

# Flap Endonuclease Disengages Dna2 Helicase/Nuclease from Okazaki Fragment Flaps\*

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Okazaki fragments contain an initiator RNA/DNA primer that must be removed before the fragments are joined. In eukaryotes, the primer region is raised into a flap by the strand displacement activity of DNA polymerase  $\delta$ . The Dna2 helicase/nuclease and then flap endonuclease 1 (FEN1) are proposed to act sequentially in flap removal. Dna2 and FEN1 both employ a tracking mechanism to enter the flap 5' end and move toward the base for cleavage. In the current model, Dna2 must enter first, but FEN1 makes the final cut at the flap base, raising the issue of how FEN1 passes the Dna2. To address this, nuclease-inactive Dna2 was incubated with a DNA flap substrate and found to bind with high affinity. FEN1 was then added, and surprisingly, there was little inhibition of FEN1 cleavage activity. FEN1 was later shown, by gel shift analysis, to remove the wild type Dna2 from the flap. RNA can be cleaved by FEN1 but not by Dna2. Pre-bound wild type Dna2 was shown to bind an RNA flap but not inhibit subsequent FEN1 cleavage. These results indicate that there is a novel interaction between the two proteins in which FEN1 disengages the Dna2 tracking mechanism. This interaction is consistent with the idea that the two proteins have evolved a special ability to cooperate in Okazaki fragment processing.

Replication of eukaryotic cellular DNA involves the synthesis and joining of Okazaki fragments on the lagging strand. These fragments are 100–150 nucleotides long in eukaryotes (1). They are initiated by polymerase  $\alpha$ /primase (pol  $\alpha$ )<sup>2</sup> complex, which synthesizes 10–12 nucleotides of RNA followed by ~20 nucleotides of DNA (2). pol  $\alpha$  is then replaced by a complex consisting of proliferating cell nuclear antigen and polymerase  $\delta$  (pol  $\delta$ ) through a process known as polymerase switching. Polymerase  $\epsilon$  (pol  $\epsilon$ ) may also play a role in lagging strand synthesis or processing, because some portion of the cellular proliferating cell nuclear antigen is bound by pol  $\epsilon$  during S-phase (3, 4). DNA synthesis continues until pol  $\delta$  encounters a down-

stream Okazaki fragment, at which time strand displacement synthesis creates a single-stranded flap (5–7). This flap must then be processed to form a continuous strand of DNA, a pathway known as Okazaki fragment processing (OFP).

Several models of OFP have been developed from reconstitution studies *in vitro* in which nuclease activity is used to remove the primer made by pol  $\alpha$  followed by ligation to form a continuous strand (6, 8, 9). One model, proposed by the Burgers group (5, 10), suggests that short flaps are created by pol  $\delta$  strand displacement synthesis and are successively cleaved by flap endonuclease 1 (FEN1). *Saccharomyces cerevisiae* FEN1, also known as Rad27p, is a 42-kDa protein with 5' to 3' single-stranded DNA (ssDNA) endonuclease activity and minor 5' to 3' exonuclease activity (11). FEN1 has been shown to cleave at the base of a 5' single-stranded flap substrate (11–13). Additionally, FEN1 has been shown to prefer short flaps to long flaps (5, 14).

*S. cerevisiae* Dna2 was first implicated in OFP when it was found to associate both genetically and physically with FEN1 (15). It has recently been shown to be associated with other components of OFP, including Exo1, RNaseH2, Rrm3, Sgs1, and Pol32 (16). Dna2 is a 172-kDa protein that is essential in yeast (17–20) and has two domains, as determined by limited proteolysis studies (21). One domain, found at the C terminus, possesses ssDNA-dependent ATPase activity and ATP-dependent 5' to 3' helicase activity (17, 22, 23). The second domain, located between residues 650 and 700, contains homology to the RecB nuclease domain. This domain contains 5' to 3', and minor 3' to 5', ssDNA nuclease activities (23, 24). The ratio of helicase and nuclease activities can be regulated *in vitro* by ATP concentration (18, 25, 26). Dna2 was further implicated in OFP when the ssDNA-binding protein, replication protein A (RPA), was shown to physically interact with Dna2 (27, 28) and stimulate Dna2 activity while repressing FEN1 activity on a flap substrate (5, 10, 14, 25, 28, 29). This led to the RPA/Dna2/FEN1 model, proposed by the Seo group (23). In this model, flaps displaced by pol  $\delta$  reach a length that allows them to be coated by RPA. Bound RPA inhibits cleavage by FEN1 but not by Dna2. Dna2 would then cleave these RPA-coated flaps, releasing RPA. Dna2 does not have cleavage specificity for the flap base (14). Instead it produces short (5–7 nucleotides), RPA-free flaps that are substrates for FEN1. The current view is that some portion of OFP occurs by the RPA/Dna2/FEN1 pathway (9).

The model above suggests that a unique interplay between Dna2 and FEN1 exists. As stated previously, FEN1 and Dna2 interact physically with each other. The experiments discussed in this paper are used to further understand the interaction

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<sup>2</sup> The abbreviations used are: pol, polymerase; FEN1, flap endonuclease 1; OFP, Okazaki fragment processing; RPA, replication protein A; ssDNA, single-stranded DNA; ssRNA, single-stranded RNA; DTT, dithiothreitol.

between these two proteins. Both FEN1 and Dna2 have been shown to employ a tracking mechanism to cleave the flap substrate (13, 30, 31). They both must interact with the 5' end of the flap and then move to the site of cleavage. Dna2 was found to act as if it were threaded onto the flap like a bead on a string (30). Because they are proposed to act in order, the Dna2 must cleave but then allow the FEN1 to have access to the 5' end of the shorter flap. In this study, we used a nuclease-inactive Dna2 to block the base of a flap substrate and then determined whether FEN1 could access the substrate. Surprisingly, FEN1 was capable of efficient cleavage. We then explored the mechanism by which FEN1 could reach its cleavage site.

