Flap endonuclease 1 (FEN1) is a structure-specific nuclease that cleaves substrates containing unannealed 5'-flaps during Okazaki fragment processing. Cleavage removes the flap at or near the point of annealing. The preferred substrate for archaeal FEN1 or the 5'-nuclease domains of bacterial DNA polymerases is a double-flap structure containing a 3'-tail on the upstream primer adjacent to the 5'-flap. We report that FEN1 in Saccharomyces cerevisiae (Rad27p) exhibits a similar specificity. Cleavage was most efficient when the upstream primer contained a 1-nucleotide 3'-tail as compared with the fully annealed upstream primer traditionally tested. The site of cleavage was exclusively at a position one nucleotide into the annealed region, allowing human DNA ligase I to seal all resulting nicks. In contrast, a portion of the products from traditional flap substrates is not ligated. The 3'-OH of the upstream primer is not critical for double-flap recognition, because Rad27p is tolerant of modifications. However, the positioning of the 3'-nucleotide defines the site of cleavage. We have tested substrates having complementary tails that equilibrate to many structures by branch migration. FEN1 only cleaved those containing a 1-nucleotide 3'-tail. Equilibrating substrates containing 12-ribonucleotides at the end of the 5'-flap simulate the situation in vivo. Rad27p cleaves this substrate in the expected 1-nucleotide 3'-tail configuration. Overall, these results suggest that the double-flap substrate is formed and cleaved during eukaryotic DNA replication in vivo.

Pathways for DNA replication, recombination, and repair are all proposed to involve DNA flap intermediates (1–8). The creation and resolution of single-stranded flap structures is a preferred method for removal of damaged or mismatched nucleotides. DNA replication requires the synthesis and joining of discontinuous segments, or Okazaki fragments. Each fragment is initiated by an RNA primer that must be removed prior to joining. Synthesis from the upstream fragment is thought to displace the RNA primer and some adjacent DNA into a single-stranded flap (8–11). Processing of the flap intermediate is carried out by the structure-specific flap endonuclease 1 or FEN1 (4).

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† The abbreviations used are: FEN1, flap endonuclease 1; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; MMS, methyl methanesulfonate; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; HhH, Helix-hairpin-Helix.

Cleavage Specificity of Saccharomyces cerevisiae Flap Endonuclease 1 Suggests a Double-Flap Structure as the Cellular Substrate*

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The current model proposed for Okazaki fragment processing indicates that it is a sequential pathway (33–35). Proliferating cell nuclear antigen (PCNA) appears to act as a coordination factor at the replication fork and interacts with different enzymes in a stepwise manner. PCNA is a trimeric, toroidal molecule that surrounds DNA to act as a sliding clamp to increase the processivity of DNA polymerase δ in eukaryotic cells. According to the model, after creation of the flap intermediate, two nucleases collaborate for initiator RNA removal. The helicase/endonuclease Dna2p interacts with FEN1 and replication protein A (RPA), and this enzyme removes a portion of the flap. Of the two endonucleases, only FEN1 has the specificity for cleavage at the base of the flap. PCNA interacts with FEN1 by tethering it to the cleavage site to stimulate catalysis. PCNA then interacts with DNA ligase I to facilitate sealing of the nick.

Cleavege specificities of the members of the FEN1 nuclease family have been examined in vitro (16, 19, 21, 24, 36). FEN1 cleaves nick-flap substrates at two locations: at the base of the flap or at a position one nucleotide into the downstream annealed region. This leaves either a nick or a 1-nucleotide gap product. The nick product is a substrate for DNA ligase I, but the gap would have to be converted to a nick before ligation could occur. This would require additional action by the polymerase and would add steps to the current model for Okazaki fragment processing.

To clarify the activity of FEN1 in DNA replication, we examined the substrate specificity of the FEN1 from Saccharomyces cerevisiae, Rad27p. Previous reports have shown higher cleavage activity and specificity for FEN1 on double-flap structures (16, 19, 21, 24, 36). Furthermore, rad27-G240Dp, the FEN1 from a mutant strain with nearly normal growth rate, cleaves the double-flap substrate but not the conventional nick-flap substrate (23). This suggests the involvement of FEN1 activity in resolving a double-flap structure during replication. However, the role of double-flap structures in facilitation of FEN1 cleavage activity has not been characterized in detail. The wild-type and mutant nucleases have been examined herein, and results support a role for the double-flap structure as a substrate for FEN1 in Okazaki fragment processing.

**EXPERIMENTAL PROCEDURES**

**Materials—**Oligonucleotides, with and without modifications, were synthesized either by Integrated DNA Technologies (Coralville, IA) or by the Midland Certified Reagent Co. (Midland, TX). Radionucleotides [γ-32P]ATP (3000 Ci/mmol) and [α-32P]dCTP (3000 Ci/mmol) were from PerkinElmer Life Sciences. The T4 polynucleotide kinase (labeling grade) and the Klenow fragment of DNA polymerase I were both from Roche Molecular Biochemicals. RNase-free solutions and reagents were from Ambion, Inc. (Austin, TX). All other reagents were the best available commercial grade.

**Cloning—**The wild-type and mutant Rad27p coding regions were PCR-amplified from Rad27-UC19 (provided by Dr. E. Alani at Cornell University). Restriction enzyme sites for EcoRI, NdeI, and XhoI were introduced to assist subcloning (New England BioLabs, MA). The PCR primer sequences used were 5’-GGCAATTCTCAATGTTAGTAAAG-3’ and 5’-GGCGAATTCCTGGTATTCGTCGAA-3’ (Integrated DNA Technologies, Coralville, IA). The amplified product fragments were directly cloned into a T-A vector (Invitrogen). Subsequently, these fragments were excised from the T-A vector by NdeI and XhoI and subcloned into a PET-24b expression vector (Novagen), which added a 6-histidine tag at the C terminus of the proteins.

