Characterization of the Enzymatic Properties of the Yeast Dna2 Helicase/Endonuclease Suggests a New Model for Okazaki Fragment Processing*

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The Saccharomyces cerevisiae Dna2, which contains single-stranded DNA-specific endonuclease activity, interacts genetically and physically with Fen-1, a structure-specific endonuclease implicated in Okazaki fragment maturation during lagging strand synthesis. In this report, we investigated the properties of the Dna2 helicase/endonuclease activities in search of their in vitro physiological functions in eukaryotes. We found that the Dna2 helicase activity translocates in the 5′ to 3′ direction and uses DNA with free ends as the preferred substrate. Furthermore, the endonucleolytic cleavage activity of Dna2 was markedly stimulated by the presence of an RNA segment at the 5′-end of single-stranded DNA and occurred within the DNA, ensuring the complete removal of the initiator RNA segment on the Okazaki fragment. In addition, we demonstrated that the removal of a pre-existing initiator 5′-terminal RNA segments depends on a displacement reaction carried out during the DNA polymerase β-catalyzed elongation of the upstream Okazaki fragments. These properties indicate that Dna2 is well suited to remove the primer RNA on the Okazaki fragment. Based on this information, we propose a new model in which Dna2 plays a direct role in Okazaki fragment maturation in conjunction with Fen-1.

Biochemical and genetic studies of DNA replication in viruses and lower eukaryotes have contributed substantially to our understanding of eukaryotic DNA synthesis (1–5). The various steps in eukaryotic DNA synthesis are basically similar to those in prokaryotes, requiring many common enzymatic functions. Despite these striking similarities, certain eukaryotic replication components differ from the prokaryotic counterparts. Most noteworthy is that three essential DNA polymerases (pol) α, δ, and ε are required for DNA replication in eukaryotes (5–7). In contrast, pol III is the only replicative enzyme in prokaryotes involved in DNA synthesis. Unlike its prokaryotic counterpart, the eukaryotic primase is complexed with DNA pol α. Thus, the role of the pol α-primase complex appears to function solely in the synthesis of a short RNA-DNA primer (referred to as primer DNA in this paper). The primer DNA are then utilized by pol δ for the initiation of leading strand synthesis and by pol δ or pol ε for each Okazaki fragment synthesis for lagging strand DNA replication.

The short and discontinuous Okazaki fragments at replication forks are processed and then joined to generate a continuous DNA strand through a series of complex enzymatic reactions that require a number of enzymes that include a 5′- to 3′-exonuclease activity for Okazaki fragment processing, unlike the well characterized prokaryotic polymerase, Escherichia coli DNA pol I (1). In the current model of replication in eukaryotes, Fen-1 provides the 5′- to 3′-exonuclease activity, and with the assistance of RNase HI removes the RNA segments on Okazaki fragments (9–11). RNase HI hydrolyzes the initiator RNA of the primer DNA leaving a single ribonucleotide at the RNA-DNA junction, which is subsequently removed by the 5′- to 3′-exonuclease activity of Fen-1. In mammalian cells, DNA pol δ, replication protein-A (RPA), proliferating cell nuclear antigen (PCNA), replication factor-C (RFC), RNase HI, Fen-1, and DNA ligase I are necessary and sufficient to reconstitute lagging strand synthesis in vitro (11). Recently, it was shown that Fen-1 contains a structure-specific endonuclease activity that cleaves the 5′-unannealed single-stranded (ss) DNA or RNA at the duplex junction (8, 12–14). In addition, mammalian RNase HI can cleave 5′ of the last ribonucleotide of ssRNA-DNA hybrid molecules (15). These findings suggest that Okazaki fragment maturation could occur through a more complex process than inferred previously. For example, Okazaki fragment maturation is likely to require formation of a 5′-tail prior to the action of Fen-1 and/or RNase HI, a process that is poorly understood at present.

Although the role of Fen-1 and RNase HI in Okazaki fragment maturation has been well established in vitro, the phenotypes observed in Saccharomyces cerevisiae strains lacking a gene for the yeast Fen-1 (yFen-1) or RNase HI (RAD27/RTH1 or RNH35, respectively) reveal that, unlike other genes whose products are essential for DNA replication, RAD27 and RNH35 are dispensable. RNase HI is a two-subunit enzyme (16), and the deletion of its catalytic subunit did not affect cell viability (17). Yeast strains carrying a deletion of RAD27 are viable at 30 °C and compromised for growth at 37 °C, producing a yeast strain with a terminally arrested phenotype, an indication of a defect in DNA replication (18, 19). At 30 °C, this strain shows elevated rates of spontaneous mutation, mitotic recombination, and chromosome loss (19–22), consistent with its critical roles...
in chromosome maintenance. In support of these findings, Fen-1 has been shown to participate in long patch base excision repair in human cells (23–27), maintenance of direct repeats (22) or CTG trinucleotide repeats (28), nonhomologous DNA end joining (29), and mitotic gene conversion (30) in yeasts. Therefore, the viability of the RAD27-deleted yeast mutant strain is puzzling if yFen-1 is the only enzyme that plays such a crucial role in the completion of DNA replication. This is especially true considering that deletion from the yeast genome of the DNA ligase I gene, which is required for the joining of Okazaki fragments processed by Fen-1/RNase H1, renders cells inviable (31). Thus, the viability of yeast cells lacking Fen-1 argues strongly for the existence of a more critical pathway for the in vivo processing of Okazaki fragments.