## EXPERIMENTAL PROCEDURES

**Materials**—Synthetic oligonucleotides, including the 5'-biotin conjugation, were produced by Integrated DNA Technologies. Radionucleotide [ $\alpha$ - $^{32}$ P]dCTP (6000 Ci/mmol) was obtained from PerkinElmer Life Sciences. The Klenow fragment of DNA polymerase I and ATP were from Roche Applied Science. All other reagents were the best available commercial grade.

**Enzyme Expression and Purification**—*S. cerevisiae* Dna2 was cloned into the Sf9 baculovirus expression vector (Invitrogen). It was then expressed and purified as described previously (18), except that High Five cells were utilized for the final expression step of the protein. *S. cerevisiae* Dna2 E675A was created by site-directed mutagenesis as described in Ref. 18. It was then expressed and purified as described above for the wild type Dna2. *S. cerevisiae* FEN1 was cloned into the T7 expression vector pET-FCH and overexpressed in *Escherichia coli*. It was then purified as described previously (26).

**Oligonucleotides**—All downstream primers were labeled at the 3' terminus with [ $\alpha$ - $^{32}$ P]dCTP (6000 Ci/mmol) by the Klenow enzyme. They were then purified on a 12% polyacrylamide gel containing 7 M urea. DNA substrates were annealed in 50 mM Tris, pH 8.0, 50 mM NaCl, and 1 mM dithiothreitol (DTT), and substrates containing RNA were annealed in 10 mM Tris, pH 8.0, 1 mM EDTA, and 50 mM NaCl. For annealing, substrates were incubated at 95 °C for 5 min and then cooled slowly to room temperature. Protector RNase Inhibitor (Roche Applied Science) was then added to RNA-containing substrates. All substrates were annealed in a 1:2:4 ratio of downstream primer to template to upstream primer. Most experiments described were done using a 53-nucleotide DNA flap substrate. This substrate consisted of the following: downstream primer-5'-/biotin/GTACCAGCTCGAATTCGCCCGTTTCACGCCTGTTAGTTAATTCAGTGGCCGTCGTTTTACAACGACGTGACTGGG-3', upstream primer-5'-CGCCAGGGTTTTCCAGTCACGACA-3', and template-5'-GCCAGTCACGTCGTTGTAAAACGGGTCGTGACTGGGAAAACCCTGGCG-3'. The experiments shown in Fig. 5 involved a 30-nucleotide RNA flap substrate, which consisted of the following primers (RNA is in bold): downstream primer-5'-**GUCACGCCUGUUAUUAUUCACUGGCCGUCCACCCGUCCACCCGACG**-3', upstream primer-5'-CGACCGTGCCAGCCTAAATTTCAAGA-3', and template-5'-GCGTCGGGTGGACGGGTGGCTTGAAATTTAGGCTGGCACGGTCG-3'.

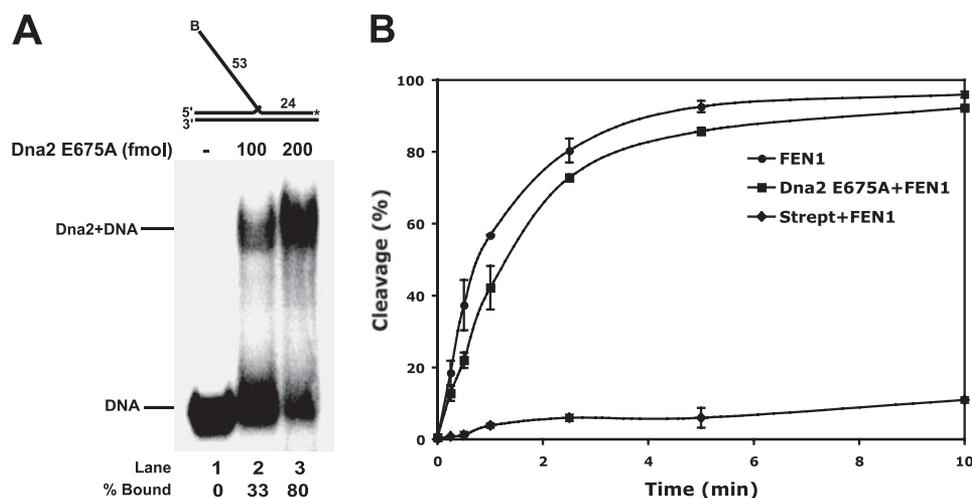
**Nuclease Assay**—The reaction buffer for both Dna2 and FEN1 reactions consisted of 50 mM Tris-HCl, pH 8.0, 30 mM NaCl, 2 mM DTT, 0.1 mg/ml bovine serum albumin, 2 mM MgCl<sub>2</sub>, 5% glycerol, and 10  $\mu$ M ATP. The reaction mixture volume was 20  $\mu$ l, which included 5 fmol of labeled substrate and various amounts of enzymes, as indicated in the figure legends. Reactions were then incubated for 10 min at 37 °C, unless otherwise indicated, and stopped with a 2 $\times$  termination dye (90% formamide (v/v), 10 mM EDTA, with 0.01% bromophenol blue and xylene cyanol). Reactions were then separated on a 15% polyacrylamide gel containing 7 M urea. Pre-binding Dna2 conditions were as follows. Dna2 and labeled substrate were mixed, incubated for 10 min on ice, and then incubated for 5 min at 37 °C. In Figs. 1B and 5B, Dna2 was pre-bound prior to FEN1 addition. In Figs. 1B and 3, streptavidin was used in 50-fold excess over substrate and was conjugated to the 5'-biotin by incubation at 37 °C for 10 min. In Fig. 2, 50 microunits of micrococcal nuclease (Fermentas) in 50 mM Tris-HCl, pH 8.0, 30 mM NaCl, 2 mM DTT, 0.1 mg/ml bovine serum albumin, and 2 mM CaCl<sub>2</sub> was added after Dna2 E675A was bound to substrate, as described previously. The reaction was then incubated at 37 °C for 10 min. For experiments using RNA, Protector RNase Inhibitor (Roche Applied Science) was added to help prevent RNA degradation.

