Multiple Roles for Members of the Long Flap Pathway in Okazaki Fragment Processing

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Dedication

To Mom, Dad, Adam, and Jen for their love, support and inspiration.

Curriculum Vitae

The author was born on July 28, 1983, in Abington, Pennsylvania. He attended Johns Hopkins University in Baltimore, Maryland, from 2001 to 2005 and earned a Bachelor of Arts degree, majoring in Biophysics with a minor in Philosophy. At Johns Hopkins, he studied the effects of the transcriptional inhibitor M_4N on the growth of various cancer cell lines in the laboratory of Dr. Ru Chih Huang. He began graduate school at the University of Rochester in the fall of 2005 in the Biochemistry, Cellular, and Molecular Biology cluster. In the fall of 2007, he joined the Biochemistry Ph.D. program in the laboratory of Dr. Robert Bambara. He earned the Master of Science degree in Biochemistry in 2008. Following graduation, he will be working as a postdoctoral fellow in the lab of Dr. Andy Andrews in the department of Cancer Biology at the Fox Chase Cancer Center in Philadelphia, Pennsylvania.

Publications

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Henry RA, Balakrishnan L, Tan Ying-Lin S, Campbell JL, Bambara RA. (2010) Components of the secondary pathway stimulate the primary pathway of eukaryotic okazaki fragment processing. *Journal of Biological Chemistry*, 285 (37): 28496-505.

Pike JE, **Henry RA,** Burgers PM, Campbell JL, Bambara RA (2010) A Third Okazaki Fragment Processing Pathway: Resolution of Fold-back Flaps by Pif1 Helicase. *Journal of Biological Chemistry,* 285 (53): 41712-23

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Abstract

The lagging strand of eukaryotic DNA is synthesized discontinuously via the creation of short segments known as Okazaki fragments. Each fragment is initiated with an RNA-DNA primer that is extended by DNA polymerase δ (pol δ) until it reaches the subsequent downstream primer, displacing a flap that must be cleaved for ligation to occur. This occurs via one of two pathways. In the short flap pathway, flap endonuclease 1 (FEN1) cleaves flaps while they are still ~1-6 nt long. In the long flap pathway, flaps are bound by replication protein A (RPA), which blocks FEN1 cleavage. A second nuclease, Dna2, can cleave RPA-coated flaps, leaving behind a short flap for subsequent FEN1 cleavage. The helicase Pif1 has been shown to play a role in long flap creation.

To better understand how protein components of the pathways interact, synthetic oligonucleotide flap substrates were created to test, *in vitro,* the activities of purified *Sacchromyces cerevisiae* (*S. cerevisiae*) proteins. Results demonstrated that Dna2, Pif1, and RPA are each able to stimulate cleavage by FEN1 on both long and short flaps, as well as on RNA flaps. Co-immunoprecipitation showed that each of Dna2, Pif1, and RPA is able to bind directly to FEN1.

Investigation of the role of Pif1 on self-complimentary (fold-back) flaps suggests that the helicase helps resolve these structures. Neither Dna2 nor FEN1 can cleave fold-back flaps. However, Pif1 was able to remove the entire fold-back-

containing fragment, allowing pol δ to synthesize again. The presence of RPA was able to partially restore pol δ synthesis, resolving fold-backs in the template.

Finally, we questioned whether replication proteins replace a greater length of primer from fragments that contain mismatches. To assess correction, we developed a novel set of reconstitution substrates utilizing a diagnostic EcoRI site. Only when generation of long flaps was augmented by reduction in FEN1 or dNTP concentrations could an increase in displacement by Pif1 be observed. In contrast, pol δ and FEN1 responded to mismatches within the first 20 nt of the downstream primer by promoting farther strand displacement.

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Abbreviations

C. elegans – Caenorhabiditis elegans

Dna2 – Dna2 endonuclease/helicase

E. coli – Escherichia coli

EMSA – Electrophoretic mobility shift assay

ExoI – exonuclease I

FEN1 – flap endonuclease 1

fmol – femtomole, 1 x 10^{-15} moles

kDa – kilodalton

LigI – DNA Ligase I

nt – nucleotide

p300 – Human acetylase

PCNA – proliferating cell nuclear antigen

Pfh1 – *S. pombe* 5'-3' helicase

Pif1 – *S. cerevisiae* and human 5'-3' helicase

Pol I– *E. coli* DNA polymerase I

pol α – DNA polymerase α/primase

pol δ – DNA polymerase δ

Pol32 – *S. cerevisiae* PCNA-interacting subunit of pol δ

pol3-L523H – polymerase and exonuclease domain switching mutant of pol δ

Rad27 – *S. cerevisiae* homolog of FEN1

RFC – replication factor C

RPA – replication protein A

Rrm3 – *S. cerevisiae* 5’-3’ helicase

S. cerevisiae – Saccharomyces cerevisiae

S. pombe – Schizosaccharomyces pombe

ssDNA – single-stranded DNA

Foreword

Parts of this document contain published figures and text. Figures 2.3, 2.4 B, and 2.5 represent original research completed by the author. Dr. Jason Pike performed the experiments presented in Figure 2.1, 2.2, and 2.4A. These figures have been adapted from the following co-authored publication:

Pike JE, Henry RA, Burgers PM, Campbell JL, Bambara RA (2010). An alternative pathway for Okazaki fragment processing: resolution of fold-back flaps by Pif1 helicase. *Journal of Biological Chemistry*, 285 (53): 41712-23. © the American Society for Biochemistry and Molecular Biology.