Although Dna2 has been implicated in Okazaki fragment maturation on the basis of its genetic and physical interaction with yFen-1 (32), its precise role in DNA replication remains unclear. Recently, we reported that Dna2 of S. cerevisiae contains a potent ssDNA-specific endonuclease activity (33). In an attempt to define the role of Dna2 in yeast DNA replication, we have carried out genetic and biochemical studies to examine whether Dna2 participated in the maturation of Okazaki fragments. In this paper, we present evidence that Dna2 is well suited to remove completely the primer RNA on Okazaki fragments. Although Dna2 has been implicated in Okazaki fragment maturation on the basis of its genetic and physical interaction with yFen-1 (32), its precise role in DNA replication remains unclear.

### EXPERIMENTAL PROCEDURES

**Oligonucleotides, DNA, and Nucleoside Triphosphates—**All oligonucleotides used for the construction of various DNA and RNA substrates were synthesized commercially (Life Technologies, Inc., and Gemini Biotech) and gel-purified prior to use. The sequences of the oligonucleotide used in this study are listed in Table I. Oligonucleotides used to prepare substrates, the position of radioisotopic labels in the substrates, and the substrate structures are described in each figure. The oligonucleotides 2 and 12 (Table I) were complementary to dX174 ssDNA (New England Biolabs) at nucleotides 702–753 and 980–1006, respectively. Nucleoside triphosphates were obtained from Roche Molecular Biochemicals and [γ-32P]ATP (>5000 Ci/mmol), [α-32P]dCTP (>5000 Ci/mmol), and [α-32P]ddATP (>6000 Ci/mmol) were purchased from Amersham Pharmacia Biotech.

**Proteins and Enzymes—**The following proteins were obtained commercially: restriction endonuclease HpaII, T4 polynucleotide kinase, and an terminal transferase were from New England Biolabs. Streptavidin- bovine serum albumin, and 15 fmol of substrate (33) unless otherwise indicated. When necessary, ATP and/or Mg2+ were included as indicated.

**RESULTS**

### Dna2 Translocates in the 5’ to 3’ Direction—**Early efforts to establish the polarity of translocation of the Dna2 helicase activity were hampered by its intrinsic endonuclease and weak helicase activities. We determined the DNA unwinding polarity of Dna2 by blocking the nuclease activity through high ratios of ATP to Mg2+ (33) and by using a partial duplex DNA substrate that consisted of linear dX174 ssDNA containing a 29-nucleotide (nt) fragment at the 3’-end and a 23-nt fragment

### Enzyme Assays—**Standard reaction mixtures (20 µl) contained 50 mM Tris-HCl (pH 7.8), 2.0 mM dithiothreitol, 0.25 mg/ml bovine serum albumin, and 15 fmol of substrate (33) unless otherwise indicated. When necessary, ATP and/or Mg2+ were included as indicated.
at the 5′-end (Fig. 1). Due to its limited unwinding activity, the HX-Dna2 protein failed to produce ssDNA from the 52-base pair duplex DNA substrate (data not shown). In contrast, the enzyme displaced a shorter duplex region in the presence of ATP (Fig. 1, lanes 4–6). HX-Dna2 displaced the 29-nt fragment at the 3′-end but not the 23-nt fragment at the 5′-end in the presence (+) or absence (−) of 2 mM ATP. The reactions were terminated, and the products were analyzed on 12% polyacrylamide gel as described (33). S and B denote substrate alone and boiled substrate controls, respectively. The arrows indicate the positions where the labeled oligonucleotides (23- and 29-nt) migrated. An open arrowhead indicates the migration position of the uncleaved oligonucleotide 1 (52-nt, Table I). The closed arrowhead denotes the migration position of cleavage products, 2–8 nt in length, containing the 3′-end label, which were formed only in the presence of ATP (33). The amount of substrate unwound and 3′-end label released (closed arrowhead) was measured with the use of a PhosphorImager (Molecular Dynamics), and the results are presented at the bottom of the figure.