**Enzyme Expression and Purification—**The PET-24b plasmids containing the wild-type and mutant proteins were transformed into Escherichia coli strain BL21(DE3) codon plus (Stratagene, CA). A single colony was picked and inoculated. Two liters of cultures was grown at 37°C with shaking until the optical density reached 0.5, and the protein was induced by adding isopropyl-1-thio-β-D-galactopyranoside (final concentration of 0.5 mM) and incubating at 37°C for 3 h. The cells were harvested by centrifugation at 1000 × g for 5 min, and the pellet was resuspended in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, 10 mM imidazole, 1 mM DTT, and 1 mM PMSF). Subsequently, the resuspension was lysed using a French press. Cell debris was removed by centrifugation at 10,000 × g for 30 min. The purification was conducted by fast-protein liquid chromatography. The collected supernatant was directly loaded onto a 1-mL TALON column (Qiagen, Chatsworth, CA) equilibrated in lysis buffer. The column was then washed with 5 column volumes of wash buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 20 mM imidazole). Rad27p was eluted with a linear gradient in 10 column volumes of elution buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl, and 250 mM imidazole). The peak fractions were pooled (20 ml), dialyzed in HI-20 mM NaH2PO4 buffer, and applied to a Ni-agarose column (Bio-Rad, CA) equilibrated with HI-20 mM NaH2PO4 buffer. The protein was eluted with a linear gradient of 10 column volumes from 250 mM NaH2PO4 to 30 mM NaH2PO4. About 2 ml of fractions were pooled and dialyzed into HI-30 mM KCl buffer (30 mM Hepes, pH 7.8, 0.5% inositol, 0.25 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, 1 mM PMSF, and 20 mM NaH2PO4). The dialyzed sample was centrifuged at 10,000 × g for 30 min to remove any proteins that were denatured during the dialysis. The supernatant material was loaded onto a 2-ml hydroxyapatite column (Bio-Rad, CA) equilibrated with HI-20 mM NaH2PO4 buffer. The protein was eluted with a linear gradient of 10 column volumes from 30 mM NaH2PO4 to 2 mM NaH2PO4. About 2 ml of fractions were pooled and dialyzed into HI-30 mM KCl buffer (30 mM Hepes, pH 7.8, 0.5% inositol, 0.25 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, and 30 mM KCl). The pooled sample was applied onto a CM-Sepharose column (Amersham Biosciences, Inc.) equilibrated with HI-30 mM KCl buffer, and the elution was carried out with a linear gradient of 10 column volumes from 30 mM KCl to 2 mM KCl.

The rad27-G240Dp preparation contained some inactive truncation products (~20%), as described previously (23). Therefore, the purification scheme was modified for the mutant protein. After the N242-agarose column, the peak fractions of rad27-G240Dp were pooled, dialyzed into HI-30 mM KCl buffer, and loaded onto the Mono-S column (Amersham Biosciences, Inc.). Subsequently, the peak fractions were collected and dialyzed into HI-2 mM (NH4)2SO4 buffer. This sample was applied onto a Phenyl-Sepharose column (Amersham Biosciences, Inc.) equilibrated with HI buffer (30 mM Hepes, pH 7.8, 0.5% inositol, 0.25 mM EDTA, 0.01% Nonidet P-40, 1 mM DTT, and 1 mM PMSF), and rad27-G240Dp was eluted with 10 column volumes of a linear gradient from 2 mM (NH4)2SO4 to 0 mM (NH4)2SO4. A hydroxyapatite column (described as above) was used as the last column. Highly purified Rad27p (>95%) and rad27-G240Dp (~80%) were obtained. The truncation contaminants in the rad27-G240Dp preparation did not possess any nuclease activities. Recombinant human DNA ligase I was a generous gift from the Hayes Laboratory at the University of Rochester.

**Oligonucleotide Substrates—**Oligomer sequences are listed in Table 1. All double-flaps were annealed as described in the figure legends to form various structures, such as nick, nick-flap, gap-flap, double-flap, gap-double-flap, or displacement substrates. For the nick substrate, two primers were annealed to a template such that the upstream and downstream primers formed a nick. Nick-flap substrates were generated because of the inclusion of sequences that are not complementary to the template at the 5′-end of the downstream primer. These sequences are complementary to the 3′-end of the upstream primer, and the upstream primer was annealed to the template to form a nick at the base of the flap. The gap-flap substrates were generated by introducing a 1-nucleotide gap into the nick-flap substrates. After annealing a downstream primer to its corresponding template, the double-flap substrates were created by annealing an upstream primer containing a 3′-end as described above. These substrates contain various lengths of upstream primers with either non-complementary or complementary tails at the 3′-end. The gap-double-flap substrate has a 1-nucleotide gap between the non-complementary 3′-tail and the unannealed downstream 5′-flap. Displacement substrates contain all complementary nucleotides to the template, which includes partial overlapping sequences. These overlapping regions at the beginning of the 5′-end of the downstream primer and the 3′-end of the upstream primer will compete for the same sites on the template. The RNA-DNA chimera displacement substrates contain 12 ribonucleotides at the 5′ region of the template, which mimics the in vitro situation of Okazaki fragments (37). Annealing and labeling conditions were the same as described previously (23). All radiolabeled primers were purified by gel isolation from 15% or 18% polyacrylamide, 7 % urea denaturing gels. ATP PO assay conditions were the same as described previously (23). The enzyme-titration assays were incubated at 30°C for 10 min. Time-course assays were initiated with 1 mM MgCl2. Cleavage-ligation assays were incubated at 30°C for 15 min in the presence of 1 mM ATP. The denatured reactions were resolved on 12% or 15% polyacrylamide, 7 % urea denaturing gels. Each gel was quantitated using a PhosphorImager (Molecular Dynamics) and analyzed using Image-
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# RESULTS

Previous analyses of FEN1 substrate specificity have usually been conducted on nick-flap substrates with a specific structure. The flaps have been fixed through the use of flap sequences that are not complementary to the template. The advantage of a fixed substrate is that the starting material for the cleavage reaction is a single species. However, such substrates differ from the natural substrate produced by strand-displacement synthesis. In the natural substrate, the flap is fully complementary to the template. Depending on the flap length, the substrate can equilibrate to a variety of structures, some with double flaps. To fully understand the substrate specificity of Rad27p, we have tested cleavage on both fixed and equilibrated flap substrates.

