Direct Visualization of RNA-DNA Primer Removal from Okazaki Fragments Provides Support for Flap Cleavage and Exonucleolytic Pathways in Eukaryotic Cells*

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During DNA replication in eukaryotic cells, short single-stranded DNA segments known as Okazaki fragments are first synthesized on the lagging strand. The Okazaki fragments originate from ~35-nucleotide-long RNA-DNA primers. After Okazaki fragment synthesis, these primers must be removed to allow fragment joining into a continuous lagging strand. To date, the models of enzymatic machinery that removes the RNA-DNA primers have come almost exclusively from biochemical reconstitution studies and some genetic interaction assays, and there is little direct evidence to confirm these models. One obstacle to elucidating Okazaki fragment processing has been the lack of methods that can directly examine primer removal in vivo. In this study, we developed an electron microscopy assay that can visualize nucleotide flap structures on DNA replication forks in fission yeast (Schizosaccharomyces pombe). With this assay, we first demonstrated the generation of flap structures during Okazaki fragment processing in vivo. The mean and median lengths of the flaps in wild-type cells were ~51 and ~41 nucleotides, respectively. We also used yeast mutants to investigate the impact of deleting key DNA replication nucleases on these flap structures. Our results provided direct in vivo evidence for a previously proposed flap cleavage pathway and the critical function of Dna2 and Fen1 in cleaving these flaps. In addition, we found evidence for another previously proposed exonucleolytic pathway involving RNA-DNA primer digestion by exonucleases RNase H2 and Exo1. Taken together, our observations suggest a dual mechanism for Okazaki fragment maturation in lagging strand synthesis and establish a new strategy for interrogation of this fascinating process.

In eukaryotic cells, DNA pol2ε and pol δ direct the synthesis of leading and lagging strand DNA, respectively (1, 2). The antiparallel nature of DNA and the unique 5’ to 3’ direction of DNA synthesis by all DNA polymerases make the synthesis of leading strand continuous and lagging strand discontinuous. In the lagging strand, Okazaki fragments are synthesized first (3). The average size of Okazaki fragments in eukaryotic cells is ~150–200 nucleotides (nt) (4). Because DNA polymerases lack de novo DNA synthesis activity, each Okazaki fragment contains an RNA-DNA primer at its 5’-end, and this primer is synthesized with low fidelity by primase-DNA pol α complex (5–7). DNA ligase I is responsible for joining Okazaki fragments together to form a continuous lagging strand. Because DNA ligase I is unable to join DNA to RNA, the RNA-DNA primers must be removed from each Okazaki fragment to complete lagging strand DNA synthesis and maintain genomic stability.

The mechanism underlying the removal of RNA-DNA primers from Okazaki fragments remains uncertain. Over the previous 20 years, three models have been proposed to explain how these primers are removed (8). In the first model, the RNA-DNA primers are hydrolyzed directly by RNase H2 and DNA exonucleases, such as Fen1 (the exonuclease pathway), while the RNA-DNA primers remain annealed to the template strand. This model is reminiscent of RNA primer removal in prokaryotes. In Escherichia coli and bacteriophage T4 and T7, the short RNA primers at the 5’-end of Okazaki fragments are hydrolyzed directly by the 5’- to 3’-exonuclease activity of DNA pol I, RNase H, and T7 gene 6 DNA exonuclease, respectively (9–11). Surprisingly, budding yeast Saccharomyces cerevisiae cells are viable when both the Fen1 and RNase H2 genes are deleted (12). This observation suggests either that S. cerevisiae cells possess redundant RNA and DNA exonucleases for primer digestion or that the exonuclease pathway is not a major pathway in the primer removal event. The second and third models (the flap pathway) suggest that the RNA-DNA primers are first displaced and generate flap structures through DNA pol δ-mediated strand displacement DNA synthesis, and the flap structures are subsequently cleaved by the flap endonucleases Fen1 and Dna2. The flap pathway is further subdivided into the short flap and long flap pathways (8). In the short flap pathway, Fen1 and pol δ work together to remove the RNA primers (13–15). In the long flap pathway, Dna2 first cleaves replication protein A (RPA)-coated flaps of ~30 nt or longer, resulting in a short flap of ~5–7 nt. This short flap is then cleaved by Fen1 (16). Support

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‡The abbreviations used are: pol, polymerase; nt, nucleotide(s); BND-cellulose, benzoylated naphthoylated diethylaminoethyl cellulose; RPA, replication protein A; ssDNA, single-stranded DNA.