Copyright permission to use the figures was granted by the American Society for Biochemistry and Molecular Biology as outlined at http://www.jbc.org/site/misc/Copyright_Permission.xhtml. Dr. Jason Pike has given permission to include his work in this dissertation. Dr. Wensheng Wang purified the recombinant Pif1 protein. Note that the text of Chapter 2 has been rewritten since publication of the manuscript and necessarily contains discussions similar to those that appear in the published manuscript.

Chapters 1 and 3 represent original research completed by the author. The contents of Chapter 1 was published previously as follows:

Henry RA, Balakrishnan L, Tan Ying-Lin S, Campbell JL, Bambara RA (2010). Components of the secondary pathway stimulate the primary pathway of eukaryotic Okazaki fragment processing. *Journal of Biological Chemistry*, 285 (37): 28496-505.

Dr. Judith Campbell was a collaborator in Chapter 1 and 2. Dr. Peter Burgers was a collaborator for Chapter 2. Dr. Peter Burgers provided the purified recombinant DNA polymerase δ and replication factor C. Dr. Judith Campbell provided the purified recombinant Dna2. Dr. Campbell and Dr. Burgers provided critical discussion during preparation of the respective manuscripts.

Introduction

DNA Replication

Complete and proper replication of DNA in eukaryotic organisms is essential for the maintenance of genome stability. Failure to properly replicate DNA leads to mutations, with the creation of multiple mutations occurring during aging. The accumulation of multiple mutations in DNA is, in turn, essential in the development of a cancerous cell. Thus, without careful regulation of the replication pathway, an organism becomes more susceptible to cancer, disease, and ultimately death. For these reasons, a complete and detailed understanding of the replication machinery is vital.

Because of the 5'-3' directionality of the polymerases involved in DNA replication, and the antiparallel structure of the DNA helix, the replication process must occur via two different methods. For what is termed the leading strand, DNA polymerase ε can synthesize a single, continuous strand of DNA that is able to grow in the direction of strand separation (1,2). However, for the lagging strand, DNA synthesis must occur discontinuously via the creation of Okazaki fragments. These fragments are initiated by DNA polymerase α/primase (pol α), which synthesizes approximately 10 nucleotides (nt) of RNA primer. Pol α then synthesizes 10-20 nt of DNA (3). Through a polymerase switching process, pol α dissociates and is replaced by DNA polymerase δ (pol δ) at the primer-template junction (4,5). As it lacks any proofreading activity, pol α has a lower fidelity of synthesis than pol δ. Pol α has a base substitution error rate of 10^{-4} (6). Pol δ possesses a 3'-5' exonuclease activity

which allows for proofreading of newly synthesized DNA, and has a lower error rate of 10^{-7} (6).

The interaction of pol δ with the template is stabilized by the presence of proliferating cell nuclear antigen (PCNA), a toroidal heterotrimer that encircles the DNA double helix (7). PCNA is loaded onto the DNA template in an ATP-dependent manner by replication factor C (RFC) (8). Pol δ, aided by PCNA, is a highly stable and processive complex, readily able to synthesize 100-200 nt of DNA, completing synthesis of the lagging strand (9).

As pol δ synthesis lengthens the upstream fragment, it will eventually encounter a subsequent downstream Okazaki fragment. At this point, pol δ begins displacing the fragment into a flap, a process known as strand displacement synthesis. In order for a final double-stranded DNA product to be completed, the entire flap must be cleaved by a nuclease to leave behind a nick. This nick is then sealed by DNA ligase I (LigI) (10-12). Because LigI is unable to ligate RNA segments, the RNA region of the primer synthesized by pol α must be fully removed by nucleolytic cleavage.

Initial work studying flap processing suggests that this RNA segment can be cleaved by RNase H (13,14). RNase H is a ribonuclease that cleaves RNA in RNA-DNA helices and is therefore able to process the RNA component of an Okazaki fragment. Cleavage by RNase H must occur before strand displacement by pol δ, as RNase H requires the RNA to exist in a duplex with DNA in order to cleave (3,15). Because RNase H cleaves the phosphodiester bond between two ribonucleotides, it is

unable to remove the final nucleotide of RNA from the primer, as it is bonded to the first deoxyribonucleotide (16-18). This also means RNase H is unable able to cleave any of the DNA primer.

The process of removing the RNA primer is completed, therefore, by another nuclease: flap endonuclease 1 (FEN1) (17). While RNase H must cleave before strand displacement synthesis occurs, FEN1 requires a 5' flap for cleavage. Therefore, it is proposed that after RNase H has performed its final cleavage, pol δ synthesizes into the primer, creating a flap. This flap can then be cleaved by FEN1, removing the final ribonucleotide from the primer (19,20).

Currently, two different pathways are believed to be responsible for the cleavage of flaps created during strand displacement synthesis (Fig. I.1). The first, referred to as the FEN1-only model, involves pol δ displacing short flaps, which are then cleaved by FEN1 alone (Fig. I.1A) (21,22). This pathway is also referred to as the short flap pathway. The second pathway, termed the two-nuclease model or long flap pathway (Fig. I.1B), involves the creation of long flaps by pol δ, which are bound by replication protein A (RPA) (23). This necessitates sequential cleavage by Dna2 nuclease followed by a final cleavage by FEN1 (24).