**Study of Fixed Double-Flap Structures**—To define structural requirements in the upstream primer, we examined cleavage efficiency and specificity on fixed substrates having various lengths of unannealed 3′-tails (Fig. 1A). To denote that both the 5′-flaps and 3′-tails are not complementary in these substrates, they are represented by gray-textured lines on the schematic representation of the substrate. A double-flap structure containing a 1-nucleotide tail at the 3′-end was cleaved with the highest efficiency (*lanes 11–15*). This substrate preference is evident throughout the range of nuclease concentrations tested. Significantly, the cleavage efficiency on the double-flap structure containing a 1-nucleotide 3′-tail was much greater than that for the conventional nick-flap structure (*lanes 6–10* and *lanes 11–15*). For example, at the highest enzyme concentration tested, the difference was about 4-fold (*lanes 10 and 15*). Henceforth, a substrate with this structure will be denoted as a “3′ 1-nucleotide double-flap.”

## TABLE I

| Oligonucleotide sequences (5′-3′) |
|----------------------------------|
| **Downstream primers**            |
| D1 (34-mer)                      |
| CCAAGGCCACCAGTCACCCCGGAGGCAGCTCTGG |
| D2 (28-mer)                      |
| CCACCGTGCAACCGGACCCACCTCTGG      |
| D3 (28-mer)                      |
| TCACCGTCCACCCGAGCCACCTCTGG       |
| D4 (33-mer)                      |
| CCTTGGCACCAGCGTCCAGAGGACAGAGAGCTCTGG |
| D5 (33-mer)                      |
| TCAATTCCACCCGTCACCCCGAGGCCACCTCTGG |
| D6 (40-mer)                      |
| AGGTCTCGACTAACCCGGCTCACCGAGGCACCTCTGG |

| **Downstream RNA-DNA chimera primers** |
|---------------------------------------|
| C1 (40-mer)                           |
| AGGUCUCGACUACCCCGCTCCACCGAGGCAACCTCTGG |

| **Upstream primers** |
|----------------------|
| U1 (25-mer)          |
| CGACCGTGGCAAGCTTTAATCTAAT |
| U2 (26-mer)          |
| CGACCGTGGCAAGCTTTAATCTG |
| U3 (27-mer)          |
| CGACCGTGGCACGCTTAAATCTG |
| U4 (27-mer)          |
| CGACCGTGGCACGCTTAAATCC |
| U5 (27-mer)          |
| CGACCGTGGCACGCTTAAATCTA |
| U6 (31-mer)          |
| CGACCGTGGCACGCTTAAATTTCAATCCACCC |
| U7 (31-mer)          |
| CGACCGTGGCACGCTTAAATTTCAATTTT |
| U8 (37-mer)          |
| CGACCGTGGCACGCTTAAATTTCAATTTTCTGCAC |
| U9 (37-mer)          |
| CGACCGTGGCACGCTTAAATTTCAATTTTCTT |
| U10 (24-mer)         |
| CGACCGTGGCACGCTTAAATTTCA |
| U11 (25-mer)         |
| CGACCGTGGCACGCTTAAATTTCAAC |
| U12 (26-mer)         |
| CGACCGTGGCACGCTTAAATTTCAAT |
| U13 (31-mer)         |
| CGACCGTGGCACGCTTAAATTTTGGTCT |
| U14 (39-mer)         |
| CGACCGTGGCACGCTTAAATTTTCAATGTCCTGACCTACC |

| **Templates** |
|---------------|
| T1 (54-mer)   |
| GCAGGAGGTGGCGTGCGTGGACGCGGTTGAGTTGAAATTTAGCTGCGACGCTG |
| T2 (68-mer)   |
| GCAGGAGGTGGCGTGCGTGGACGCGGTTGAGTTGAAATTTAGCTGCGACGCTG |

As observed previously for forms of FEN1 (4, 16, 21, 23), Rad27p-directed endonucleolytic cleavage of the nick-flap substrate resulted in two products (*lanes 6–10*). A portion of the substrate molecules was cut at the base of the 5′-flap, releasing a 6-nucleotide oligomer. Another portion was cut at a position one nucleotide into the annealed region, releasing a 7-nucleotide oligomer. The latter cut left a 1-nucleotide gap between the upstream and the downstream primers. This population of products requires synthesis to fill in the gap before ligation would be possible.

The 3′ 1-nucleotide double-flap structure was cut in a single position between the first 2 base pairs in the downstream duplex. This makes a nick product. The substrate with a 2-nucleotide 3′-tail was cut to generate 7- and 8-nucleotide products (*lanes 16–20*) with a slight preference for the 7-mer. An interpretation of this result is that the 2-nucleotide 3′-tail is recognized, albeit inefficiently, as a characteristic of the substrate for cleavage to make the 7-nucleotide oligomer. The 8-nucleotide product may be a consequence of an effort by the nuclease to force the formation of the 3′ 1-nucleotide tail structure to cut at the position defined by that configuration. If the 2-nucleotide 3′-tail substrates were cut with the same specificity in *vivo*, a portion of the products could only be ligated after further synthesis.