**Fig. 1. Directionality of Dna2 helicase unwinding of duplex DNA substrates.** The structures of the linearized φX174 partial duplex substrates are shown at the top of the figure. The asterisks indicate 32P-labeled ends. The indicated amount of Dna2 in a 20-μl reaction mixture containing 1 mM MgCl2 was incubated with 15 fmol of either 3′-end- or 5′-end-labeled substrate at 37 °C for 10 min in the presence (+) or absence (−) of 2 mM ATP. The reactions were terminated, and the products were analyzed on 12% polyacrylamide gel as described (33). S and B denote substrate alone and boiled substrate controls, respectively. The arrows indicate the positions where the labeled oligonucleotides (23- and 29-nt) migrated. An open arrowhead indicates the migration position of the uncleaved oligonucleotide 1 (52-nt, Table I). The closed arrowhead denotes the migration position of cleavage products, 2–8 nt in length, containing the 3′-end label, which were formed only in the presence of ATP (33). The amount of substrate unwound and 3′-end label released (closed arrowhead) was measured with the use of a PhosphorImager (Molecular Dynamics), and the results are presented at the bottom of the figure.

**Enzymatic Role of Dna2 in Okazaki Fragment Maturation**

The enzyme degraded the 5′-ssDNA tails most efficiently. The amount of the 5′-tail (69 nt) substrate cleaved with 0.5 ng of HX-Dna2 was 10.4 fmol (Fig. 2A, lane 4), whereas the amount of 3′-tail (69 nt) substrate cleaved with the same amount of enzyme was slightly reduced (7.2 fmol) (Fig. 2A, lane 10). We estimated that the efficiency of cleavage of the 5′-tail substrate by HX-Dna2 was about 2-fold greater than that of the 3′-tail (Fig. 2, lanes 5 and 12). In contrast, cleavage of internal ssDNA regions flanked by partial duplexes at both ends was extremely poor (Fig. 2A, lanes 16–18), requiring much higher enzyme levels (>200-fold) to accumulate comparable amounts of products. The addition of 100 ng of HX-Dna2 to the reaction resulted in the cleavage of 9.3 fmol of the substrate with partial duplexes at both ends (Fig. 2A, lane 18). This is not because the internal 48-nt ssDNA between the two partial duplexes is too short for Dna2 to utilize. The labeled, but unannealed, 21-nt ssDNA present as background in the substrate-alone control (Fig. 2A, lanes 13 and 15) was degraded efficiently by HX-Dna2 to yield faster migrating products, indicated by a closed arrowhead (Fig. 2A, lanes 16–18; see also Figs. 3 and 4). We repeated this in an identical way but analyzed the reaction products formed using a high resolution sequencing gel. As shown in Fig. 2B, the cleavage products that migrated as a uniform band in the low resolution gel (Fig. 2A) consisted of oligonucleotides of varying sizes that were separated on the high resolution sequencing gel. In addition, we noted that Dna2 cleaved the 5′-overhang ssDNA more efficiently than the 3′-overhang DNA justifying from the amount of substrate uncleaved (Fig. 2B), in keeping with the observations described in Fig. 2A. These results demonstrate that Dna2 prefers to cleave ssDNA with free ends to the internal ssDNA regions flanked by duplex regions.

This finding suggests that Dna2 binds to or uses free ssDNA ends as an entry site for its subsequent endonucleolytic action. This prompted us to examine whether modification of the ends of ssDNA affected the DNA-dependent ATPase activity of Dna2. For this purpose, we used 5′- or 3′-biotinylated oligonucleotides coupled to streptavidin in order to block access of Dna2 to the ends of ssDNA substrate (Fig. 2C). In control experiments, the addition of free streptavidin to reaction mixtures containing nonbiotinylated oligonucleotides did not affect ATP hydrolysis by Dna2 (Fig. 2C, open squares). However, in the presence of the 5′-biotinylated oligonucleotide, ATPase activity was inhibited partially by the addition of streptavidin (Fig. 2C, open circles). Due to the relatively slight difference in activity, this experiment was repeated four times, and the average of the results obtained are presented with error bars that represent the standard deviation about the mean. Inhibition was greatest (2–2.5-fold lower than control values) at >50 ng of streptavidin, which saturated ≥1 pmol of the oligonucleotide substrate used. In contrast, the binding of streptavidin at the 3′-end of the oligonucleotide stimulated the ATPase activity of HX-Dna2 nearly 2-fold over control values (Fig. 2C, closed circles). ATP hydrolysis by HX-Dna2 was inhibited when we created a partial duplex at the 5′-end of the ssDNA template but stimulated by a partial duplex at the 3′-end (data not shown). If this is not due to the fact that the partial duplex present at the 3′-end of the ssDNA effector is not really a large bulky complex, one alternative explanation for the increased ATP hydrolysis is the inability of Dna2 to interact with a DNA duplex. Since Dna2 does not utilize double-stranded DNA as an effector for ATP hydrolysis (data not shown), it is likely that the presence of a partial duplex at the 3′-end causes Dna2 to interact more efficiently with the available 5′-ssDNA end, resulting in the increased ATP hydrolysis. We conclude that the
binding of streptavidin to the 5’-end or the presence of a partial duplex at the 5’-end blocks the entry of Dna2 onto the DNA substrate, thus decreasing ATP hydrolysis. On the other hand, formation of a large complex with streptavidin or a partial duplex structure at the 3’-end of the template could increase the effective concentration of available 5’-ends of ssDNA, thereby stimulating the ATPase activity of Dna2. This notion is consistent with the observations that the enzyme prefers free 5’-ends of ssDNA as substrate (Fig. 2 A) and that Dna2 translocates in the 5’ to 3’ direction (Fig. 1).