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A

What exonuclease(s)?
Are the RNA-DNA primers displaced to become flap structures in vivo?
Are flaps cleaved by Dna2 and Fen1?
DNA ligase I

B

wt

C

D

E

F

G

H

fen1⁻

I

J

K

L

dna2⁻

M

N

O

P

fen1⁻dna2⁻
for the flap pathway is largely based on the following evidence. 1) Fen1 and Dna2 prefer to cleave flap structures in vitro (16–22). 2) DNA replication appears to be defective in fen1 and dna2 cells (12, 23–27).

Although significant progress has been achieved in understanding the processing of Okazaki fragments, the exact pathway involved in the removal of the RNA-DNA primers in vivo has not been finally determined, and several critical questions relevant to this event still remain to be answered (28). If the exonuclease pathway plays a role in removing the RNA-DNA primers, then the DNA exonucleases responsible for hydrolyzing the DNA portion of the RNA-DNA primers have not been definitively identified. In yeast, Dna2 and Fen1 do not appear to participate in the exonuclease pathway because yeast Dna2 and Fen1 lack or have very weak double-stranded DNA exonuclease activity (29, 30). Regarding the flap pathway, direct in vivo evidence demonstrating that the RNA-DNA primers are displaced to form flap structures and that the flap structures are subsequently cleaved by Dna2 and Fen1 is lacking. Furthermore, the mechanism to completely remove the mutation-prone RNA-DNA primers also remains to be resolved.

In the present study, an electronic microscopy (EM) approach was used to investigate the mechanism underlying Okazaki fragment processing. EM imaging is a relatively powerful technique for observing the fine structures of replication forks (31, 32). Here, we first demonstrated that flap structures are generated on the lagging strand in replication forks. We found that the flap structures in forks significantly and progressively increased from wild type (WT) to rnh201, exo1, fen1, exo1–rnh201, dna2, and fen1–dna2 cells. The flap structures were almost exclusively located on one strand of the fork. The median/mean length of these flaps in WT cells was ~41/51 nt. Taking these results together with Fen1 and Dna2 being flap endonucleases and Rnase H2 and Exo1 being RNA and DNA exonucleases, respectively, we conclude that the RNase H2- and Exo1-mediated exonucleolytic digestion and the Dna2- and Fen1-mediated flap cleavage are utilized by eukaryotic cells to remove the RNA-DNA primers.
Results

A Flap-like Structure Is Observed by EM in Replication Forks—To determine whether the flap pathway is indeed used by eukaryotic cells to remove RNA-DNA primers from Okazaki fragments (Fig. 1A), we first examined whether flap structures are formed in the replication forks in WT fission yeast cells. To describe the bona fide features of the flap structures, all of the examined replication forks were observed in a form that was as close to the natural state as possible by ensuring the following. 1) All of the genomic DNA used to examine the replication forks was prepared from unsynchronized cells. 2) The replication forks were not enriched with benzoylated naphthoylated diethylaminoethyl cellulose (BND-cellulose) to avoid possible fork enrichment bias. Fig. 1, B–D, depicts EM images of replication forks from WT cells harboring flap structures. The flap structures are indicated by black arrows.

Flap Structures Significantly Increased in Replication Forks of fen1−/H11002, dna2−/H11002, and fen1−/H11002-dna2−/H11002 Cells Compared with WT Cells—Next, we examined the flap structures in replication forks from fen1−/H11002, dna2−/H11002, and fen1−/H11002-dna2−/H11002 cells. Dna2 is essential for cell growth; therefore, dna2− and fen1−-dna2− replication forks were obtained from germinated dna2− and fen1−-dna2− spores. The results are presented in Fig. 1, E–P. The number of flap structures in the replication forks significantly and progressively increased from WT cells to fen1−/H11002, dna2−/H11002, and fen1−/H11002-dna2−/H11002 cells. Only ~10% of forks from WT cells exhibited flap structures, whereas the percentage of forks that possessed flap structures increased to ~23, ~32, and ~43% in fen1−/H11002, dna2−/H11002, and fen1−-dna2−/H11002 cells, respectively (Fig. 2A). Approximately one flap was noted every 78 kb of DNA in replication forks in WT cells. The flap density increased to one flap every ~24.7, ~6.5, and ~3.0 kb of DNA in fen1−/H11002, dna2−/H11002, and fen1−-dna2−/H11002 forks, respectively (Fig. 2B). The mean and median lengths of the flaps in WT cells were ~51 and ~41 nt, respectively. The flap lengths increased to ~131/89 (mean/median), ~146/138, and ~203/179 nt in fen1−/H11002, dna2−/H11002, and fen1−-dna2−/H11002 forks, respectively (Fig. 2C). The distribution of flap lengths in WT and the mutant cells is also shown (Fig. 2C). The statistical significance of the difference for flap lengths among WT and the mutant cells is shown in the table (Fig. 2C).