In contrast, substrates with 3-nucleotide tails exhibit similar cleavage patterns as the substrates with 6- and 12-nucleotide tails (data not shown). Tails that are 3 nucleotides or longer become “invisible” to Rad27p. The moderate production of 7-nucleotide product (*lanes 21–30*) resembled the results of cleaving the substrate with a gap between a fully annealed upstream primer and the downstream flap (gap-flap substrates in *lanes 1–5*). Overall, both cleavage efficiency and biologically relevant specificity argue that Rad27p has evolved to cleave a 3′ 1-nucleotide double-flap substrate. This is not a sequence-dependent phenomenon as we have tested Rad27p with structures containing different sequences (data not shown).
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Fig. 1. Wild-type Rad27p prefers a double-flap structure containing a 1-nucleotide 3′-tail. A, enzyme titrations of Rad27p (0, 2.5, 5, 7.5, and 10 fmol, as indicated by the triangles) were performed with 5 fmol of 5′-32P-radiolabeled substrates in 20-μl reactions as described under “Experimental Procedures.” The sizes of the substrates and products are listed on both sides of the gel. The same downstream primer (D1) and template (T1) were used in each substrate tested, but different upstream primers containing various lengths of 3′-tails were annealed to D1:T1 to create different double-flap structures (see the schematic representations of the substrates). Lanes 1, 6, 11, 16, 21, and 26 are control lanes without Rad27p. Lanes 1–5 contain gap-flap substrates (D1,T1,U3,D1,T1:U11), lanes 6–10 contain nick-flap substrates (D1,T1:U1), lanes 11–15, 16–20, 21–25, and 26–30 contain double-flap substrates with 1-, 2-, 6-, and 12-nucleotide 3′-tails, respectively (D1,T1:U3,D1,T1:U5,D1,T1:U7,D1,T1:U9). All the 5′-flaps and the 3′-tails in these substrates (labeled with gray-textured lines) were designed to be non-complementary to the template so that they would form fixed structures for Rad27p recognition and cleavage. B, a time-course assay (0, 1, 3, 5, 7, and 10 min, as indicated by the triangles) was performed with 5 fmol of Rad27p and 5 fmol of 5′-radiolabeled substrates in 20-μl reactions as described under “Experimental Procedures.” Lanes 1, 1′, 13, and 19 are control lanes at 0 min without 1 mM MgCl2. Lanes 1–6 contain the conventional nick-flap substrate (D1,T1:U3,D1,T1:U5,D1,T1:U7,D1,T1:U9). Lanes 7–12 contain a gap double-flap substrate having a 1-nucleotide gap between the 5′-flap and the 3′-tail (D1,T1:U5,D1,T1:U7,D1,T1:U9). Lanes 13–18 contain a 1-nucleotide double-flap substrate with a non-complementary tail (D1,T1:U5,D1,T1:U7), and lanes 19–24 contain a substrate with a complementary 3′-terminal nucleotide tail (D1,T1:U5,D1,T1:U7). This complementary tail is illustrated as a solid black line to differentiate it from the other tails, all of which are non-complementary to the template.

Cleavage of Substrates with 1-Nucleotide 3′-Tails—Because initial results highlighted a substrate having an upstream primer with a 1-nucleotide 3′-tail, we further examined substrates having the 3′-tail but with other structural variations. In one variation, we introduced a 1-nucleotide gap between the 3′-tail and the 5′-flap on the downstream primer, called a gap double-flap structure. In another, the 3′-1-nucleotide flap was made complementary to the template. A time-course assay was performed with these substrates (Fig. 1B). The cleavage pattern of the gap double-flap structure (lanes 7–12) was similar to that of a nick-flap substrate (lanes 1–6). Both 6- and 7-nucleotide products were formed in both cases; however, the rate of cleavage was about 2-fold higher with the gap double-flap substrate (lanes 6 and 12). The non-complementarity of the 3′-terminal nucleotide of the gap double-flap substrate apparently enhances the level of cleavage as compared with the nick-flap substrate, but cleavage does not generate specifically one product.

For overlapping double flaps, the cleavage specificity was the same irrespective of the complementarity at the 3′-end. Interestingly, the cleavage rate of the substrate with the non-complementary terminus was slightly faster (compare lanes 14–18 to 20–24). This phenomenon probably occurs because a complementary tail will compete with the downstream-annealed region to create different alternative structures, but a non-complementary tail will fix the substrate in the configuration preferred by the nuclease for cleavage.

Examination of Substrates Likely to Be Found in Vivo—Flaps generated during Okazaki fragment processing in vivo are proposed to result from strand-displacement synthesis. Consequently, they should be complementary to the template. To test substrates of this type, we designed various upstream primers to be fully complementary to the template but with sequences that overlapped those of fully complementary downstream primers. The appearance of the substrate diagrammed in Fig. 2A shows the configuration immediately after annealing an upstream primer to a substrate with a downstream primer. This initial structure is expected to equilibrate to all of the possible annealing configurations including double flaps and nick 5′-flaps, and the cleavage specificity of Rad27p reflects that equilibration.

Overall, as the complementary flap length increases, the level of cleavage decreases. For example, the percent product conversion drops from −15% to 8% as the length of the 3′-tail is increased from one nucleotide to twelve nucleotides at the 2.5-fmol level of enzyme (Fig. 2A). This effect is most easily seen from the amount of 28-nucleotide starting material degraded at each nuclease concentration (lanes 11–30). When the levels of cleavage with the fixed double-flap (as shown in Fig. 1A) and the equilibrating substrates were quantitated and compared, the fixed double-flap substrates exhibited higher cleavage efficiencies. Therefore, fixing both flaps with non-complementary sequences on a substrate appears to contribute to the enhanced cleavage efficiency. Presumably, the equilibration among flap configurations that are either favorable or unfavorable leads to a relatively lower cleavage efficiency. Furthermore, in every case of these equilibrating substrates, there is a single predominant cleavage product. A logical interpretation of these observations is that the substrates equilibrate by branch migration, but for each substrate, only one intermediate allows cleavage. Because the substrate with the longer flaps can form more intermediates, the steady-state concentration of the cleavable intermediate is the lowest in that substrate. The lower concentration would then account for the observed slight decrease in cleavage rate.