Short Flap Pathway

As the name implies, the first pathway utilized for flap processing occurs through the creation of short flaps (Fig. I.1A) (21,22). This model proposes that as

pol δ is performing strand displacement synthesis, FEN1 is frequently cleaving the flaps that are being created, allowing the flaps to only reach several nucleotides in length. The process of displacing the downstream primer slows synthesis by pol δ, thus quick and efficient cleavage by FEN1 aids pol δ in continued synthesis through the downstream primer. Cleavage of a flap by FEN1 leaves behind a nick, which can be ligated by LigI. This creates a finished double-stranded product, which is the final step in synthesis of the lagging strand.

FEN1 is a 42 kDa, magnesium-dependent nuclease that specifically recognizes the base of a flap (12,25,26). It was initially believed that FEN1 operated through a tracking mechanism, threading the 5' end of a flap through itself while moving itself toward the base of the flap (26-28). This model was based on the fact that blockage of the 5' end of a flap inhibits FEN1 cleavage (26). However, current research suggests that FEN1 actually operates by first binding to the base of the flap and then threading the flap through itself (25). FEN1, therefore, has two binding modes: the first is binding the flap without threading, which can be considered unproductive binding. The second mode of binding is after the flap has been fully threaded, allowing for FEN1 cleavage at the base of the flap (25). Although the primary mode of cleavage for FEN1 is endonuclease activity, FEN1 also possesses a weak exonuclease activity (20,29).

FEN1 is homologous to the 5' to 3' nuclease domain present in *E. coli* DNA polymerase I (Pol I) (30). A number of prokaryotic polymerases have both a polymerase and an intrinsic 5'-3' nuclease activity, essentially performing the roles of

both pol δ and FEN1 (15). Thus, Pol I is able to perform nick translation, the process of extending an Okazaki fragment via synthesis while simultaneously coordinating cleavage (31). While both synthesis and cleavage are performed by a single polymerase holoenzyme in prokaryotes, this function seems to have been split between two enzymes during eukaryotic evolution.

FEN1 has been found in numerous eukaryotes and is highly conserved from yeast to human (12). FEN1 was titled as such (flap endonuclease 1) after observing the ability of purified mouse FEN1 to cleave single-stranded flaps of DNA (20). The yeast homologue was discovered after noting sequence and function conservation between FEN1 in *Saccharomyces cerevisiae* and in mice (32,33).

The preferred substrate for FEN1 is the double flap created during synthesis by pol δ. As pol δ lengthens an upstream primer, it will displace the adjacent downstream primer in a reversible manner. As the displaced flap and newly synthesized DNA possess the same sequence, either can become bound to the template. This allows the two to equilibrate to produce a double flap structure, a structure containing both a 5' and 3' flap (34,35). The ideal structure for FEN1 cleavage involves a 5' flap with an additional 3' flap containing only a single nucleotide (36). A 3' flap any longer than one nucleotide leads to a decrease in FEN1 cleavage activity, as will the absence of a 3' flap (37). This behavior is consistent with structural studies of FEN1 that found that there is a binding pocket on FEN1 for a short 3' flap (38,39).

In addition to the ability of FEN1 to cleave DNA flaps, FEN1 is also able to cleave flaps composed of RNA (40). This is an important function for FEN1, as in order to properly process the flaps created during lagging strand synthesis, the RNA portion of the primer created by pol α must be removed. Although other nucleases are also capable of removing this RNA segment, like RNase H mentioned above, it has been shown that FEN1 is sufficient in cleaving both RNA and DNA flaps in order to create a ligatable nicked substrate (22).

The involvement of FEN1 in lagging strand replication is further supported by its ability to interact with PCNA. PCNA, in addition to being able to bind pol δ and DNA ligase I, is able to bind FEN1, stimulating the catalytic rates of each (41). This implies the formation of a complex that is capable of quick, efficient Okazaki fragment processing through careful coordination of synthesis and cleavage.

Long Flap Pathway

Despite efficient cleavage by FEN1, occasionally flaps will grow long (Fig. I.1B) (11,42). Once these flaps reach a length of ~22-30 nt, they can be stably bound by RPA (43). RPA is a single stranded binding protein that, once bound to a flap, inhibits FEN1 cleavage (23). Therefore, the creation of flaps long enough to be bound by RPA necessitates cleavage by a second nuclease, specifically Dna2. Unlike FEN1, Dna2 is able to dissociate RPA from DNA in order to cleave RPA coated flaps (44). While Dna2 cleaves a flap multiple times, it cannot cleave at the base of the

flap and must leave behind a short, ~5-6 nt residual flap (24). This short flap can no longer be bound by RPA, leaving it available to FEN1 for a final cleavage.

Dna2 was originally discovered in *S. cerevisiae* in an attempt to identify DNA replication mutants (45). Homologs of Dna2 have since been found in multiple organisms including *Schizosaccharomyces pombe* (46), *C. elegans* (47), and humans (48-50). Dna2 was found to be essential in *S. pombe* and *S. cerevisiae,* as its deletion was lethal (46,51,52). Recent work has demonstrated a key role for Dna2 in the DNA damage checkpoint (53).