**ATP Alters Cleavage of the 5’-ssDNA Tail by Dna2 but Not the 3’-End ssDNA Tail**—In order to investigate further whether Dna2 cleaves 5’-ssDNA tails more efficiently than 3’-ssDNA tails in the presence of ATP, we prepared a Y-structured partial duplex substrate that contained 5’- and 3’-oligo(dT) tails. Only one of the two tails present in the Y-structured substrate was labeled as shown in Fig. 3. In order to follow only the fate of labeled strands, we used a limited amount (3 fmol) of HX-Dna2 in the presence of excess substrate (15 fmol) and preincubated the enzyme with substrates in the absence of Mg\(^{2+}\) to allow only the binding events. Under this condition, it was expected that most of the enzyme would be bound to the substrate and, therefore, the addition of Mg\(^{2+}\) would cause initial cleavage of only the Dna2-bound strand. We first examined the products generated by the action of HX-Dna2 with the 5’-end-labeled substrate, either in the absence or presence of ATP. The DNA cleavage reaction was completely dependent on the addition of Mg\(^{2+}\) (Fig. 3A, lane 1). In the absence of ATP, the 5’-tail of the substrate was cleaved, yielding predominantly oligonucleotides that were 7–12 nt in length (Fig. 3A, lanes 2–4). Higher levels of Mg\(^{2+}\) affected the cleavage reaction qualitatively, resulting in a slight decrease in the average size of the products (Fig. 3A, lanes 2–4). The addition of 2 mM ATP resulted in a significant increase in the size of the cleavage products, which varied from 16 to 22 nt in length (Fig. 3A, lanes 5–7). At a higher ratio of ATP to Mg\(^{2+}\) (Fig. 3A, lane...
of 32P-labeled ends (denoted as asterisks) are shown at the top of the figure. The oligonucleotides used (indicated as circled numbers, see Table I) to construct the substrates contain a 25-nt oligo(dT) (dT)25 homopolymer at either the 5’- or 3’-end. The 5’-end-labeled (lanes 1–7) or the 3’-end-labeled (lanes 8–14) substrate (15 fmol each) was preincubated with 0.5 ng of Dna2 at 37 °C for 5 min in the presence (+) or absence (−) of 2 mM ATP. Reactions were initiated by the addition of the indicated amount of MgCl₂ and incubated at 37 °C for an additional 2 min; products were then analyzed as described in Fig. 2B. M denotes molecular size markers. The numbers shown on the left and right of the figure refer to the size of markers. B, analysis of the cleavage products generated by the mutant Dna2K1080E protein (devoid of both ATPase and helicase activities) in the presence and absence of ATP. The reactions were carried out, and the products were analyzed as described in A.

Fig. 3. ATP differentially influences the Dna2-mediated endonucleolytic cleavage of unannealed 5’- or 3’-overhangs in a Y-structured substrate. A, the Y-structured substrates and the position of 32P-labeled ends (denoted as asterisks) are shown at the top of the figure. The oligonucleotides used (indicated as circled numbers, see Table I) to construct the substrates contain a 25-nt oligo(dT) (dT)25 homopolymer at either the 5’- or 3’-end. The 5’-end-labeled (lanes 1–7) or the 3’-end-labeled (lanes 8–14) substrate (15 fmol each) was preincubated with 0.5 ng of Dna2 at 37 °C for 5 min in the presence (+) or absence (−) of 2 mM ATP. Reactions were initiated by the addition of the indicated amount of MgCl₂ and incubated at 37 °C for an additional 2 min; products were then analyzed as described in Fig. 2B. M denotes molecular size markers. The numbers shown on the left and right of the figure refer to the size of markers. B, analysis of the cleavage products generated by the mutant Dna2K1080E protein (devoid of both ATPase and helicase activities) in the presence and absence of ATP. The reactions were carried out, and the products were analyzed as described in A.
greater than or at least equivalent to its binding to ssDNA (data not shown).

