also prefers double-flap structures with both a long 5′-flap and long 3′-tail that are 18 nucleotides in length. Dna2p mutants also exhibit sensitivity to ionizing radiation, and it colocalizes with Sir3p at the telomere (30, 61). Upon double strand break induction, Ku and Dna2p mobilize throughout cells, which implies the participation in double strand break repair (30, 61). Dna2p biology is still evolving, and many more questions still remain to be investigated to understand its roles in other DNA metabolic pathways.

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On the Roles of *Saccharomyces cerevisiae* Dna2p and Flap Endonuclease 1 in Okazaki Fragment Processing
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