Dna2 possesses multiple activities, including an ATP-dependent 5'-3' helicase activity in addition to a single-stranded DNA nuclease and ATPase activities (54-56). Although the helicase ability of Dna2 was originally believed to be its primary activity, it has since been shown that only its nuclease activity is essential *in vivo* (52,57). In fact, in higher organisms such as humans, Dna2 exhibits minimal helicase activity (49). Dna2 also has strand annealing and strand exchange properties, and in humans Dna2 possesses a 3'-5' endonuclease activity (49,58). Each of these activities has been proposed to aid in the processing of equilibrating flaps.

Dna2 is able to cleave long flaps created during pol δ strand displacement synthesis. Although Dna2 can bind to single stranded DNA, competition assays have shown that Dna2 has a much higher binding affinity when it recognizes a junction point between single-stranded DNA and double-stranded DNA (59). After binding, Dna2 is able to thread the ssDNA and cleave. To do so, Dna2 requires the single-stranded DNA to have an unblocked 5' end (55). Unlike FEN1, which can both bind

and cleave an RNA flap, Dna2 can only bind (24). Therefore, in order for Dna2 to cleave, the RNA section of the primer must either be removed, or the flap must grow long enough for Dna2 cleave within the DNA region.

Dna2 has been shown to interact biochemically with RPA, as binding of RPA to a flap influences the ability of Dna2 to cleave (23,56,60). When RPA binds an unstructured flap, it alters the frequency at which Dna2 cleaves: in the presence of RPA, Dna2 will cleave more often, creating shorter cleavage products (60). On a flap containing weak secondary structure, RPA is able to melt out the structure that is normally inhibitory to Dna2 cleavage. In this way, the presence of RPA is able to stimulate Dna2 cleavage (60,61). RPA does not inhibit Dna2 cleavage as it does with FEN1 because Dna2 is able to actively dissociate RPA from a flap (60).

Dna2 also interacts with FEN1. Immunoprecipitation of the proteins shows a physical interaction between FEN1 and Dna2 (62). Importantly, just as Dna2 can dissociate RPA from a flap, FEN1 is able to remove Dna2 from both a DNA and RNA flap (63). As noted above, Dna2 cannot cleave RNA flaps, so the ability of FEN1 to remove unproductive Dna2 from the base of a flap is important to allow efficient cleavage. This ability is also important to ensure that Dna2 is disengaged from the flap after Dna2 has made its final cleavage. The ability of these proteins to disengage each other from a flap is important for the coordination of the long flap pathway: after RPA is bound, it is removed by Dna2, which cleaves, leaving behind a short flap. Dna2 is then dissociated by FEN1 to allow for the final cleavage event.

The single-stranded binding protein RPA, in addition to interacting with Dna2, prevents the formation of secondary structure during flap creation, as such structure is inhibitory to flap processing. In *S. cerevisiae*, RPA consists of three polypeptides, which are 14, 30, and 70 kDa (43,64). The major binding interactions of RPA occur between the DNA and RPA70. This subunit of RPA contains three binding domains (A, B, C) as well as an N-terminal domain, which has a weak DNA binding affinity. The N-terminal domain is mainly involved in protein-protein interactions (65). RPA30 contains a fourth DNA binding domain (D) and a C-terminal region that serves as another site for protein-protein interactions. Finally, RPA14 has a weak DNA binding domain, with its main role being to stabilize the interactions between RPA70 and RPA30. In humans, RPA has been shown to contain a fifth and sixth potential DNA binding domain, although the necessity of these domains for proper RPA function are unclear (66).

The mechanism for RPA binding to ssDNA has been well characterized. RPA has three different binding modes, which are dependent on the length of DNA that RPA is binding (43,67). The weakest binding mode occurs on short segments of ssDNA that are approximately 8 to 10 nt in length. In this first mode of binding, the high affinity domains A and B of RPA70 form the initial contacts with the ssDNA. This binding mode requires RPA to bind in a compact conformation. The second mode of binding occurs on segments of ssDNA that are 12 to 23 nt in length. On segments of this length, domains A, B, and C are able to bind the DNA, causing RPA to assume a less compact conformation. The most stable binding mode, which occurs

at lengths greater than 23 nt allows all four binding domains of RPA to contact the DNA. Fully extended RPA has been shown to occupy approximately 30 nt of ssDNA (43).

Recently, the importance of the long flap pathway has been under scrutiny. There are two main concerns, the first of which is whether human Dna2 is present in the nucleus. A report demonstrated that tagging of Dna2 *in vivo* via immunofluorescence showed Dna2-specific signals only in the mitochondria, not the nucleus (68). However, a later report that utilized immunofluorescence as well as other biochemical techniques found Dna2 present in both the nucleus and the mitochondria (69). These differing results may have derived from the use of different cell lines or the use of an antibody that did not bind the nuclear isoform of Dna2 in the original study. Additional evidence demonstrates that human Dna2 is acetylated by p300, a protein that localizes solely to the nucleus (70,71).

The second concern is the frequency of long flap formation *in vivo*. Experiments demonstrating the coordination between pol δ and FEN1 suggest that flaps are efficiently cleaved by FEN1 before they become long (34,35,72). However, this observation conflicts with reports demonstrating that Dna2 nuclease activity is essential to the cell (51,52). Additionally, acetylation by p300 inhibits FEN1 and stimulates Dna2 in humans (70), suggesting that regulation may be responsible for the use of the long flap pathway. It seems likely that at least a small percentage of flaps become long *in vivo*, and this process may be further regulated by other proteins, such as p300 or Pif1.