The size of the product implies the structure of the cleaved substrate. In each case, the substrate could equilibrate to a double-flap structure containing a 3′-1-nucleotide tail (lanes 11–30), which is then efficiently cleaved. This is consistent with the substrate specificity indicated by analyses with non-complementary flaps described above. We used oligomeric size standards to verify the size of each product (data not shown) and found that each oligonucleotide released from the downstream primer is the same length as the invading flap from the
stream primers (D2:T1:U10), and substrate with a 1-nucleotide gap between the upstream and the downstream displacement substrate (D2:T1:U2) as in part A. Annealing of the upstream primers before the upstream primers. The complementary flap structure is formed by the sequences tested here are complementary to the template (as illustrated by the black lines), and they were annealed according to the ‘Experimental Procedures’ in which the downstream primers were annealed before the upstream primers. The complementary flap structure allows displacement and branch migration. Lanes 1–6, 11, 16, 21, and 26 are the control lanes without Rad27p. Lanes 1–5 contain a gap substrate with a 1-nucleotide gap between the upstream and the downstream primers (D2:T1:U10), and lanes 6–10 contain a nick substrate (D2:T1:U11). Lanes 11–15, 16–20, 21–25, and 26–30 contain displacement substrates with 1-, 2-, 6-, and 12-nucleotide 3'-tails, respectively (D2:T1:U3 and D3:T1:U2, respectively) that would result from the equilibration or branch migration of the 1-nucleotide displacement substrate. We then tested these substrates in a time-course assay with a nick substrate control (Fig. 2B). As expected, we found that the cleavage pattern of the displacement substrate (lanes 7–12) is the same as the fixed intermediate containing a 3'-tail (lanes 13–18). The presence of the 3'-tail improved the efficiency of cleavage (comparison of lanes 1–6 to 7–12 or 13–18). The nick-flap intermediate (lanes 19–24) produces both 1- and 2-nucleotide products as observed previously. Because a non-complementary nucleotide had to be introduced to fix the 5'-end of the downstream primer in a single conformation, the mononucleotide migrates differently than the product of the other substrates (lanes 19–24). Products of the complementary 5'-end are designated as (C) on the left side of the gel whereas the ones for the non-complementary end are labeled (NC) on the right side of the gel.

The rad27-G240Dp Mutant Only Cleaves a 3'-1-Nucleotide Double-Flap Substrate—The rad27-G240Dp mutant was originally isolated from a mutagenesis screen (23). Unlike the rad27A null mutant, which was temperature-sensitive for growth and hypersensitive to methyl methanesulfonate (MMS), the rad27-G240Dp mutant displayed wild-type colony sizes without temperature-sensitive phenotypes and was relatively insensitive to MMS. In previous biochemical studies, rad27-G240Dp lacked significant exonuclease activity and exhibited very low endonuclease activity on a conventional nick-flap substrate. However, rad27-G240Dp effectively cleaved a double-flap substrate (23). To define the activity of rad27-G240Dp on double-flap substrates in more detail, we performed an enzyme-titration assay with rad27-G240Dp and the fixed flap substrates as described in Fig. 1A. The rad27-G240Dp only cleaved a double-flap structure containing a 3'-1-nucleotide tail (Fig. 3A, lanes 12–15). This substrate stimulates rad27-G240Dp-directed cleavage by almost 40-fold as compared with the cleavage level with the traditional nick-flap substrate at the highest titration point, whereas wild-type Rad27p only exhibits a 3-fold stimulation (Fig. 3A, lanes 10 and 15 as compared with Fig. 1A, lanes 10 and 15).

The rad27-G240Dp was then tested on the same set of displacement substrates as used in Fig. 2A. Substrates with a variety of flap lengths were cleaved (Fig. 3B, lanes 11–25). As observed with the wild-type enzyme, cleavage efficiency decreased as the flap length increased. For instance, the percent product formation decreases from ~67% to 9% as the 3'-tail changes in length from one nucleotide to twelve nucleotides at the highest level of enzyme tested. This decrease was more severe with the mutant protein (approximate 7-fold between 1- and 12-nucleotide displacing substrates with rad27-G240Dp and 1.5-fold with Rad27p). This is best observed when comparing the disappearance of the starting material on the gel. Because results with the fixed substrates showed that rad27-G240Dp only cleaves a 3'-1-nucleotide tail substrate, cleavage specificity of FEN1 suggests the cellular substrate.
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Assessment of Double-Flap Substrates in a Cleavage-Ligation Assay—To further understand the role of the double-flap substrates in Okazaki fragment processing, we reconstituted a partial system in vitro with model substrates, Rad27p, and human DNA ligase I. Both nick-flap and double-flap substrates, having either complementary or non-complementary 3’-tails, were tested (Fig. 4). In this system, the formation of the ligation product is dependent upon the Rad27p cleavage. We performed an assay with a titration of Rad27p with or without human DNA ligase I. The Rad27p titration level was controlled to exhibit minimal exonuclease activity. This allowed any nicks produced by exonuclease cleavage to support ligation. The final amount of ligation was then dependent on the efficiency of exonuclease cleavage and the proportion of cleavage products that could be ligated.

After Rad27p was incubated with the 3’-radiolabeled nick-flap substrate (Fig. 4A, lanes 1–5), 27-, 28-, and 29-nucleotide products were visible on the denaturing gel. Because of the 3’-radiolabel, the lengths of these products suggest the structure of the annealed downstream primer after cleavages and allow the structure of the double-stranded product to be inferred. The 29- and some of the 28-nucleotide products were the results of endonucleolytic cleavage at either the base of the flap or one nucleotide into the annealed region, respectively. The 27- and the remaining 28-nucleotide products were the consequences of exonucleolytic cleavage of either the 28- or 29-nucleotide fragment. Thus, the 29-nucleotide product is the downstream primer of the nick product from the nick-flap substrate, and it is this structure that is recognized and sealed by DNA ligase I. When DNA ligase I was added into the nick-flap reactions (Fig. 4A, lanes 6–10), 28- and 29-nucleotide products accumulated while the 29-nucleotide product was ligated to form the 54-nucleotide product. The presence of DNA ligase I slightly stimulated Rad27p cleavage activity as indicated by a comparison of lanes 1–5 and 6–10. FEN1 is known to

Fig. 3. The rad27-G240Dp mutant can only cleave the fixed double-flap structure containing a 1-nucleotide 3’-tail and the displacement substrates. A, the same assay conditions and substrates were utilized as in Fig. 1A, except that the enzyme titration is higher for rad27-G240Dp in 20-μl reactions (0, 5, 10, 50, and 100 fmol, as indicated by the triangles). Lanes 1, 6, 11, 16, 21, and 26 contain substrates that have not been reacted with rad27-G240Dp. B, the same assay conditions and substrates were used as in Fig. 2A and in part A of this figure, except that the gap substrate was not included. Lanes 1, 6, 11, 16, and 21 are substrate-only controls.
dissociate slowly from its cleavage product (38). Perhaps DNA ligase I competes with FEN1 for the nick substrate, which allows FEN1 to cycle more efficiently to the next substrate.