**Gel Shift Assay**—This assay was used to determine the binding of Dna2 and/or FEN1 to various labeled substrates. Reactions were performed in 50 mM Tris-HCl, pH 8.0, 30 mM NaCl, 2 mM DTT, 0.1 mg/ml bovine serum albumin, 5% glycerol, 10  $\mu$ M ATP, and either 2 mM MgCl<sub>2</sub>, with Dna2 E675A, or 4 mM CaCl<sub>2</sub>, with wild type Dna2. The reaction mixture volume was 20  $\mu$ l, which included 5 fmol of labeled substrate and various amounts of enzymes, as indicated in the figure legends. In Figs. 1A and 5A, various amounts of Dna2 were pre-bound, as described above. In Fig. 4, Dna2 was pre-bound, and then FEN1 was added to the reaction mix at various concentrations and incubated for 5 min at 37 °C. The 5' terminus of the substrate was then blocked by the addition of 250 fmol of streptavidin, which was conjugated to the 5'-biotin on the flap. In Fig. 6, streptavidin (250 fmol) was incubated with substrate for 10 min at 37 °C. Dna2 was then added, and reactions were incubated again at 37 °C for 10 min. Reactions were then run on a pre-run 5% (Bio-Rad) or 6% (Invitrogen) Tris borate-EDTA gel at 200 V for 20–40 min.

**Gel Analysis**—All experiments were done at least in duplicate, and representative gels are shown. All gels, after running conditions, were transferred to filter paper and dried on a gel dryer (Bio-Rad) with vacuum (Savant) for 1 h at 80 °C. They were then exposed to a Phosphor screen and imaged using a PhosphorImager (GE Healthcare). Gel analysis was then performed using ImageQuantMac, version 1.2 (GE Healthcare).

## RESULTS

Dna2 is expected to precede FEN1 in tracking on a flap during OFP. Even after cleavage by Dna2 its helicase activity would be expected to drive it onto the flap so that it would block the entry of FEN1. It is known that FEN1 can access the flap after Dna2 cleavage. We hypothesized that the two proteins are designed to allow efficient, successive action. To understand



**FIGURE 1. FEN1 cleavage is not inhibited by Dna2 E675A-bound flaps.** *A*, a gel shift assay was performed with 100 fmol (*lane 2*) and 200 fmol (*lane 3*) of Dna2 E675A and 5 fmol of the 53-nucleotide flap substrate as indicated under "Experimental Procedures." *Lane 1* is the substrate alone control. Percent bound, shown below the gel, is defined as:  $(\text{bound}/(\text{bound} + \text{unbound})) \times 100$ . *B*, 5 fmol of the 53-nucleotide flap substrate was incubated under the following conditions (described under "Experimental Procedures"): *circles*, 5 fmol of FEN1 was added at time zero; *squares*, 200 fmol of Dna2 E675A was pre-bound with substrate prior to FEN1 addition at time zero; *diamonds*, 250 fmol of streptavidin was pre-bound with substrate prior to FEN1 addition at time zero. Time points were taken at 0, 0.25, 0.5, 1, 2.5, 5, and 10 min. Percent cleavage is defined as:  $(\text{cleaved}/(\text{cleaved} + \text{uncleaved})) \times 100$ . The graph is an average of two experiments, and *error bars* indicate S.D. The substrate, used in both *A* and *B*, is depicted above the gel in *A*, where the *asterisk* indicates the site of the 3'  $^{32}\text{P}$  radiolabel and *B* indicates the 5'-biotin.

how Dna2 works with FEN1, we assessed FEN1 binding and cleavage to a flap already occupied by a Dna2 molecule.

**Dna2 E675A Binds Flaps but Does Not Inhibit FEN1**—Dna2 E675A was characterized previously and shown to be defective in nuclease activity while retaining helicase activity (18). We confirmed both a defect in cleavage activity and retention of helicase activity of Dna2 E675A on a flap substrate (data not shown). It was previously shown that Dna2 binds to flap substrates by gel shift assay (28). We tested whether Dna2 E675A could bind to a flap substrate and block FEN1 cleavage. Because we have previously shown that FEN1 requires a free 5' end for tracking to the base of the flap and cleavage (13), we hypothesized that Dna2 E675A binding would inhibit FEN1 cleavage at the base of the flap. The approach was to load Dna2 so that it tracks or binds to the base of the flap but does not cleave. We used an ATP concentration that allows the Dna2 to bind onto the flap but does not make the helicase function so potently that it displaces the flap and dissociates the primer from the template (data not shown). Fig. 1*A* shows native PAGE separation of the 53-nucleotide flap substrate with Dna2 E675A present. Upon addition of purified Dna2 E675A (Fig. 1*A*, *lanes 2* and *3*), we saw a gel shift from the position of the DNA only band (Fig. 1*A*, *lane 1*) to a higher molecular weight band signifying binding. The fraction of shifted DNA was dependent on Dna2 E675A concentration. At 200 fmol of Dna2 E675A (Fig. 1*A*, *lane 3*), at least 80% of the DNA was bound with protein, as some portion of the protein may have been dissociated during electrophoresis.

Dna2 E675A was then pre-bound, under the same conditions shown in Fig. 1*A*, *lane 3*, to the 53-nucleotide flap substrate. This should have prevented FEN1 access to at least 80% of the substrate molecules. FEN1 was added to the reaction mixture at the end of the pre-binding period. When FEN1 was incubated