### TABLE 1

The distribution of flaps in replication forks in WT, fen1−/H11546, dna2−/H11546, and fen1−-dna2−/H11546 cells

| Distance (kb) | 0.5-1.0 | 1.0-1.5 | 1.5-2.0 | 2.0-2.5 | 2.5-3.0 | 3.0-3.5 | 3.5-4.0 | 4.0-4.5 |
|--------------|---------|---------|---------|---------|---------|---------|---------|---------|
| WT           | 38.1%   | 28.6%   | 6.3%    | 11.7%   | 4.8%    | 1.6%    | 1.6%    | 1.6%    |
| fen1−        | 11.7%   | 10.5%   | 9.4%    | 6.4%    | 7.5%    | 6.4%    | 6.0%    | 5.6%    |
| dna2−        | 3.7%    | 6.2%    | 5.8%    | 7.1%    | 6.2%    | 6.2%    | 5.8%    | 6.2%    |
| fen1−-dna2−  | 3.1%    | 3.9%    | 3.1%    | 7.3%    | 4.5%    | 6.2%    | 7.6%    | 6.2%    |

| Distance (kb) | 4.5-5.0 | 5.0-5.5 | 5.5-6.0 | 6.0-6.5 | 6.5-7.0 | 7.0-7.5 | 7.5-8.0 | 8.0-8.5 | 8.5-9.0 |
|--------------|---------|---------|---------|---------|---------|---------|---------|---------|---------|
| WT           | 1.6%    | 3.2%    | 1.6%    | 1.6%    | 0.0%    | 0.0%    | 0.0%    | 0.0%    | 1.6%    |
| fen1−        | 3.4%    | 3.4%    | 2.6%    | 1.9%    | 2.6%    | 2.3%    | 1.9%    | 1.1%    | 0.8%    |
| dna2−        | 4.6%    | 5.2%    | 3.4%    | 4.6%    | 3.1%    | 3.1%    | 2.5%    | 3.4%    | 1.5%    |
| fen1−-dna2−  | 5.4%    | 3.7%    | 3.7%    | 3.7%    | 2.3%    | 3.7%    | 2.3%    | 2.3%    | 4.8%    |

| Distance (kb) | 9.0-9.5 | 9.5-10.0 | 10.0-10.5 | 10.5-11.0 | 11.0-11.5 | 11.5-12.0 | 12.0-12.5 | 12.5-13.0 | 13.0-13.5 |
|--------------|---------|----------|------------|-----------|------------|-----------|-----------|-----------|-----------|
| WT           | 1.6%    | 0.0%     | 0.0%       | 0.0%      | 0.0%       | 1.6%      | 0.0%      | 0.0%      | 0.0%      |
| fen1−        | 1.1%    | 0.4%     | 1.5%       | 0.4%      | 1.1%       | 1.5%      | 0.8%      | 1.5%      | 0.4%      |
| dna2−        | 1.8%    | 1.8%     | 1.5%       | 1.5%      | 1.2%       | 0.6%      | 1.5%      | 0.9%      | 0.6%      |
| fen1−-dna2−  | 3.1%    | 2.3%     | 3.1%       | 1.1%      | 0.8%       | 0.6%      | 0.3%      | 2.0%      | 1.4%      |