From these preliminary results, one possible prediction was that the 5′-end of an ssRNA segment present in an RNA-DNA chimeric flap structure would inhibit the endonucleolytic activity of Dna2. If this were the case, the 5′-terminal ssRNA would probably render such a flap structure resistant to endonucleolytic degradation by Dna2. In order to test this prediction, we prepared a substrate containing a 12-nt oligo(U) segment at its 5′-end in the RNA-DNA chimeric flap (Fig. 4A). Contrary to the prediction, this substrate was efficiently cleaved within the DNA sequence. Cleavage occurred exclusively within the DNA sequence of the RNA-DNA chimeric flap. Although the products formed with the RNA tail are mostly longer than 19 nt (Fig. 4A, lanes 2 and 3), this does not indicate that the nucleas is likely to cleave off a segment of that length from an Okazaki fragment, since Dna2 is able to cleave starting 2 nt from the RNA-DNA junction (Fig. 4A, lanes 2 and 3). When we used a substrate containing a flap consisting of a random sequence instead of homopolymeric (U-dT), the endonucleolytic cleavage occurred preferentially 2–3 nt from the RNA-DNA junction (data not shown), indicating that the cleavage site can be varied in the sequence context of DNA present in the flap. These findings suggest that Dna2 can completely remove the primer RNA segment in Okazaki fragments, resulting in the complete removal of ribonucleotides in the newly replicated DNA. Noteworthy was the observation that the chimeric flap tail region was cleaved more efficiently by HX-Dna2 than was the flap tail that solely contained DNA (Fig. 4A, compare lanes 2 and 3, and 5 and 6). The simplest explanation for this observation is that the RNA segment provided a more efficient initial binding or entry site for Dna2. The chimeric flap substrate was also efficiently cleaved within the DNA sequence in the presence of 2 mM ATP (data not shown) and the cleavage extended into the duplex region in the presence of 2 mM ATP. This indicates that the presence of RNA at the terminus of the flap did not inhibit the unwinding of the strand by Dna2 (data not shown).

In order to confirm further the stimulatory effects of a terminal RNA on the Dna2 cleavage reaction, we used two levels (15 and 30 fmol) of the two substrates in the presence of the fixed amount of Dna2 (0.25 ng and 1.5 fmol), and we determined the rate of substrate hydrolysis. In the presence of 0.5 mM Mg2+, the chimeric flap tail substrate was cleaved 5–7 times more efficiently than the ssDNA flap substrate (Fig. 4B), regardless of the amount of substrates used. As shown, 1.5 fmol of enzyme cleaved more than 9 fmol of substrate in 1 min in the presence of 30 fmol of substrate, indicating that Dna2 cycles efficiently especially with an RNA-initiated substrate. At lower Mg2+ (<0.1 mM), the disparity between the rates of cleavage of the two substrates was even greater (>10-fold) (data not shown). These results suggest that a displaced RNA segment in an Okazaki fragment may provide a highly efficient entry site for Dna2.

**Dna2 Cleaves the 5′-End of Duplex DNA in Conjunction with a DNA Polymerase Capable of Displacement DNA Synthesis**—We have shown that Dna2, like Fen-1, can remove an RNA segment from a flap structure consisting of an RNA-DNA chimeric segment from a flap structure consisting of an RNA-DNA chimera. If this structure were a preferred substrate in vivo for the two enzymes, how could an unannealed flap structure be generated? Several mechanisms have been proposed in which the 5′-end regions of pre-existing Okazaki fragments are displaced for processing by Fen-1 (5, 8). One postulated mechanism suggested that Dna2 generates the flap structure from the 5′-end region of a pre-existing Okazaki fragment through its ability to unwind in the 3′ to 5′ direction along the template, preceding the action of pol δ, which extends the newly synthesized upstream Okazaki fragment. The 5′ to 3′ directionality ofHX-Dna2 demonstrated in this study, however, questions this mechanism. Another possible mechanism postulated is strand displacement synthesis by a polymerase that extends the newly synthesized pre-Okazaki fragment.

We decided to examine whether Dna2, in conjunction with a polymerase capable of displacement DNA synthesis, could remove the 5′-end label of duplex DNA that is normally resistant
Fig. 5. Dna2 cleaves 5'-end-labeled duplex DNA in conjunction with a DNA polymerase capable of displacement synthesis. The primed partial duplex substrate used consisted of two oligonucleotides (2 and 12) annealed to øX174 ssDNA separated by a 226-nt gap, as shown at the left of the figure. The first 5' 12 nucleotides of the downstream annealed oligonucleotide were substituted with ribonucleotides (wavy line) to simulate a downstream Okazaki fragment. The asterisk indicates the 32P-labeled 5'-end of the downstream oligonucleotide. Pol denotes the DNA polymerase used (Klenow or T4 DNA pol). The reaction mixtures (20 µl) contained 15 fmol of the substrate, 5 mM MgCl₂, dNTPs (20 µM each), and increasing amounts of Dna2 as indicated. The reactions were carried out in the absence of a DNA pol (lanes 1–4) and in the presence of either the Klenow fragment (+Klenow, 0.2 unit; lanes 5–8) or T4 DNA pol (+T4 DNA Pol, 0.2 unit; lanes 9–12) at 37 °C for 10 min. The products were analyzed in a high resolution sequencing gel (20% polyacrylamide plus 7M urea) as described in Fig. 2B. The closed arrowheads indicate cleavage products formed after incubation with HX-Dna2. The open arrowheads indicate the products generated in the absence of Dna2. The size markers (M) were as described in Fig. 2B. The position of labels observed at the top of the figure is the origin of the gel.