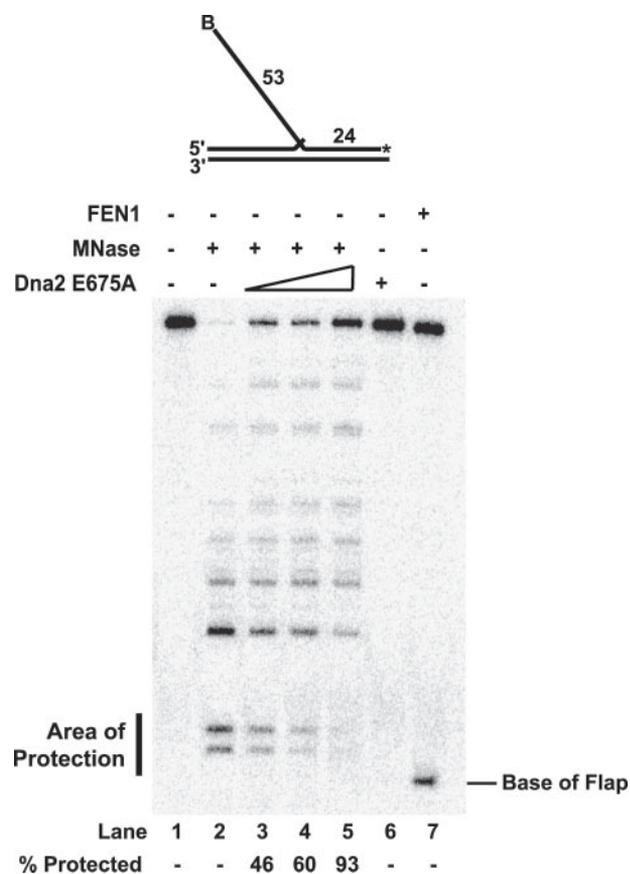
with the substrate in the absence of Dna2 E675A (Fig. 1*B*, *circles*), an increasing amount of flaps were cleaved over time. Upon addition of FEN1 to pre-bound substrate (Fig. 1*B*, *squares*), the flaps were also cleaved efficiently. Surprisingly, the presence of the Dna2 E675A made only a slight difference in the amount of cleavage, indicating that the FEN1 had access to its cleavage site on nearly all of the substrates. Inhibition of FEN1 cleavage by Dna2 E675A was compared with that of a 5' streptavidin-biotin-blocked flap (Fig. 1*B*, *diamonds*). The almost complete elimination of cleavage activity verifies that prevention of FEN1 entry to the flap is an effective deterrent to cleavage.

The early time points can be used to compare relative rates of cleavage (Fig. 1*B*, 0.5 and 1 min). Blockage of the flaps by Dna2 E675A resulted in a less than 2-fold decrease in cleavage rate (compare Fig. 1*B*, *squares* and *circles*), strikingly less inhibitory than the effect of streptavidin (Fig. 1*B*, *diamonds*).

The difference in rate was reduced to only 4% by 10 min, simply because cleavage goes to near completion even in the presence of Dna2 E675A. Moreover, cleavage products made by FEN1, as seen on the gel, appeared identical on both unblocked and Dna2 E675A-blocked substrates (data not shown). This demonstrates that FEN1 reached the base of the flap for cleavage at its preferred site in both cases. Titrations of Dna2 E675A up to 200 fmol were performed in the pre-binding reaction and showed no significant effect on the amount of FEN1 cleavage (data not shown). Overall, these results show that FEN1 can bypass Dna2 bound on a flap, thus gaining access to the flap base for cleavage.

**Dna2 E675A Should Block FEN1 Access to the Flap**—Because Dna2 appears to thread onto the flap, it is difficult to visualize how FEN1 could cleave around a bound Dna2 molecule. We determined whether FEN1 would have access to the flap during brief absences of Dna2, as might occur if Dna2 proteins occasionally slide off the 5' end of the flap. Dna2 E675A was bound to the 53-nucleotide labeled DNA flap substrate, and micrococcal nuclease was then added (Fig. 2). Micrococcal nuclease is an endonuclease, which, unlike Dna2 or FEN1, has no loading requirement and can readily cleave exposed DNA. In addition, it prefers ssDNA over double-stranded DNA. In Fig. 2, Dna2 E675A was bound prior to micrococcal nuclease addition. This allowed us to evaluate the percent of substrates that were inaccessible to micrococcal nuclease and thus protected by bound Dna2 E675A. Fig. 2, *lane 2*, shows the amount of micrococcal nuclease cleavage without Dna2 E675A present. In contrast, when Dna2 E675A was bound to the substrate prior to micrococcal nuclease addition, there was a decrease in the amount of products formed after a 10-min incubation with micrococcal nuclease (Fig. 2, *lanes 3–5*). Upon quantitation of