Two cleavage products were seen when either of the two double-flap substrates was assayed. The 28-nucleotide fragment was from the nick product resulting from cleavage of one nucleotide into the annealed downstream region, and the 27-nucleotide segment was an exoneucleolytic product. As expected, the 28-nucleotide product did not accumulate in the presence of DNA ligase I, because most of it had been ligated to the nick product containing a non-complementary 3'-tail (Fig. 1A, lanes 6–20). A possible explanation is that rad27-G240Dp lacks exonuclease activity and does not bind well to nicks (data not shown). Therefore, it could be allowing the DNA ligase I more access to the substrate.

**Determination of the Roles of the 3'-Tail in Rad27p Recognition and Cleavage**—We were interested in examining the significance of the 3'-hydroxyl group and the position of the sugar ring in the cleavage reaction. Therefore, we designed and assayed substrates with various modifications to the 3'-carbon located on the last nucleotide of a complementary 3'-tail. The structures of the modifications are given in Fig. 5A. The control experiment with a nick-flap substrate containing a 5'-6-nucleotide flap produced the expected 6- or 7-nucleotide products (lanes 1–5, Fig. 5B). The corresponding unmodified double-flap substrate exhibited a higher level of cleavage and specificity (lanes 6–10). The existence of the 3'-hydroxyl group is important, but this moiety is not required for the recognition and cleavage of the structure because the deoxy modification on the last nucleotide did not affect enzyme specificity (lanes 36–40). However, the hydroxyl group was found to moderately increase the level of cleavage. The percent product formation decreased with the deoxy modification from ~91% to 68% at the 5-flap level of enzyme. This level was restored to 82% with the modification containing a hydroxyl group at the 2'-position instead of the 3'-position (comparison between lanes 6–10 and 36–40 and between lanes 6–10 and 41–45). In addition to the hydroxyl group, the correct positioning of the sugar ring from the 3'-tail in the recognition site of the enzyme contributes to the increased level of cleavage. The modification containing a 3' to 5' polarity on the last nucleotide of the upstream primer causes the cleavage level to decrease as compared with the unmodified double-flap substrate (lanes 6–10 and 21–25).

Cleavage specificity and the site of cleavage are determined by the spatial occupancy with any structure that mimics the nucleotide on the 3'-tail within the enzyme binding site. When bulky adducts on the 3'-carbon were tested with Rad27p, the site of cleavage changed to make an 8-nucleotide product (lanes 11–15, 16–20, 26–30, and 31–35). This suggests that the enzyme attempts to fit the substituent into the binding site by forcing the formation of a “pseudodouble-flap” structure. This structure consists of a 7-nucleotide flap on the downstream primer and a 3'-adduct that forms a 3'-tail on the upstream primer. This makes the enzyme cut between the first two base pairs of the annealed region to make the 8-nucleotide fragment. Particularly for the longer modifiers, i.e. thiol and C7am, the primary product has the length of 8 nucleotides. This indicates that both the thiol and C7am adducts behave more like nucleotides than the other modifications.

To characterize this phenomenon further, we utilized the C7am modifier and constructed different possible combinations of structures containing C7am at the 3' terminus of the upstream primer (Fig. 5C). Nick-flap and complementary (on the 3'-tail, not the 5'-flap) double-flap substrates were used as controls in this enzyme-titration assay (lanes 1–5 and 6–10). The complementary double-flap substrate containing a C7am modification at the 3'-end produces primarily an 8-nucleotide fragment (lanes 11–15). This observation is consistent with the previous results. When the tail consisted only of the modifier without the last nucleotide, a 7-nucleotide fragment was generated (lanes 16–20). The non-complementary 5'-flap in lanes 16–20 contains two nucleotides that are different from the ones in lanes 1–15. Therefore, the 7-nucleotide fragment migrates slightly slower. A non-complementary 3'-tail containing one non-complementary nucleotide and a C7am modifier in a double-flap substrate was also tested (lanes 21–25). The level of cleavage for this pseudo double-flap substrate containing one non-complementary nucleotide and a C7am modifier at the 3'-end was low, with specificity resembling that of a substrate containing a non-complementary 2-nucleotide 3'-tail (Fig. 1A, lanes 16–20 and Fig. 5C, lanes 21–25). This confirms that a bulky adduct can indeed act like a nucleotide to define the cleavage site. However, the presence of the modifier causes the enzyme to lose cleavage efficiency (compare the cleavage levels between lanes 6–10 and 16–20 in Fig. 5C and the cleavage levels between lanes 16–20 in Fig. 1A and lanes 21–25 in Fig. 5C).

We also considered whether the modifier would behave like a normal nucleotide in a displacement substrate. Interestingly, the modifier does not behave like a normal nucleotide in facilitating the formation of the double-flap structure, as indicated by a nonspecific cleavage pattern (lanes 26–30). When we assayed the same substrate with no modifier, we obtained the same cleavage pattern as shown in Fig. 2A where only one product is formed (data not shown). This implies that Rad27p is participating in the formation of a double-flap substrate by recognizing, positioning, and binding to the 3' terminus of the upstream primer. Complementary sequences were introduced into the displacement substrate in lanes 26–30. This caused altered product migration.

In summary, the correct orientation of the sugar ring and the existence of the 3'-hydroxyl group on the tail both contribute to the efficiency of cleavage. The length and general shape of the 3'-end region of the upstream primer strongly influences cleavage specificity. A genuine nucleotide at the 3' terminus appears necessary for the nuclease to effectively recognize or facilitate formation of the double-flap structure in displacement substrates.

**Comparison between DNA-DNA and RNA-DNA Displacement**—Because Okazaki fragments are initiated by RNA, and FEN1 might participate in its removal, we assessed the cleavage specificity of FEN1 on RNA-initiated substrates. Natural RNA primers have been estimated to be 8–12 nucleotides long (37). For this study, 12 ribonucleotides have been used to initiate the downstream primer (depicted in Fig. 6 as the textured box in the schematic representation of the substrates), and a
corresponding DNA sequence has been generated to act as a control. Different lengths of 3'-invading primers have been employed, and they are 1-, 6-, and 14-nucleotide fragments long. Rad27p was expected to cleave within the RNA except in the substrate with the 14-nucleotide upstream primer.