| Distance (kb) | 13.5-14.0 | 14.0-14.5 | 14.5-15.0 | 15.0-15.5 | >15.5 |
|--------------|------------|------------|------------|-----------|-------|
| WT           | 0.0%       | 0.0%       | 0.0%       | 0.0%      | 0.0%  |
| fen1−        | 0.4%       | 0.8%       | 0.4%       | 0.4%      | 1.1%  |
| dna2−        | 0.9%       | 0.6%       | 0.3%       | 0.6%      | 2.2%  |
| fen1−-dna2−  | 0.8%       | 0.8%       | 0.3%       | 0.8%      | 3.1%  |
FIGURE 3. RPA foci in WT, fen1−/−, and dna2ts strains. A, a schematic of cells in M-G1, S, early G2, and late G2 phases. The cell phases were determined based on the cell length and number of nuclei present in an S. pombe cell. B, RPA foci (10×100-fold amplification) measurements in WT, fen1−/−, and dna2ts strains. SSB1, the largest subunit of RPA, was tagged by YFP. The ssb1-yfp gene was integrated into the ssb1 gene locus of the WT, fen1−/−, and dna2ts strains. WT, fen1−/−, and dna2ts cells expressing SSB1-YFP were cultured to log phase at 30 °C, and RPA foci were examined. C–F, RPA foci were examined during M-G1, S, early G2, and late G2 phases in WT, fen1−/−, and dna2ts cells. Cell growth conditions and the number of cells examined are indicated. For each statistic, at least four independent experiments were conducted. Error bars indicate the standard error. DIC, differential interference contrast.
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This result indicates that displacement DNA synthesis occurs at non-processed flap sites, which increases the length of these non-processed flaps.

The flap structures were almost exclusively located on one strand of replication forks (Fig. 1, B–P). The statistics indicated that ~95–97.5% of the forks exhibited flap structures that were present on only one strand (Fig. 2D). In addition, the major fraction of the flap structures in WT cell replication forks were located very close to the end of the fork (the conjunction point of the three strands) and that ~70% of the flaps in forks were located within ~1-kb distance from the end of the fork (Fig. 2E and Table 1). In fen1-, dna2-, and fen1-dna2- cells, the flap structures were widely distributed in a 10–15-kb DNA region (Fig. 2E and Table 1). These results demonstrated that the flap structures are indeed generated in replication forks, and both Dna2 and Fen1 are required for cleavage of the flap structures.

**RPA Foci Increase in fen1- and dna2- Cells in M-G1, S, and Early and Late G2 Phases Compared with WT Cells**—RPA is a single-stranded DNA (ssDNA)-binding protein that is required for DNA replication, recombination, and repair. RPA foci are frequently used as an indicator of the presence of ssDNA regions in cells. An expected result, based on what is known, is that the number and fluorescence intensity of RPA foci are proportionally related to the number and length of ssDNA fragments in cells. Therefore, we used analysis of RPA foci as another method to quantify relatively the number and length of the flaps in WT, fen1-, and dna2- cells. For this assay, three strains were constructed wherein the sbb1-yfp gene was integrated into the original sbb1 gene locus of WT, fen1-, and dna2- cells to achieve an equivalent level of SSB1-YFP expression in the three strains. SSB1 is the largest subunit of RPA. Western blotting analysis confirmed similar levels of SSB1-YFP expression in these strains (data not shown). The cell cycle phases of the *Schizosaccharomyces pombe* cells were determined based on cell length, appearance of septa, and number of nuclei per cell (Fig. 3A). Fig. 3B depicts RPA foci in unsynchronized WT, fen1-, and dna2- cells. Both RPA foci and fluorescence intensity increased significantly in fen1- and dna2- cells compared with WT cells. Fig. 3, C–F, present the percentage of nuclei with RPA foci and the number of RPA foci per nucleus in the M-G1, S, and early and late G2 phases in WT, fen1-, and dna2- cells. In all four stages of cell growth, both the percentage of nuclei with RPA foci and the number of RPA foci per nucleus increased from the WT cells to the fen1- cells and dna2- cells. The increased number of RPA foci in fen1- and dna2- cells is consistent with an increased number of flap structures in the Dna2 and Fen1 function-defective cells compared with WT cells. The increasing fluorescence intensity in fen1- and dna2- cells compared with WT cells is also consistent with the longer flaps observed in the fen1- and dna2- cells. RPA binds to ssDNA; thus, the results obtained from the assay of RPA foci provided further support that the flap structures observed by EM represented ssDNA.