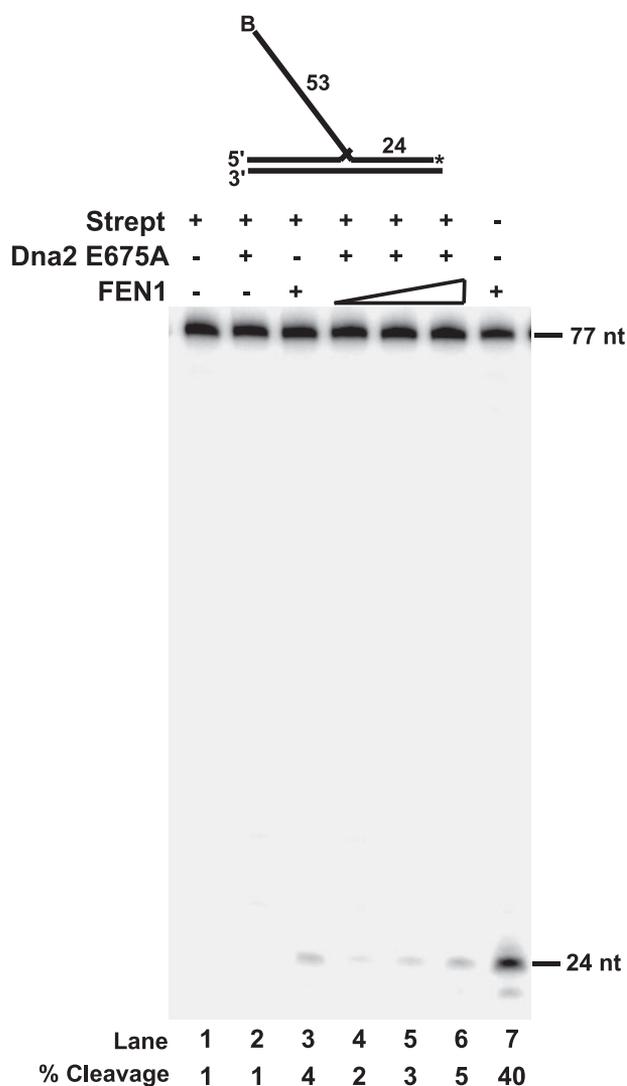
## FEN1 Disengages Dna2



**FIGURE 2. Dna2 blocks micrococcal nuclease cleavage.** Dna2 E675A, 100 fmol (*lane 3*), 200 fmol (*lane 4*), and 500 fmol (*lane 5*), was pre-bound to 5 fmol of the labeled 53-nucleotide flap, and micrococcal nuclease (*MNase*) was then added at time zero as described under "Experimental Procedures." The reaction was then incubated at 37 °C for 10 min. *Lane 1* is the substrate alone control, *lane 2* is micrococcal nuclease without Dna2 present, and *lane 6* is the highest Dna2 E675A concentration without micrococcal nuclease. In *lane 7*, FEN1 was used to identify the base of the flap. Percent protection is defined as: (area of protection/(area of protection + uncleaved substrates)) × 100. The substrate used is depicted above the gel, the asterisk indicates the site of the 3' <sup>32</sup>P radiolabel, and *B* indicates the 5'-biotin.

the protected area (indicated in Fig. 2), we determined that Dna2 E675A protected ~60% of the substrates from cleavage at the base of the flap when 200 fmol of Dna2 E675A was used. This corresponds to the concentration of Dna2 E675A used in Fig. 1*B*. Similar results were seen with wild type Dna2 (data not shown). Overall, these results suggest that Dna2 does not transiently leave the flap in a way that allows the flap to become accessible to FEN1.

**FEN1 Tracking Is Not Bypassed by Dna2**—We next considered several hypotheses to explain the ability of FEN1 to bypass bound Dna2. Because FEN1 and Dna2 interact (15), it is possible that FEN1 binds to Dna2, bound to the flap, from solution and can cleave without tracking. We tested this idea by using the 5' streptavidin-biotin-blocked substrate. Dna2 E675A was pre-bound to the substrate followed by conjugation with streptavidin to form the streptavidin-biotin block. FEN1 was then added. When FEN1 was incubated alone with blocked substrates that contained no bound Dna2 E675A (Fig. 3, *lane 3*), there was almost complete suppression of the substantial cleavage observed with the unblocked substrate (Fig. 3, *lane 7*). The small residual amount of cleavage is most likely because of a



**FIGURE 3. Bound Dna2 E675A does not allow bypass of FEN1 tracking.** A nuclease assay was used to assess the cleavage of FEN1 on the 53-nucleotide flap as described under "Experimental Procedures." Streptavidin (*lanes 1–6*) and Dna2 E675A (*lanes 2 and 4–6*) were preincubated with the substrate as described under "Experimental Procedures." Dna2 E675A (500 fmol) was pre-bound to 5 fmol of substrate, which was then blocked with streptavidin. 0.1 (*lane 4*), 0.5 (*lane 5*), and 1 fmol (*lane 6*) of FEN1 was then added. *Lane 7* indicates the amount of FEN1 (1 fmol) cleavage without pre-bound streptavidin compared with *lane 3* with streptavidin. *Lane 1* is substrate alone, and *lane 2* is substrate bound by Dna2 E675A (500 fmol) without FEN1 added. Percent cleavage, shown below the gel, is defined as: (cleaved/(cleaved + uncleaved)) × 100. The substrate is depicted above the gel, the asterisk indicates the site of the 3' <sup>32</sup>P radiolabel, and *B* indicates the 5'-biotin.

contamination of substrates lacking biotin, suggested by a failure to bind streptavidin (see Fig. 4, *lane 2*). In Fig. 3, *lanes 4–6*, Dna2 E675A was pre-bound prior to the flap blockage and subsequent FEN1 addition. We observed no additional cleavage in these reactions (Fig. 3, *lanes 4–6*) compared with the reaction without Dna2 (Fig. 3, *lane 3*). These data show that FEN1 is unable to employ Dna2 E675A bound on the flap as a means to bypass the FEN1 5' end entry and tracking requirements.

**FEN1 Accomplishes Direct Removal of Dna2**—We next set out to determine whether FEN1 is able to disengage Dna2 from the flap, enabling FEN1 to gain access to the base of the flap. This experiment required FEN1 and Dna2 to bind but not cleave the substrate. Both FEN1 and Dna2 use Mg<sup>2+</sup> for nucle-

ase activity. However, substitution of  $\text{CaCl}_2$  for  $\text{MgCl}_2$  was found to inhibit nuclease activity while still allowing effective binding of the substrate (31, 32) (see Fig. 4). This also allowed for the use of wild type Dna2 in this experiment. Binding of

Dna2 and FEN1 to the biotinylated flap substrate was assessed by gel shift. Streptavidin was used to prevent rebinding and allow FEN1 to be trapped onto the substrate, because FEN1, unlike Dna2, appears to equilibrate rapidly between unblocked substrates (data not shown). In the first set of experiments, increasing amounts of FEN1 were added to the substrate followed by addition of streptavidin (Fig. 4, lanes 3–5). Results showed that progressively more FEN1 was bound. Dna2 was then pre-bound followed by the addition of FEN1 and finally streptavidin (Fig. 4, lanes 7–9); lanes 7–9 show a progressive increase in binding of FEN1 to the substrate as the concentration of FEN1 was increased. Moreover, there was a progressive decrease in the density of the band representing bound Dna2, indicating that it was being removed from the flap. Although this displacement was not completely quantitative, as a minor amount of Dna2 appears to remain bound (Fig. 4, lane 9), the majority of Dna2 was efficiently removed from the flap. These results show that the addition of FEN1 to a reaction containing substrate with pre-bound Dna2 caused the removal of Dna2 from the flap. Surprisingly, even though the Dna2 appeared to thread onto the flap on entry, a FEN1 molecule following behind it could directly disengage it from the flap.