We observed cleavage products in both RNA-terminated and full DNA substrates corresponding to the formation and cleavage of a 3'-1-nucleotide double-flap intermediate (Fig. 6). Cleavage within the RNA occurred with similar efficiency and specificity as within the DNA (lanes 16–25 and 26–30). The RNA-
containing substrates are distinguished by sensitivity to a low level of RNase contamination (lanes 16–30), which is particularly evident in the 14-nucleotide displacement substrate (lanes 26–30). However, this contaminating activity is minimal as compared with the level of FEN1 cleavage, so the result should be valid. The product fragments of DNA and RNA have different mobilities on a polyacrylamide gel. They have been labeled accordingly in Fig. 6.

**DISCUSSION**

We found that Rad27p, a flap endonuclease from *S. cerevisiae*, prefers a double-flap substrate containing a 1-nucleotide 3'-tail (Fig. 1A). The rate of cleavage is higher than that with any other tested substrates, including the nick-flap substrate traditionally used to assay this nuclease. Rad27p exhibits precise cleavage with a double-flap structure regardless of sequence, cutting exclusively one nucleotide into the annealed region of the downstream primer. In contrast, the conventional nick-flap structure is cut at two different sites, only one of which is a substrate for ligation.

To examine the substrate recognition mechanism in a more biologically relevant context, we have tested substrates that contain complementary flaps. Reynaldo et al. (39) have recently shown, when one oligonucleotide primer replaces another on a template in vitro at temperatures below Tm, the replacement occurs by sequential nucleotide displacement, causing branch migration. Because each equilibrating intermediate contains the same number of base pairs, they are presumably at similar energy levels. This suggests that they can readily interconvert, and each configuration is present in similar concentrations. We have observed that Rad27p only recognizes one intermediate that contains a 1-nucleotide 3'-tail, and the cleavage site is exclusively between the first two base pairs of the downstream duplex (Fig. 2A).

Because the cleavable substrate is consumed by the enzyme, we assume that it is replenished by the equilibration process. However, we do not know the rate at which the replenishment occurs. If equilibration is rapid as compared with the cleavage reaction, all of the substrates can be converted readily to product. In contrast, only a portion of the substrates will be cleaved rapidly if the equilibration rate is much slower than the cleavage rate. Moreover, the amount of rapidly formed product should be inversely proportional to the length of the displacing flaps. From our observations, it appears that the equilibration is faster than the cleavage reaction because the nuclease cleaves equilibrating substrates with similar efficiencies irrespective of flap lengths.

There are many factors that could influence equilibration in *vivo*, such as temperature, facilitation of a particular structure by FEN1, or the presence of other proteins. Both the nuclease and the DNA polymerase are likely to influence the substrate configuration. Research on the 5'-nuclease domain of *Taq* DNA polymerase in bacteria suggests that the polymerase domain regulates the 5'-nuclease activity by adding stringency to substrate recognition and enhancing binding (19, 20). Ma et al. (40) have discovered a new substrate binding mode for the thermostable DNA polymerases called the 5'-nuclease mode. This is different from the polymerizing and editing mode described previously and infers a close interaction between the nuclease and polymerase domains in prokaryotes. This model suggests that the transition of the enzyme-substrate complex from the polymerizing mode to the 5'-nuclease mode requires the presence of the 5'-nuclease domain and the double-flap substrate (40). Thus, from bacterial homologues of FEN1, we know that the nuclease and the polymerase work in close coordination. This indirectly implies a role for a polymerase in participating in the equilibration step to form the preferred substrate for the nuclease.

Among fixed substrates, the mutant nuclease rad27-G240Dp cleaves only a 3'-1-nucleotide double-flap structure (Fig. 3A). Because it can cleave different displacement substrates with the same specificity as the wild-type nuclease (Fig. 3B), the intermediate captured and cleaved by Rad27p is also the 3'-1-nucleotide double flap. The cleavage level decreases noticeably with increasing flap length for the rad27-G240Dp mutant. One possible explanation is that the G240D mutation is located at the conserved Helix-hairpin-Helix (HHH) motif found in proteins that mediate non-sequence-specific interactions with DNA (41, 27). The HhH motif has been found in all DNA-binding proteins. A structural study of the *Pyrococcus furiosus* FEN1 reveals a H3TH motif (similar to the HhH motif), which was proposed to bind to double-stranded DNA upon reaching the flap junction (27). This suggests that the G240D mutation causes a defect in the ability to recognize the 1-nucleotide intermediate during the branch migration process. It explains why the cleavage of longer flaps is adversely affected.

Previous studies using a 5'-nuclease domain of *Taq* DNA polymerase demonstrated that the last two base pairs of an upstream duplex and a 3'-unannealed tail comprise the preferred substrate (20). To analyze the role of the 3'-tail structure on substrate recognition by FEN1, we examined Rad27p cleavage specificity on substrates with chemically modified 3'-ends (Fig. 5A). Unlike FEN1 homologues in eubacteria and archaea (19), the 3'-OH is not absolutely required for Rad27p cleavage. However, we demonstrated that both the presence of the 3'-OH and the correct positioning of the sugar ring on the tail increase the level of cleavage by Rad27p. When bulker adducts were assayed with Rad27p, the cleavage sites were altered, generating longer fragments. This suggests that the substituent is acting as a “pseudo nucleotide” and fits into the catalytic cleft of the enzyme, which changes the cleavage specificity. We concluded that being able to occupy the enzyme binding site spatially with any moiety that mimics the length of a real nucleotide is sufficient to determine the cleavage site on a substrate (Fig. 5C).

However, an equilibration substrate containing a modified 3'-end (Fig. 5C) was cleaved with less specificity as compared with the unmodified equilibration substrate, which was
cleaved at only one site. Perhaps the 3′-nucleotide on the tail is required for the nuclease to recognize or facilitate double-flap substrate formation. Spatial occupancy of the enzyme binding site with other structures is not sufficient to allow FEN1 to recognize the correct cleavage configuration with complete accuracy. Further examination will be needed to elucidate this mechanistic hypothesis.