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Fig. 6. Dna2 cleaves 5'-end-labeled duplex DNA in conjunction with eukaryotic DNA pol δ. The substrate used was as described in Fig. 5. Pol δ denotes S. pombe DNA pol δ. The other enzymes were derived from S. cerevisiae. The reaction mixtures (40 µl) contained 15 fmol of the substrate, 5 mM MgCl₂, dNTP (80 µM each), 0.5 mM ATP, and the indicated amounts of HX-Dna2. Reactions were incubated in the absence (−) and presence (+) of RPA (200 ng), RFC (20 ng), PCNA (50 ng), and pol δ (1 unit) (35) at 37 °C for 20 min, and products formed were analyzed in a high resolution sequencing gel (20% polyacrylamide plus 7 M urea) as described in Fig. 2B. The cleavage products formed in the presence (closed arrowheads) or absence (open arrowheads) of Dna2 are indicated. The size markers (M) were as described in Fig. 2B. Dna2, wild type HX-Dna2, Dna2K1080E, a mutant Dna2 devoid of ATPase and helicase activities (33).

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In order to determine whether our observations with the prokaryotic polymerases also applied to a eukaryotic system, we determined whether Dna2 could cleave an RNA segment conjointly with the extension of the upstream Okazaki fragment by pol δ (Fig. 6). In this experiment, we used recombinant pol δ of S. pombe (a gift from Dr. J. Hurwitz) in conjunction with RFC, PCNA, and RPA purified from S. cerevisiae. DNA synthesis by S. pombe pol δ was dependent on S. cerevisiae RFC and PCNA with either singly primed (Fig. 6, lanes 4 and 5) or multiply primed DNA templates (data not shown), similar to previous findings that calf thymus pol δ was interchangeable with human pol δ (43). As shown, HX-Dna2 removed the 5’-labeled RNA segment by cleaving 3–4 nucleotides beyond the RNA-DNA junction (Fig. 6, lanes 8–10, closed arrowheads). This was hardly observed when the elongation of the primer was dependent, as the Klenow fragment alone produced this signal (Fig. 6, lane 5, open arrowheads). In the presence of T4 DNA pol, cleavage was hardly observed (Fig. 6, lanes 9–12), despite the fact that more efficient DNA synthesis was observed with this polymerase than with the Klenow enzyme. This demonstrates that cleavage at the 5’-end requires a displacement reaction and that Dna2 can efficiently cleave displaced 5’-ends as soon as they are generated.

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cooperative binding of RPA required for inhibition of Dna2 endonuclease activity. Thus, RPA binding to ssDNA may protect the template ssDNA in either lagging or leading strand from the endonucleolytic action of Dna2, which is critical to preserve the integrity of replication forks.

**DISCUSSION**

In this study, we have examined the properties of the ssDNA-specific endonuclease activity of Dna2 in order to evaluate its role in Okazaki fragment maturation. By demonstrating that Dna2 translocates in the 5′ to 3′ direction, we have ruled out the possibility that Dna2 acts to create an unannealed flap structure that facilitates Fen-1-catalyzed removal of the 5′-terminal RNA-DNA primer segment in the Okazaki fragments. The properties of the endonuclease activity of Dna2 described here suggest that Dna2 plays a more direct role in Okazaki fragment maturation. This is supported by the following observations. (i) Dna2 cleaved unannealed ssDNA tails more efficiently (>200-fold) than ssDNA regions flanked by duplex DNA at both ends. (ii) Dna2 acted preferentially on unannealed 5′-tail ssDNA in the presence of ATP. (iii) Dna2 completely removed the ssDNA tail from the 5′-end but not from the 3′-end of a duplex DNA structure (33). (iv) The Dna2-catalyzed cleavage reaction was significantly stimulated by the presence of an RNA segment at the displaced 5′-end of the ssDNA tail; RNA alone neither stimulated the ATPase activity of Dna2 nor was cleaved by the enzyme. (v) Finally, when combined with a DNA polymerase capable of displacement synthesis, Dna2 catalytically and efficiently removed the 5′-end-labeled region. These data imply that Dna2 alone can remove an RNA-DNA primer from a duplex DNA structure without the participation of other nucleases such as Fen-1 or RNase HI.