*Pre-bound Wild Type Dna2 Does Not Inhibit FEN1 Activity*—It was also important to demonstrate that the ability of FEN1 to cleave a flap with bound Dna2 was not only characteristic of the Dna2 E675A mutant but also of the wild type. To measure FEN1 cleavage with the wild type Dna2, we needed to inhibit Dna2 nuclease function while retaining FEN1 cleavage activity. This was accomplished by exploiting a key difference in substrate specificities between the two nucleases, namely the ability of FEN1, but not Dna2, to cleave an RNA substrate (23, 33). We created a flap substrate in which the 30-nucleotide flap and the

first 8 nucleotides annealed to the template were all ribonucleotides (Fig. 5). The annealed RNA was included to prevent possible cleavage by Dna2 resulting from its helicase activity or transient unannealing of the annealed portion of the substrate.

We first determined whether Dna2 bound to the RNA flap in the same fashion as shown previously for a DNA flap (Fig. 1A). Fig. 5A shows the results of the gel shift assay used to determine binding. The RNA flap substrate shifted to a higher molecular weight complex upon addition of Dna2, indicating binding. The amount of shifted product increased with the amount of Dna2 added. At 200 fmol of Dna2 over 90% of the substrate was shifted into a Dna2-dependent band (Fig. 5A, lane 3). We then determined FEN1 cleavage over time with and without pre-bound Dna2

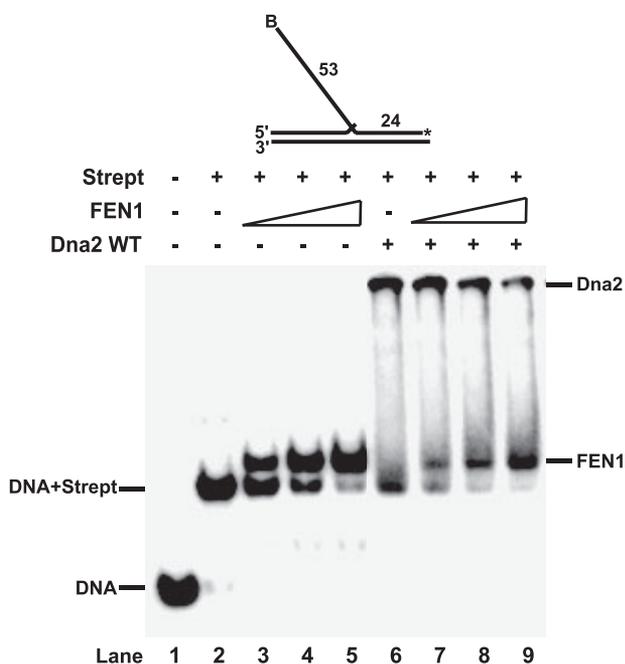


FIGURE 4. FEN1 removes Dna2 from the flap substrate. Dna2 (200 fmol) was pre-bound to 5 fmol of labeled substrate; 10 (lane 7), 25 (lane 8), and 50 fmol (lane 9) of FEN1 was then added followed by the addition of streptavidin as described under "Experimental Procedures." Lanes 3–5 indicate binding of FEN1 alone at the same concentrations as in lanes 7–9, respectively. Lane 6 is a control of pre-bound Dna2 (200 fmol) without FEN1 addition. Lane 1 is the substrate alone, and lane 2 the substrate plus streptavidin. The substrate is depicted above the gel, the asterisk indicates the site of the 3'  $^{32}\text{P}$  radiolabel, and B indicates the 5'-biotin. WT, wild type.

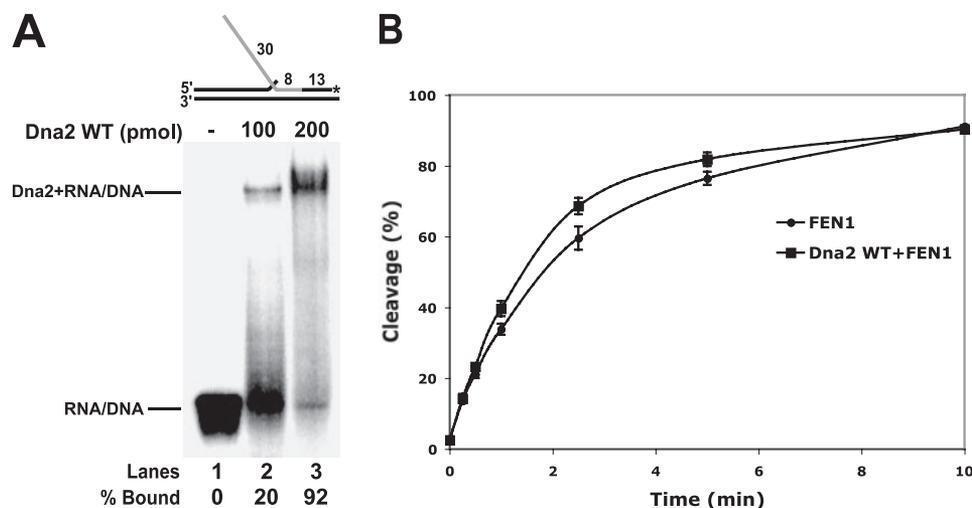


FIGURE 5. Wild type (WT) Dna2 does not inhibit FEN1 cleavage on an RNA flap. A, binding of Dna2 was assessed on a 30-nucleotide RNA flap substrate as described under "Experimental Procedures." Dna2 was bound at 100 fmol (lane 2) and 200 fmol (lane 3). Lane 1 is the substrate alone control. Percent bound, shown below the gel, is defined as:  $(\text{bound}/(\text{bound} + \text{unbound})) \times 100$ . B, 5 fmol of the 30-nucleotide RNA flap substrate was incubated under the following conditions (described under "Experimental Procedures"): circles, 5 fmol of FEN1 was added at time zero; squares, 200 fmol of Dna2 E675A was pre-bound with substrate prior to FEN1 addition at time zero. Time points were taken at 0, 0.25, 0.5, 1, 2.5, 5, and 10 min. Percent cleavage is defined as:  $(\text{cleaved}/(\text{cleaved} + \text{uncleaved})) \times 100$ . The graph is an average of two experiments, and error bars indicate S.D. The substrate, used in both A and B, is depicted above A, where RNA is labeled in gray, DNA is labeled in black, and the asterisk indicates the site of the 3'  $^{32}\text{P}$  radiolabel.