**RNase H2 and Exo1 Function in Removing the RNA-DNA Primers**—Following the above assays, we further examined whether an exonuclease pathway is utilized to remove the RNA-DNA primers. Thus, the role of RNase H2 and Exo1 in removing the RNA-DNA primers was examined by measuring the number of flap structures presented in replication forks in RNase H2- or Exo1-deficient cells. It is logically assumed that if an exonuclease pathway indeed functions to remove the RNA-DNA primers a block of this pathway should increase flap structures presented in replication forks.

It was reported that a double knock-out of rad27 and exo1 is lethal in budding yeast (the *rad27* gene encodes Fen1 in *S. cerevisiae*), suggesting that they are functionally related (33). A genetic study also showed that human Exo1, a 5’–3’ double-stranded DNA exonuclease, suppresses the conditional lethality of a budding yeast *S. cerevisiae rad27Δ* mutant (34). Although the precise reason for the observed suppression is unknown, one possibility is that Fen1 and Exo1 both function in Okazaki fragment processing so that their function in this event can complement each other. RNase H2, a ribonuclease, can digest the RNA portion in reconstituted DNA synthesis systems (28, 35, 36), suggesting that RNase H2 possesses an intrinsic enzymatic activity to digest the RNA portion of RNA-DNA primers. In some recent studies, RNase H2 is demonstrated to play a critical role in ribonucleotide excision repair (37–40). In mammalian cells, the level of RNase H2 is enhanced in proliferative cells, and its activity is correlated with DNA replication (41, 42). In budding yeast, the homologue of mammalian RNase H2 is RNase H(35); deletion of both Rad27 and RNase H(35) severely impairs cell viability, but overexpression of RNase H(35) suppresses the poor growth of the Rad27 deletion mutant (12). Furthermore, RNase H2 is co-purified with a number of replication proteins (43–47), and RNase H2 is localized to replication foci (48). All these results strongly suggest that RNase H2 and Exo1 participate in DNA replication, and very possibly they act at the step of removing the RNA-DNA primers. Here, we used the EM assay to examine whether RNase H2 and Exo1 are directly involved in the processing of Okazaki fragments.

The *rnh201* gene in *S. pombe* encodes the catalytic subunit of RNase H2 that is the homologue of budding yeast RNase H(35). We constructed the four strains *rnh201*- , *exo1*- , *exo1*- *rnh201* - , and *fen1*- *rnh201* - to measure the frequency of flap structures presented in replication forks. The results are presented in Fig. 4. Fig. 4, A–I, display the EM images of replication forks that possess flap structures. The statistics of flap structures in these replication forks are presented in Fig. 5, A–E. A moderate increase of flap structures in the *rnh201* - , *exo1*- *rnh201* - , and *fen1*- *rnh201* - forks is observed compared with the WT, *exo1* - , and *fen1* - cells, respectively (Figs. 2, A and B, and 5, A and B). Although the increase in flap frequency is not significant, the length distribution of the long flaps is significantly different when the *rnh201* gene was deleted (Figs. 2C and 5C). These results suggest that RNase H2 functions in primer removal in vivo. Furthermore, the Exo1-deficient cells had a rate of flap structures comparable with that in the *fen1* - cells (Figs. 4, C and D; 5, A–C; and 2, A–C), indicating that Exo1 has a critical role in removing the RNA-DNA primers. As expected, the double deletion of Exo1 and RNase H2 or Fen1 and RNase H2 increased the rate of flap structures and the length of flaps as well in forks in comparison with a single deletion of Exo1 or Fen1 (Figs. 5, A–C, and 2, A–C). Similar to *fen1* - or dna2- cells, the majority of flap structures were also located on one strand of replication forks (Fig. 5D). The distribution of flaps on the lagging
strand is shown in Fig. 5E and Table 2, indicating that the location of flaps in the rnh201− forks are close to the end of replication forks, but the flaps in the exo1−, exo1−rnh201−, and fen1−rnh201− forks are more evenly distributed in the lagging strand.