**Genetic Evidence of DNA2 Involvement in Okazaki Fragment Maturation**—In an S. cerevisiae DNA2 temperature-sensitive mutant strain (dna2-1), low molecular weight DNA accumulates at the nonpermissive temperature (41). This observation was interpreted as a potential defect in the elongation stage of DNA replication due to the lack of fork propagation. An equally likely interpretation is that the accumulation of low molecular DNA is due to a defect in the joining of Okazaki fragments. Our data support the latter possibility. Consistent with this possibility, other DNA2 mutant alleles (for example, dna2-2) under nonpermissive conditions do not affect bulk DNA synthesis but arrest cells at late S-phase of cell cycle (46). When the temperature-sensitive allele of S. pombe dna2− was combined with a mutant allele (hus1-14; see Ref. 47) of a DNA replication checkpoint gene, the double mutant cells underwent aberrant mitosis at the restrictive temperature, resulting in a catastrophic consequence (34). This result suggests that the replicated DNA cannot be converted to the complete duplex DNA in the absence of a functional Dna2. In addition, the temperature lethality of the dna2 mutant cells was specifically rescued by the presence of multiple copies of genes such as cdc17− (S. pombe DNA ligase I), cdc1−, or cdc27− (subunits of S. pombe pol b), and rad22− (S. pombe Fen-1) that are involved directly in Okazaki fragment synthesis/maturation (34). These genetic observations, together with enzymatic properties of Dna2 described above, support the hypothesis that Dna2 plays a direct and essential role in Okazaki fragment maturation.
pol denotes the initiator RNA and DNA, respectively, synthesized by DNA wavy line Fen-1. See text for details. The both Dna2 and Fen-1. ment maturation that involves the endonuclease activities of pol segment is accomplished by Dna2 after displacement DNA synthesis by pol arrows cleavage sites of Dna2 (38). The remaining flap DNA region is then cleaved primarily by Fen-1. See text for details. The wavy line followed by a linear thin line denotes the initiator RNA and DNA, respectively, synthesized by DNA pol α-primase. The circle denotes a pol δ complex, and wedges indicate cleavage sites of Dna2 (open) and Fen-1 (closed). The arrowhead indicates the direction of Okazaki fragment extension by pol δ. RFC and PCNA are not shown for clarity of the model.

Based on these distinct properties and the results presented here, we propose a novel model for Okazaki fragment processing (Fig. 8) involving the sequential action of these two processing enzymes for the removal of primer RNA and DNA. In this model, (i) pol δ first extends the newly synthesized Okazaki fragment, and (ii) Dna2 displaces the RNA segment of the pre-existing downstream Okazaki fragment, creating the intermediate flap structure through displacement synthesis until the RNA segment is completely displaced. Dna2 is then targeted to the displaced RNA-DNA junction prior to the removal of the primer RNA. (iii) Dna2 releases the RNA segment of the downstream Okazaki fragment by cleaving several nucleotides away from the RNA-DNA junction. It would cleave ssDNA as well if available. The fact that Dna2 is unable to digest the RNA segment, but cleaves within ssDNA, ensures the complete removal of the RNA segment. In this step, the flap junction generated while DNA is being displaced by pol δ may not be sensitive to cleavage by Fen-1 for the following reasons. (a) Steric hindrance caused by the replication proteins engaged at the junction might prevent Fen-1 from recognizing the ssDNA-dsDNA junction. (b) The presence of an ssDNA gap of a few nucleotides between the 3'-end extended by pol δ and the flap junction generated ahead may not be suitable for cleavage by Fen-1. These suggestions are hypothetical at present and await future biochemical analysis. If this is the case, replication proteins that prevent the access of Fen-1 have to be disassembled prior to the action of Fen-1. The disassembly could also allow the annealing of displaced strand to the ssDNA template, if present, restoring a flap structure consisting only of DNA. (iv) Subsequently, the flap junction becomes susceptible to Fen-1, and then Fen-1 cleaves the remaining flap DNA. The second cleavage reaction in this step, however, could be accomplished by Dna2 or another enzyme possessing 5' to 3' exonuclease activity. (v) Finally, DNA ligase I is recruited to complete Okazaki fragment maturation by sealing the resulting nick, generating a continuous DNA strand to complete lagging strand maturation. Our model suggests that the endonuclease activity of Dna2 is a critical function of the enzyme. In support of this, it was recently shown that point mutations that either abolished or reduced the endonuclease activity of Dna2 rendered the mutant cells inviable (42, 45).