Pif1 Helicase

Pif1 is a 95 kD, 5' to 3' ATP-dependent helicase (73). Pif1 is found in both the nucleus and mitochondria. In *S. cerevisiae*, the *PIF1* gene contains two alternative translation start sites that determine mitochondrial or nuclear localization (74). Pif1 was first discovered in *S. cerevisiae* where it was determined to have a role in mitochondrial DNA maintenance (75). In *S. cerevisiae*, a second Pif1-like helicase exists: Rrm3 (76). Multiple members of the Pif1 helicase family have been found in numerous eukaryotes (77), with significant work being done on four members of the family in particular: *S. cerevisiae* Pif1 and Rrm3, *S. pombe* Pfh1, and human Pif1 (78).

Each member of the Pif1 family loads in a single-stranded region of DNA and unwinds in a 5' to 3' direction (79-81). Both Pfh1 and Pif1 have been shown to have a low processivity (82,83). Pfh1 was shown to unwind substrates up to 30 nt, demonstrating significantly less activity on double-stranded DNA of 40 nt or more (82). Although *S. cerevisiae* and human Pif1 cannot bind to RNA, they are able to unwind RNA-DNA duplexes if there is ssDNA present to load on (84,85). In fact, Pif1 has been shown to be more efficient at unwinding RNA-DNA duplexes than it is at unwinding DNA-DNA duplexes (84). This is an important activity for the role Pif1 plays in telomere maintenance.

Chromatin immunoprecipitations have shown that Pif1 interacts directly with telomeric DNA, and Pif1 has been shown to be an important regulator of proper

telomere length (86,87). Pif1 inhibits the processivity of telomerase *in vitro*: it is believed that Pif1 is able to unwind the telomerase RNA-telomeric DNA hybrid from the ends of telomeres (84,85). This in turn prevents excessive telomere lengthening by telomerase. Thus, one of the most noticeable phenotypes of a *PIF1* deletion is long telomeres (86). This mechanism for telomere length regulation is supported by the fact that Pif1 helicase activity is required for telomerase dissociation, with a helicase-deficient mutant of Pif1 unable to rescue the long telomere phenotype found in a *pif1Δ* mutant (84,85). Additionally, Pif1 is stimulated on a forked RNA-DNA hybrid, which is the structure adopted by the telomerase RNA-telomeric DNA duplex (88).

Experiments in *S. pombe* utilizing Pfh1 suggest that telomere maintenance is not the only essential function of the Pif1 family of helicases. When a *pfh1-* mutant was created it was lethal, even in a strain that did not possess telomeres, suggesting another vital role for Pfh1 (80). One possibility is the role of the Pif1 family of helicases in mitochondrial DNA maintenance. It has been shown that the deletion of Pif1 causes loss of mitochondrial DNA (75,89). This suggests that the mitochondrial isoform plays an important role in mitochondrial DNA replication and/or repair.

Interestingly, deletion of Pif1 is known to partially rescue the lethality of a *dna2Δ* mutation in *S. cerevisiae* (90,91). While the *dna2Δ pif1Δ* mutant retained temperature sensitivity, full growth was restored by additionally deleting *POL32*, the subunit of pol δ that interacts with PCNA (90,91). The deletion of POL32 decreases the ability of pol δ to perform strand displacement synthesis, resulting in the

formation of shorter flaps (92,93). These results suggest that deletion of *PIF1* and/or *POL32* inhibits long flap formation, eliminating the requirement for Dna2. This implies a role for Pif1 in the creation of long flaps during Okazaki fragment processing.

Biochemical studies support the genetic evidence suggesting that Pif1 creates long flaps. In Okazaki fragment reconstitution experiments that examined the flap length of downstream primers after synthesis by pol δ and subsequent cleavage, flaps rarely became more than a few nucleotides in length (72), supporting the idea that FEN1 can quickly and efficiently cleave flaps while they are still short. However, addition of Pif1 led to the creation of a small fraction of long flaps (~28-32 nt in length). In the presence of RPA these flaps inhibited FEN1 cleavage, again suggesting that the creation of long flaps by Pif1 could create a requirement for Dna2 (72). Additionally, Pif1 has been shown to lengthen a portion of flaps during Okazaki fragment reconstitution experiments, creating a need for Dna2 (94).

Genetic studies further implicate a role for Pif1 in long flap creation. The pol3-L523H mutant is defective at switching between polymerase and exonuclease activity. As such, this mutant exhibits a greater level of strand displacement activity (91). When this mutant is coupled with *rad27Δ*, the double mutant is inviable. Evidently the cell is not capable of processing all of the long flaps created by the pol δ mutant in the absence of FEN1. Significantly, an additional deletion of PIF1 rescues the lethality of the double mutant (91). It is believed that the absence of Pif1

results in fewer long flaps being formed, which allows nucleases that can substitute for FEN1, such as ExoI, to cleave the flaps before they become too long.

Studies of the Pif1 homolog, Pfh1, demonstrate its role in DNA replication. Pfh1 is able to unwind flap structures that contain a 5' RNA region, much like the structures that form during Okazaki fragment processing (82). Pfh1 can also remove primers that contain fold-back flaps from the template DNA (82). Fold-back flaps are flaps that have formed secondary structures that are resistant to cleavage by both FEN1 and Dna2. This activity could be useful for restoring stalled pol δ synthesis, a possibility that is investigated in Chapter 2 of this document.