Discussion
How Okazaki fragments are processed to remove the RNA-DNA primers is a long standing question in the field of chromosomal DNA replication in eukaryotic cells (28). The present
study provides the first direct in vivo evidence supporting the flap cleavage pathway: some of the RNA-DNA primers are displaced to generate flap structures, and the flap structures are subsequently cleaved by flap endonucleases Dna2 and Fen1 (Fig. 6, ii and iii). We also provide direct in vivo evidence supporting the exonucleolytic pathway: some of the RNA-DNA primers are directly digested by exonucleases RNase H2 and Exo1 (Fig. 6i). The experimental evidence acquired herein also demonstrates that Dna2, Fen1, Exo1, and RNase H2 play a direct role in removing the RNA-DNA primers in vivo (Fig. 6). Okazaki fragment processing is one of the fundamental processes of life and constitutes the single most abundant DNA transaction event in DNA metabolism, and the elucidation of its mechanism is critical in understanding how chromosomal DNA is replicated in eukaryotes. The knowledge acquired herein clarifies two important issues related to Okazaki fragment maturation and lagging strand synthesis: one is which enzymes directly participate in removing the RNA-DNA primers, and the other is by which pathways these primers are removed.

By directly observing replication forks under EM, we visualized flap structures in replication forks (Figs. 1, B–P, and 4, A–I). These fork images revealed the following. 1) The flaps were almost exclusively located on one strand in the forks regardless of whether the forks were from WT, fen1−, dna2−, exo1−, and rnh201− cells (Figs. 1, B–P; 2D; 4, A–I; and 5D and Tables 1 and 2). 2) The flap density in the forks increased significantly and progressively from WT to rnh201−, exo1−, fen1−, dna2−, and fen1−-dna2− cells (Figs. 1, B–P; 2, A–C; 4, A–I; and 5, A–C). 3) The mean and median lengths of the flaps in the WT forks were ~51 and ~41 nt, which is marginally longer than the RNA-DNA primer length (~35 nt) (6, 49). Considering these results together with previous findings demonstrating that Dna2 and Fen1 are flap endonucleases, we suggest that the flaps observed under EM are displaced RNA-DNA primers and are located on the lagging strand. This conclusion was reinforced by assaying RPA foci. Both RPA foci and the fluorescence intensity increased significantly in fen1− and dna2− cells compared with WT cells (Fig. 3, B–F). This observation is consistent with increases in flap number and flap...
length in fen1− and dna2− cells compared with WT cells. Therefore, it is highly likely that these RPA foci result from the binding of RPA to these flap structures and are an indicator of ssDNA characteristic of these flap structures.

RNA-DNA primers are synthesized with low fidelity. The mechanism by which a eukaryotic cell completely removes the RNA-DNA primers from Okazaki fragments remains uncharacterized. This issue is critical because it has a significant effect on genomic integrity. The length of RNA-DNA primers is 35 nt based on the measurements conducted in reconstituted replication systems in vitro (6, 49). Although the exact length of RNA-DNA primers in eukaryotic cells remains unknown, ~35 nt is likely to be close to the actual length of the RNA-DNA primers because the primer length appears to be determined by the intrinsic processivity of the DNA pol α-primase complex (6). Based on the measurement of flaps that can be observed under EM, the median and mean lengths of the flaps in WT cell replication forks were ~41/51 nt (median/mean) (Fig. 2C). A flap length of ~41/51 nt suggests that the entire RNA-DNA primer sequence is displaced for subsequent removal by the flap endonucleases Dna2 and Fen1. Because a flap under 30 nt is difficult to be observed under EM, it is possible that an average flap length of ~41/51 nt may be slightly overestimated.

The results presented in Figs. 1, B−D, and 2, A and B, indicate that not all of the flaps in replication forks of WT cells are immediately removed. Approximately 1 × 10⁵ Okazaki frag-
ments are produced during each round of DNA replication with a genome size of $1.35 \times 10^7$ bp in S. pombe cells; this number translates into $\sim 10^5$ flaps if all of the RNA-DNA primers are displaced. By estimation, $\sim 1$ of 1000 flaps escapes cleavage during the first 40 s after they are generated in WT cells, assuming a fork movement rate of $\sim 25$ bp/s. This estimation is based on the observation of one flap every $\sim 78$ kb of DNA in WT cell forks and that $\sim 67\%$ of these unremoved flaps are located within $\sim 1$ kb of DNA from the fork end (Fig. 2 and E). However, the majority of these unprocessed flaps are removed later despite escaping the initial cleavage; nearly 95% of these remaining flaps are removed before they were 6 kb away from the fork end in WT cells (Fig. 2E and Table 1). At present, it is unknown whether every flap is eventually removed after the completion of DNA replication or whether a few flaps still remain on DNA and will be cleaved before the next round of DNA replication. In contrast to WT cells, unremoved flaps are more evenly distributed throughout a $\sim 10-15$-kb DNA region in $fen1^{-}$, $exo1^{-}$, $dna2^{-}$, and $fen1^{-} - dna2^{-}$ cells (Figs. 1, E–P; 2E, and 4, C–F, and Tables 1 and 2), suggesting that a large number of flaps are not removed in the presence of defective Fen1, Exo1, or Dna2.