Although our model appears to require redundant enzymatic functions, it is supported by many of the genetic and biochemical observations obtained to date. First of all, the finding that Dna2 formed a physical complex with Fen-1 in carrying out its essential function (32) strongly favors our model that Dna2 and Fen-1 act in a concerted fashion. Our model is also consistent with genetic observations that overexpression of Fen-1 suppressed the temperature sensitivity of the dna2-1 allele, and overexpression of Dna2 partially compensated for the growth defect of the rad27 deletion at the restrictive temperature (32, 44). This could account for the synthetic lethality observed when a rad27 deletion was combined with several mutant alleles of dna2 (32, 44), which suggests that Fen-1 becomes essential in the absence of active Dna2 protein. Recently, the structures of DNA lesions were assessed in yeast strains deficient in lagging strand DNA synthesis (48). This study showed that dna2 and rad27Δ mutant strains contained DNA lesions characteristic of aberrant lagging strand synthesis, in keeping with our model that Dna2 and Fen-1 collaborate in the processing of Okazaki fragments. The hypothesis that more than two enzymes could be involved in Okazaki fragment maturation is also supported by recent in vivo studies that demonstrated that double-strand break (DSB) repair in yeast requires both leading and lagging strand DNA synthesis (30). In this study, DSB-induced gene conversion at the MAT locus of S. cerevisiae was analyzed in mutant strains thermosensitive for essential replication factors. Virtually all of the known replication fork proteins affected DSB-induced gene conversion events, although to varying degrees. Gene conversion was decreased 50% in RAD27 null strains, compared with wild type strains, regardless of growth conditions. This confirms that Fen-1 is required for Okazaki fragment processing but at the same time presents strong evidence for the existence of redundant functions for this process, most likely Dna2. Recent genetic analyses of rad27 mutant alleles showed that rad27-n (a nuclease-deficient allele of RAD27) inhibited the growth of mutant cells, whereas rad27-p (a PCNA-binding defective allele) did not (49). Interestingly, intragenic combination of both mutations (rad27-n,p) nullified the deleterious effect of rad27-n on cell growth. These results suggest that the interaction of Rad27-n with PCNA allows the mutant protein to occupy its normal position within a multiprotein complex, preventing access of an alternative enzyme capable of processing the Okazaki fragment to the incompletely processed DNA, whereas Rad27-n,p cannot be assembled into the complex, allowing free access of the redundant enzyme. These results support the model that the two processing enzymes collaborate to process efficiently Okazaki fragments.

The Possibility of Parallel Pathways of Dna2 Versus Fen-1 RNase HI in Okazaki Fragment Processing—The deletion of rad2Δ in S. pombe did not result in the temperature-sensitive
Enzymatic Role of Dna2 in Okazaki Fragment Maturation

In this model, we can postulate that Dna2 and other proteins that act in lagging strand synthesis.

...ability to interact with pol δ were inefficient, for example, Okazaki fragments could be susceptible to RNase HI and Fen-1 before the upstream polymerase arrives. In this case, the 5′ to 3′ exonuclease activity of Fen-1 comes into play and can contribute to the removal of the RNA-initiated primer with the aid of exonuclease activity of Fen-1 comes into play and can contribute to the removal of the RNA-initiated primer with the aid of exonuclease activity of Fen-1.

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**Possible Roles of Dna2 Helicase Activity in Okazaki Fragment Maturation**—In this model, we can postulate that Dna2 plays a number of roles. First of all, the unique feature of Dna2 is its limited unwinding activity (Ref. 33 and this study) and its ability to interact with pol δ (34). The unwinding activity of the Dna2 helicase is likely to be tightly coordinated with displacement synthesis by pol δ, regulating the extent of unwinding of the 5′ region of the Okazaki fragment. In fact, pol δ can catalyze DNA displacement synthesis up to 274 base pairs (24, 50), longer than what is required for Okazaki fragment processing. Considering the relatively small size (100–150 nt) of an average Okazaki fragment, the limited unwinding activity of Dna2 is probably an important feature of the enzyme. This activity could facilitate the timely disassembly of the pol δ complex from the nascent DNA, when no further displacement is required. If this is not the case, uncontrolled displacement synthesis catalyzed by pol δ would lead to (i) unnecessary extensive degradation of the pre-existing Okazaki fragment and (ii) the formation of lengthy ssDNA segments with the potential to form secondary structures that would be resistant to cleavage by Dna2 or Fen-1. Thus, one essential role of Dna2 could be to prevent unnecessary displacement synthesis by pol δ. Another possible role of the Dna2 helicase activity could be to help remove transient secondary structures in displaced strands, which in turn could facilitate the sliding of Fen-1 to the duplex junction (51). It may also help to load Fen-1 onto cleaved ends of ssDNA generated by Dna2, thus coordinating and coupling the two endonucleolytic reactions. The unwinding activity of Dna2 may be required for such a coordination for maximal efficiency, since inactivation of Dna2 leads to cell death (52) only when cells are grown in rich media (44). The roles for Dna2 described above may not be substituted by other nucleases, accounting for the fact that Dna2 is essential in vivo, whereas Fen-1 is not. In order to test our model, we plan to develop an in vitro system for Okazaki fragment maturation. Such a system may clarify the role played by Dna2 in Okazaki fragment synthesis as well as help to define the functional interactions between Dna2 and other proteins that act in lagging strand synthesis.

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