Finally, Pif1 has been shown to be important in resolving G-quadruplexes that form during replication (95-97). G-quadruplexes are highly stable regions of secondary structure held together by noncanonical G-G base pairs. G-quadruplexe formation slows DNA replication and, in the absence of Pif1, leads to strand breakage. Initial studies demonstrated that, *in vitro*, Pif1 readily unwinds G-quadruplexes (95,97). Further investigation utilizing chromatin immunoprecipitation confirmed that high concentrations of Pif1 are present at G-quadruplex motifs (96). The localization of Pif1 at these regions is important in restoring proper replication and preventing strand breakage.

Overview

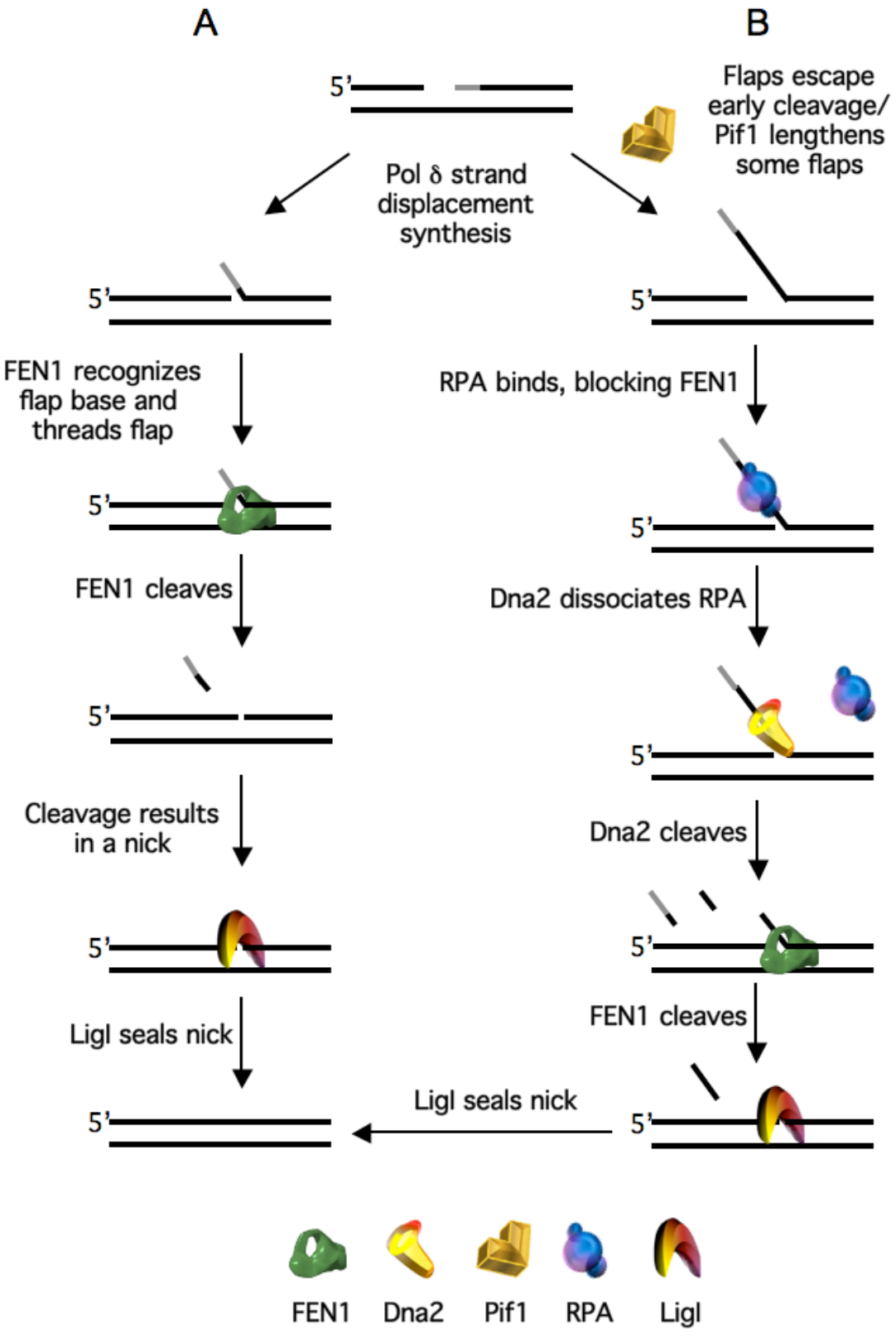
The processing of flaps created by pol δ during lagging strand synthesis occurs via one of two pathways. The FEN1-only, or short flap pathway, requires cleavage by only one nuclease: FEN1. The long flap pathway also involves the single stranded binding protein RPA and Dna2 nuclease/helicase. The helicase Pif1 has been implicated in, among other roles, creation of long flaps. While the basic actions of these four proteins have been well established in their respective pathways, we were interested in elucidating how members of the long and short flap pathways interact and what other roles these proteins may play in flap processing.