According to the current model for Okazaki fragment processing, PCNA acts as a targeting factor for replication proteins. The model suggests that each enzymatic reaction occurs in a sequential manner (34, 35). FEN1 has to process the displaced flap intermediate before a DNA ligase can seal the nick. FEN1 cleavage of a nick-flap substrate in vitro leaves a 1-nucleotide gap in a population of products. This implies that another round of synthesis would be required prior to ligation. After specific cleavage of the double flap at a position one nucleotide into the annealed region of the downstream primer, the 3′-tail can anneal and generate a nick, which is suitable for ligation every time. Studies of FEN1 homologues have also shown that the double-flap structure is the most efficiently cut and that cleavage occurs in a unique position that produces a ligatable nick (16, 19, 24, 36). Kaiser et al. (19) also showed that all of the cleavage products by various FEN1 homologues on a single double-flap substrate could be joined by T4 DNA ligase. We have found that the Rad27p cleavage product of the 3′-quence double-flap substrate allows the highest ligation efficiency by human DNA ligase I as compared with other structures tested.

The rad27-G240Dp also displayed the highest cleavage efficiency with a double-flap substrate (Fig. 4B). The traditional nick-flap substrate was cut very poorly by rad27-G240Dp, preventing subsequent ligation. Because rad27-G240Dp has a defect in exonuclease activity and has lower binding to nick substrates (data not shown), it should not compete with DNA ligase for the nick product made by cleavage of the double-flap substrate. This explanation is consistent with the high level of ligation observed with the mutant FEN1. It also provides another reason why the mutant protein supports growth and does not exhibit any temperature-sensitive phenotypes. The near normal growth rate of the rad27-G240D mutant strain, together with the specificity of rad27-G240Dp for cleaving only a double-flap substrate, strongly suggests that the double-flap structure is the biological substrate of FEN1.

There is also structural evidence that the double flap is the in vivo substrate for FEN1 nucleases. A study of the T5 5′-nuclease structure has suggested that there could be room for an additional base at the primer terminus (26). Recent analysis of the interaction between the 5′-nucleotide domain of bacterial DNA polymerase I and different 3′-tailed substrates also supports the binding of 1–2 nucleotides at the 3′ terminus in the enzyme binding pocket (15).

We have shown that Rad27p can cleave flaps that are initiated by RNA. Cleavage can occur within or adjacent to the RNA segment. The RNA has little effect on the efficiency or specificity of the cleavage reaction (Fig. 6). This demonstrates that FEN1 is capable of removing initiator RNA in addition to DNA. In vivo, FEN1-directed RNA removal may be only one of several redundant pathways. Previous studies indicated that RNase H is responsible for RNA primer removal (42), but recent genetic studies show that the deletion of the RNase H catalytic subunit did not affect cell viability in yeast (43). This suggests that other enzymes are also involved in RNA removal, one of which is Dna2p.

Dna2p is a multifunctional protein that possesses helicase, ATPase, and single-stranded DNA-specific endonuclease activities. S. cerevisiae Dna2p genetically interacts with FEN1, polymerase α, and its accessory factor, Cth4 (44, 45). Biochemically, Dna2p and FEN1 coimmunoprecipitate, and the nuclease activity of Dna2p is essential in vivo (44, 46). Like FEN1, Dna2p also recognizes a flap structure; however, it cleaves within the single-stranded DNA region (47, 46). Although Dna2p does not cleave RNA, flaps that contain RNA enhance endonuclease activity at the adjacent DNA, and this suggests that Dna2p employs its endonuclease activity to participate in removing the RNA primer during Okazaki fragment processing (47). Recently, Dna2p was shown to be stimulated by replication protein A (RPA) in S. cerevisiae (34). In a newly proposed RPA-governed nuclease switching model for Okazaki fragment processing (34), displacement of a flap to lengths greater than 27 nucleotides is envisioned to attract RPA, which
inhibits FEN1 but stimulates Dna2p for cleavage. This generates a short flap (about 5–7 nucleotides) that resists binding by RPA and cleavage by Dna2p. FEN1 can remove the remaining flap to produce a substrate for ligation. The RNase capacity of FEN1 suggests that some RNA-containing flaps, not attaining lengths sufficient for the switching pathway, are cleaved by FEN1.

Our studies have led us to suggest refinements to the model of eukaryotic Okazaki fragment processing (34). As the polymerase δ holoenzyme extends the upstream fragment, the initiator RNA and DNA of the downstream fragment are displaced to form a flap intermediate. Sufficiently long flaps will be coated by RPA, allowing for recruitment of Dna2p, which cleaves within the flap. Dna2p cleavage generates a shorter displaced flap intermediate for FEN1. Because the displaced flap is complementary to the template, it is capable of branch migrating to form numerous interconverting structures. Because the equilibrating process is likely to be rapid, the presence of replication proteins, such as the nucleosome itself or polymerase, may play a role in facilitating the formation of the preferred substrate. Our model suggests that both RNA-initiated flaps and those without RNA exhibit similar interconversions. FEN1 then recognizes the double-flap intermediate containing a 1-nucleotide 3′-tail and cleaves to make a nick substrate for the final ligation step (Fig. 7).

In summary, we report here that a 3′-1-nucleotide double-flap structure is the preferred substrate for eukaryotic FEN1 in vitro, as determined by ligation activity and specificity. The rad27-G240D mutant strain exhibits near-normal growth, but the mutant protein cleaves only the double-flap substrate, suggesting that the double-flap structure is a key substrate in vivo. The formation and cleavage of the double-flap substrate may require the coordination of displacement synthesis and nuclease action. This has been observed in prokaryotic DNA polymerases with 5′-nuclease domains. In the eukaryotic system, FEN1 and the polymerase are two separate enzymes. Whether or not the same type of mechanistic interaction between the 5′-nuclease and the polymerase is conserved in eukaryotes remains to be determined. In addition, the eukaryotic replication machinery is far more complex than the prokaryotic system. Involvement of other proteins besides the polymerase might contribute to the formation of the double-flap substrate in vivo. With the discovery that Dna2p and Rad27p interact both physically and functionally in Okazaki fragment processing, many more mechanistic details need to be clarified.

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