The cleavage of flaps requires nucleases. The experiments presented herein demonstrate that Dna2 and Fen1 are required to cleave these flap structures for Okazaki fragment maturation (Fig. 1, E–P). The requirement for both Dna2 and Fen1 for the removal of flap structures in vivo should support the Dna2-Fen1 long flap model wherein Dna2 and Fen1 are thought to act sequentially in the cleavage of long flap structures (16) (Fig. 6ii). The increasing RPA foci from the $fen1^{-}$ to $dna2^{-}$ cells also support the presence of RPA-coated flaps and the long flap model. However, the experimental evidence provided here also clearly indicates that Fen1 is an important nuclease in Okazaki fragment processing in support of two recent in vitro assays (50, 51). By comparing the average length between flaps in the $dna2^{-}$ and $fen1^{-} - dna2^{-}$ cells, unremoved flaps in replication forks increased by $\sim 116\%$ in the double mutant cells. This result suggests that at least half of flap structures are cleaved by Fen1 alone. This result suggests that the Fen1-only short flap model is also used by cells to remove the RNA-DNA primers (52) (Fig. 6iii). Furthermore, two recent studies showed that Dna2 alone is capable of removing entire flap structures (31, 53). This result, together with the present studies shown in Figs. 1 and 4, also suggests that the Fen1 acting alone or Fen1-only model should exist in vivo.

Our results also indicate that an exonucleolytic digestion pathway plays a critical role in removing the RNA-DNA primers. First, we demonstrated that both RNase H2 and Exo1 are required for removing the RNA-DNA primers (Figs. 4, A–I, and 5, A–E). Second, in the replication forks of $fen1^{-} - dna2^{-}$ cells, the average distance between two flaps is $\sim 3.0$ kb (Fig. 2B); theoretically, a 3-kb DNA region on the lagging strand contains approximately $\sim 20$ Okazaki fragments, translating into $\sim 20$ flaps if each RNA-DNA primer is displaced. Based on this estimation, most of the RNA-DNA primers could be removed by the exonuclease pathway in normal cell growth. However, if the following factors apply, the number of flaps observed in the $fen1^{-} - dna2^{-}$ cells will be reduced, resulting in the overestimation of the role of the exonuclease pathway in removing the RNA-DNA primers. These factors include that 1) some flaps are shorter than $\sim 30$ nt even though the average length of the flaps in $fen1^{-} - dna2^{-}$ cells is $\sim 203$ nt (Fig. 2C), 2) some unknown nuclease(s) comes to cleave flap structures when Dna2 and Fen1 are absent, and 3) an extensive displacement DNA synthesis occurs at the unligated Okazaki fragment sites, and this event could remove most of those unprocessed Okazaki fragments as well as flap structures. In the exonuclease pathway, Exo1 appears to be the major exonuclease.
Experimental Procedures

Strains Used in This Study—The strains are listed in Table 3.

Electron Microscopy Assay—For EM examination of replication forks, EM samples were prepared as described previously (31) with some modifications. To observe flap structures in the forks, high resolution EM images are required because the flaps are generally short (a few dozen nucleotides). Several modifications were made to prepare the EM samples. 1) The thickness of the carbon film was 4.5–6.5 nm. 2) No supporting film was placed under the carbon film. 3) The nucleic acids on the carbon film were not stained with uranyl acetate. 4) The angle for the carbon film was 4.5–6.5 nm. 2) No supporting film was placed under the carbon film. 4) The angle for the carbon film was 4.5–6.5 nm.

Fluorescence Microscopy—For fluorescence microscopy, the cells were washed twice with PBS buffer.

Assay of RPA Foci—To examine RPA foci, the cells were washed twice with PBS buffer.

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