To accomplish this, we began by utilizing biochemical assays to determine how members of the long flap pathway affect FEN1 cleavage. We found that each of Dna2, Pif1, and RPA was able to stimulate FEN1 cleavage of short and long DNA flaps and also RNA flaps. Co-immunoprecipitation revealed that each long flap pathway protein is able to directly interact with FEN1. Previous work with Pif1 has shown that its activity is insufficient to allow cleavage of strongly structured fold-back flaps by either FEN1 or Dna2. However, using a reconstituted Okazaki fragment processing system, we investigated the possibility that Pif1 can remove fold-back-containing primers entirely. The presence of Pif1 does indeed allow for removal of a fold-back primer, restoring the ability of pol δ to synthesize through the fold-back region. Additionally, RPA helps pol δ to synthesize through fold-back regions present in the template. Finally, we investigated the effect of Pif1 flap

lengthening on correcting mismatches found in the downstream primer during Okazaki fragment processing. We observe that under conditions that encourage long flap formation, Pif1 helps to unwind farther into the downstream primer to aid pol δ in correcting mismatches. Interestingly, we found that pol δ and FEN1 inherently respond to the presence of mismatches by displacing and correcting farther into the downstream primer. These results provide a fuller understanding of the multiple roles for the members of the long flap pathway.

Figure I.1. The long and short flap pathways of Okazaki fragment processing.

As pol δ encounters the downstream fragment, it displaces the primer into a flap and continues synthesizing. One of two pathways is utilized to remove the flap: *A*, The short flap model proposes that FEN1 efficiently cleaves short flaps as they are displaced by pol δ. After removal of the primer, DNA ligase I seals the resulting nick, generating a continuous double-stranded DNA product. *B*, The long flap model proposes that some flaps escape immediate FEN1 cleavage and become long. Additionally, Pif1 has been shown to play a role in flap lengthening. Long flaps are bound by RPA, which inhibits FEN1. Dna2 nuclease displaces RPA, cleaves the flap, and leaves a short flap that RPA can no longer bind. FEN1 then cleaves the remaining flap at the base and LigI seals the nick.


A
B
5'
Flaps escape early cleavage/ Pif1 lengthens some flaps
Pol δ strand displacement synthesis
FEN1 recognizes flap base and threads flap
RPA binds, blocking FEN1
FEN1 cleaves
Dna2 dissociates RPA
Cleavage results in a nick
Dna2 cleaves
FEN1 cleaves
LigI seals nick
LigI seals nick
FEN1
Dna2
Pif1
RPA
LigI

Chapter 1. Components of the Secondary Pathway Stimulate the Primary Pathway of Eukaryotic Okazaki Fragment Processing

Abstract

Reconstitution of eukaryotic Okazaki fragment processing implicates both one and two-nuclease pathways for processing flap intermediates. In most cases, flap endonuclease 1 (FEN1) is able to efficiently cleave short flaps as they form. However, flaps escaping cleavage bind replication protein A (RPA) inhibiting FEN1. The flaps must then be cleaved by Dna2 nuclease/helicase before FEN1 can act. Pif1 helicase aids creation of long flaps. The pathways were considered connected only in that the products of Dna2 cleavage are substrates for FEN1. However, results presented here show that Dna2, Pif1, and RPA, the unique proteins of the two-nuclease pathway from *Saccharomyces cerevisiae*, all stimulate FEN1 acting in the one-nuclease pathway. Stimulation is observed on RNA flaps representing the initial displacement and on short DNA flaps, subsequently displaced. Neither the RNA nor the short DNA flaps can bind the two-nuclease pathway proteins. Instead, direct interactions between FEN1 and the two-nuclease pathway proteins have been detected. These results suggest that the proteins are either part of a complex or interact successively with FEN1, as the level of stimulation would be similar either way. Proteins bound to FEN1 could be tethered to the flap base by the interaction of FEN1 with PCNA, potentially improving their availability when flaps become long. These findings also support a model in which cleavage by FEN1 alone is the preferred pathway, with the first opportunity to complete cleavage, and is stimulated by components of the backup pathway.

Introduction

Double stranded DNA in eukaryotes is synthesized through elongation of leading and lagging strands copied using the original DNA as a template. The leading strand DNA is made in a continuous manner by DNA polymerase ε, which adds nucleotides in the same direction of the opening replication fork (1). The lagging strand, which grows in the opposite direction, must be made discontinuously, through the creation of short (~150 nt) oligonucleotides known as Okazaki fragments (15). This process begins with DNA polymerase α/primase (pol α), which makes a mixed primer initiating with 10 to 12 nt of RNA, then adding 10 to 20 nt of DNA (3). The primer is then lengthened by DNA polymerase δ (pol δ). After adding deoxynucleotides to make the bulk of the fragment, pol δ encounters the adjacent downstream Okazaki fragment. At this point, pol δ will displace the downstream fragment into a flap while continuing to synthesize DNA, a process called strand displacement synthesis (10,12). These flaps are cleaved by specific endonucleases, creating a nick that will then be sealed by DNA ligase I. The ligation event finishes the production of the complete DNA strand.

Two pathways have been suggested for flap processing. The first pathway, proposed by Peter Burgers and colleagues based on reconstitutions *in vitro*, is referred to as the FEN1-only pathway, or "one-nuclease pathway" (21,22,34). As the name suggests, in this pathway the only nuclease involved in flap cleavage is flap endonuclease 1 (FEN1). FEN1 is an endonuclease with a preferred substrate having a

5’ flap with a 1 nucleotide long 3’ flap. After pol δ displaces a short flap of only a few nucleotides, FEN1 is able to bind to the 5’ end of the flap and track to its base, where it proceeds to cleave off the entire flap, leaving behind only a nick (12,20,26).