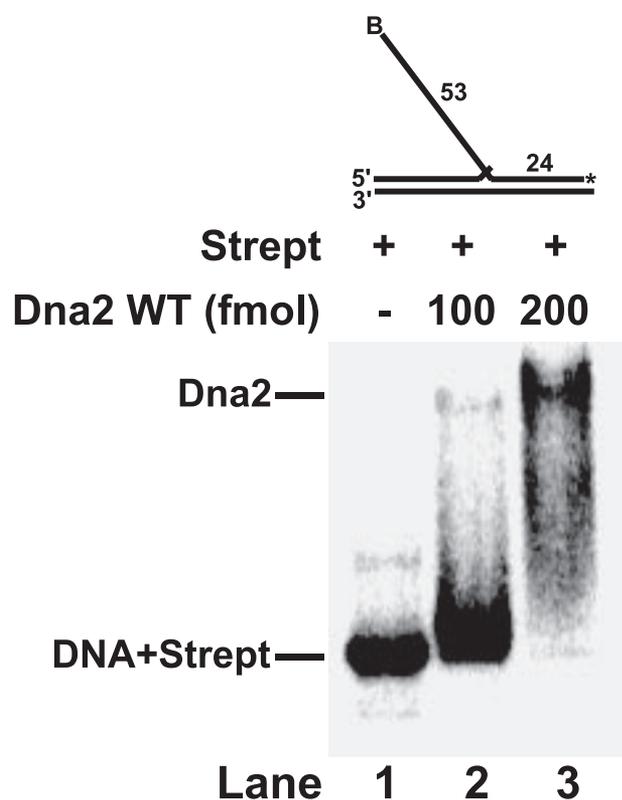


FIGURE 6. **Dna2 binds to blocked flap substrates.** 5 fmol of the 53-nucleotide substrate was incubated with 250 fmol of streptavidin for 10 min at 37 °C. Dna2 was then added to the reaction at 100 fmol (lane 2) and 200 fmol (lane 3), followed by incubated at 37 °C for 10 min. Reactions were then run by gel shift, as described under "Experimental Procedures," to assess Dna2 binding. Lane 1 is the substrate plus streptavidin control. The substrate is depicted above the gel, the asterisk indicates the site of the 3' <sup>32</sup>P radiolabel, and B indicates the 5'-biotin.

(Fig. 5B). As expected, the presence of bound Dna2 did not produce a significant change in the cleavage rate. In fact, these two curves match up almost identically (Fig. 5B, circles and squares). This demonstrates that the wild type Dna2, just as the Dna2 E675A, allows access of FEN1 to its cleavage site.

*Dna2 Binds to Blocked Flap Substrates in a Non-tracking Mode*—Dna2 cleavage activity is prevented when the 5' end of the flap is blocked (30). In view of this, we tested whether Dna2 could still bind a blocked flap. In Fig. 6, the substrate was blocked with streptavidin prior to the addition of Dna2 to the reaction. Dna2 was then added in increasing amounts. A gel shift assay was then performed to assess binding. Dna2 was found to bind even though the flaps were blocked (Fig. 6, lanes 2 and 3). It was then verified that Dna2 cleavage was still inhibited on streptavidin blocked flaps by denaturing PAGE (data not shown). This shows that Dna2 requires tracking for nuclease activity but can bind, in a non-tracking fashion, even when the flap is blocked at the 5' end. This observation is consistent with previous reports that Dna2 is active, at low levels, as a helicase on circular and primer-blocked substrates (23, 25). When Dna2 is bound to an unblocked flap, some fraction may be bound in the non-tracking mode. FEN1 was able to dissociate both types of bound Dna2 from the flap, as cleavage remained greater than 90% on Dna2-blocked flaps (see Figs. 1B and 5B).

## DISCUSSION

In this report, we have shown that a nuclease-inactive Dna2 could bind a 53-nucleotide DNA flap substrate almost quantitatively, but its presence did not inhibit FEN1 cleavage. Further analysis showed that the Dna2 did not allow FEN1 to bypass its tracking requirement. Instead, the Dna2 was observed to dissociate from the substrate. Use of an RNA flap substrate allowed the same type of experiment to be performed with wild type Dna2, which cannot cleave RNA. Again, FEN1 was active on an RNA flap substrate bound by wild type Dna2.

FEN1 and Dna2 have been shown to interact both physically and genetically, but the direct role of Dna2 in OFP still remains uncertain (15). Results from reconstituted systems suggest that mainly short flaps arise during lagging strand replication and that only FEN1 would be required to cleave such flaps (5, 34–36). FEN1 is envisioned to cut repeatedly into the RNA and into the DNA primer as the flap is displaced. Once cleavage occurs in the DNA, the FEN1 product forms a nicked substrate for the joining reaction. Dna2 has been proposed to be required for flaps that escape FEN1 cleavage and become long enough to be coated by RPA (9, 23). The RPA inhibits FEN1 but stimulates Dna2 (14, 28). Dna2 can slide onto long flaps, moving beyond the RNA into the DNA (23, 28). There it cleaves to make a short flap, which RPA cannot bind. This short flap is then a good substrate for FEN1 (5, 14).

The interaction of FEN1 with a flap already bound by Dna2 had not been addressed previously. Surprisingly, we found that FEN1 acts to remove the Dna2 bound to the flap (Fig. 4). This removal is so efficient that cleavage rates are comparable to those without Dna2 present (Figs. 1 and 5). This is the first evidence that the physical interaction of the two proteins is relevant for OFP.

Dna2 is unable to cleave a flap blocked at the 5' end with biotin-streptavidin (30, 37). In addition, Dna2 is unable to cleave past a bound primer or a branch point of a modified flap (30). In this manner the Dna2 behaves like a ring threaded over the end of the flap. Although the mechanism of Dna2 removal has not been elucidated here, our current knowledge of the two proteins allows some informed speculation. When both Dna2 and FEN1 are present, our results show that binding of FEN1 to the substrate at the expense of Dna2 must be highly favored, energetically. FEN1 is not a helicase or even an ATPase. It is not known whether FEN1 binds the substrate with higher affinity than Dna2. Although further binding studies of both FEN1 and Dna2 are required, a reasonable hypothesis is that the binding energy of FEN1 to a complex of Dna2 and substrate induces a conformational change that results in Dna2 dissociation. Although this proposal is consistent with experimental data, crystallographic studies are needed to verify the exact structural features accounting for the threading behavior and disengagement.

Because FEN1 is also obligated to track from the 5' end of the flap to the flap base for cleavage, it is not surprising that FEN1 is inhibited by proteins bound to the flap. Both RPA and *E. coli* single-stranded binding protein have been shown to stop FEN1 cleavage (13). Also FEN1 activity is stopped by primers annealed to the flap and by a biotin-streptavidin block at the 5'

end (13). As with these proteins and primers, bound Dna2, approximately three times the size of FEN1, was expected to block the smaller nuclease. The facile ability of FEN1 to bypass Dna2 implies that a removal mechanism has evolved between the two proteins.

The FEN1-Dna2 disengagement mechanism may be functionally similar to the interaction between Dna2 and RPA. Although Dna2 cleavage on the flap is also blocked by primers, streptavidin, and single-stranded binding protein, it is not inhibited, but rather is stimulated, by the presence of RPA (28, 30). It is not known whether motion of Dna2 disengages the RPA. Because this phenomenon is an important element of the proposed RPA/Dna2/FEN1 pathway of long-flap removal, it deserves additional investigation. Both of these special protein-protein interactions support the role of Dna2 in OFF.

We have shown that if Dna2 is bound to a flap and cannot cleave, it is disengaged by FEN1. Why might this disengagement be necessary *in vivo*? Although Dna2 is a helicase and endonuclease, it has different properties from most enzymes with these activities (30). Helicases generally bind to any single-stranded region large enough to accommodate binding before beginning ATP-dependent tracking (38, 39). Similarly, endonucleases generally bind and cleave directly at their cleavage sites. It has been speculated that both FEN1 and Dna2 have evolved their tracking mechanisms to protect the single-stranded regions of template between Okazaki fragments from endonucleolytic cleavage (30, 40). Because Dna2 and FEN1 are both abundant, soluble proteins, they should compete for newly created flaps. FEN1 has been proposed to cleave most flaps without the aid of Dna2 (5, 34–36). It is possible that the disengagement mechanism ensures that FEN1 enters first, unless the flaps become long enough to be coated by RPA.

Another possible reason is that Dna2 disengagement allows FEN1 to be the dominant nuclease during displacement of the RNA portion of the flap. If Dna2 threads onto a flap ahead of FEN1 while the base of the flap is still only RNA, Dna2 would block FEN1 cleavage. This is likely because Dna2 binding to ssRNA is reported to be slightly greater or at least equal to that of ssDNA (23). The capacity for disengagement would allow FEN1 to cleave within the RNA, instead of having to wait for further displacement to drive Dna2 into the DNA. Also Dna2 is not effective as a nuclease at the very base of a flap (14, 23). If it were threaded onto the flap ahead of the FEN1, it would have to wait until five or more nucleotides of DNA had been displaced before it could make a cut. The FEN1 could be active throughout this process of RNA and DNA removal if it easily disengages any Dna2 that precedes it.

Another possibility is that Dna2 does not readily dissociate from the DNA after it makes a cut in the long-flap pathway. In the cell the helicase activity would constantly be driving Dna2 toward the base of the flap. Because it does not cleave close to the base, it would always have a short segment of the flap on which to bind. If FEN1 were unable to access this short flap because Dna2 remains bound (see Fig. 2), the ligatable product would not be formed unless FEN1 had a way to promote Dna2 dissociation. Frequent failure to generate a ligatable nick would be dangerous, because unprocessed Okazaki fragments are

potential recombination intermediates that can lead to repeat sequence expansion and genome instability (2, 9, 35, 41, 42).

We showed that Dna2 binds unblocked flaps in a tracking mode that allows cleavage (30). It also binds blocked flaps in a non-tracking mode that does not allow cleavage. Some fraction of the Dna2 bound to an unblocked flap may be in the non-tracking mode. FEN1 has the ability to dissociate both from the flaps (see Fig. 4). Dna2 binding in the non-tracking mode would cause potential problems *in vivo* if not removed by FEN1. If Dna2 were to remain bound, it would block FEN1 cleavage. Removal of Dna2 bound in the non-tracking mode is another reason why FEN1 had to evolve a Dna2 dissociation mechanism. RPA may also play a role in regulating the binding modes of Dna2. Further studies will help to uncover the mechanism and regulation of Dna2 binding in both the tracking and non-tracking modes.

Although our results show that FEN1 causes the dissociation of Dna2, they do not definitively prove that Dna2 must dissociate prior to cleavage by FEN1. Recalling that FEN1 and Dna2 interact (15), it is formally possible that a complex can form between the proteins that allow FEN1 to cleave past a Dna2 bound on the flap. Although an unlikely scenario, this would mean that FEN1 could slide down the flap, interact with Dna2 in a way that allows FEN1 cleavage, and then induce the dissociation of Dna2.

It has been proposed previously that RPA stimulation of Dna2 and inhibition of FEN1 orders the reactions of the two nucleases on long flaps (28). Here we propose that the inability of Dna2 to block FEN1 cleavage also properly orders their reactions on short flaps. This allows FEN1 to cleave in an unobstructed manner on RNA flaps and allows the completion of flap removal by FEN1 to produce the nick for ligation. Because Dna2 and FEN1 interact, removal of Dna2 may position FEN1 to carry out RNA or DNA flap cleavage more efficiently. Overall our results support a role for Dna2 in processing at least some fraction of Okazaki fragments. They also suggest that the interplay of Dna2, RPA, and FEN1 is even more tightly orchestrated than previously believed.

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