Reconstitutions of Okazaki fragment processing *in vitro* suggest that FEN1 cleaves most flaps when they are only a few nucleotides long. However, in these reactions, a small fraction of flaps escape FEN1 cleavage and achieve greater lengths (42,72). Once this occurs, the flaps can be bound by replication protein A (RPA), the eukaryotic single stranded binding protein. RPA is able to stably bind flaps greater than 22 nt in length, and doing so has been shown to inhibit FEN1 cleavage (23,43).

Sung-Ho Bae and Yeon-Soo Seo proposed a second pathway through which they suggested flaps are cleaved *in vivo* (24). In the two-nuclease pathway, cleavage by the endonuclease Dna2 is promoted on RPA-coated flaps (23). Dna2 exhibits both a directional 5’-3’ endonuclease activity and a 5’-3’ helicase activity which is specific for forked substrates (24,54). Like FEN1, Dna2 enters flaps from the 5’ end. However, where FEN1 cleaves a single time, Dna2 cleaves multiple times while tracking on the flap. Also unlike FEN1, Dna2 is not able to cleave the flap entirely, but leaves a terminal flap of 5-7 nt in length (56). RPA is no longer able to bind these short flaps, allowing FEN1 to freely bind and cleave the remainder of the flap. While the two-nuclease pathway was originally proposed to predominate, later reconstitutions suggest that its role is to complete processing of the small fraction of flaps that are missed by FEN1 (24,42,72).

An additional protein, Pif1, has been implicated in lagging strand synthesis. Pif1 is a 5'-3' helicase that has been shown to have an important role in telomere and mitochondrial DNA maintenance (78). Interestingly, Pif1 has been shown to have a genetic interaction with Dna2; deletion of *PIF1* rescues the lethality created by a Dna2 nuclease deficient mutant in yeast (90). Previous work from our research group has also suggested that Pif1 plays a role in the two-nuclease pathway by aiding the creation of longer flaps during Okazaki fragment processing (72,94).

Although originally formulated as two independent pathways, some evidence suggests that the one- and two-nuclease pathways have relevant interactions. Previous characterization of Dna2 suggests that it is able to stimulate FEN1, though indirectly. By cleaving long flaps bound by RPA, Dna2 not only removes the RPA block but also leaves short flaps that are more readily processed by FEN1 (44,63).

We therefore questioned here whether the two pathways truly operate independently. That is, whether the protein components of the two-nuclease pathway performed no function until a flap grew to the length that would serve as a substrate for the two-nuclease pathway. In such a model, relative processing of substrates through either of the two pathways would depend solely on the fraction of flaps missed for cleavage by FEN1. Alternatively, the two pathways might be interactive, with components of one affecting the efficiency of the other. For example, the components of the two-nuclease pathway might interfere with FEN1 cleavage, helping to generate more substrate for their pathway. To test this concept we measured the effect of the unique two-nuclease protein components on the ability of

FEN1 to cleave substrates of the one-nuclease pathway. Surprising results showed that the two-nuclease proteins all stimulate FEN1 activity in a way that suppresses formation of the substrate for their own pathway.

Experimental Procedures

Materials– [α-^{32}P]dCTP and [γ-^{32}P]ATP were obtained from PerkinElmer Life Sciences. Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA). T4 Polynucleotide kinase and *E. coli* DNA polymerase I Klenow were obtained from Roche Applied Science. Other reagents were the best grade commercially available.

Protein Expression and Purification– *S. cerevisiae* Rad27 (FEN1) was cloned into the T7 expression vector pET-24b (Novagen/EMD Biosciences, Madison, WI), expressed in *E. coli* BL21(DE3) codon plus strain (Stratagene) and purified as previously described (19). *S. cerevisiae* Pif1 and Pif1 K264A were cloned into the pET-28b bacterial expression vector (Novagen/EMD Biosciences), expressed in the *E. coli* Rosetta strain (Novagen/EMD Biosciences), and purified as previously described (84). *S. cerevisiae* RPA was overexpressed and purified from *E. coli* as previously described (98). *S. cerevisiae* Dna2 E675A was produced by site-directed mutagenesis as previously described. Wild-type Dna2 and Dna2 E675A were overexpressed and purified from baculovirus High Five cells as previously described (52).

Oligonucleotide Substrates– Oligonucleotides were used to design substrates that

simulate Okazaki fragment processing intermediates. Downstream primers of 28, 32, and 56 nt in length were annealed at their 3'-ends to a 20-nt labeling template with a 5'-GCTA overhang and radiolabeled using [α-^{32}P]dCTP and Klenow polymerase. For 5' labeling, the downstream primer of 28 nt was labeled with [γ-^{32}P]ATP using polynucleotide kinase. Radiolabeled primers were separated by electrophoresis on a 15%/7 M urea polyacrylamide gel and then gel-purified. Substrates were then created by annealing primer components in annealing buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol), heating at 95 °C for 5 min, transferring to 70 °C, and slowly cooling to room temperature. The annealing ratio was 1:2:4 of downstream primer to template to upstream primer. Each downstream primer contains a region of 26 nt complementary to the template, resulting in 2, 6, and 30 nt unannealed 5' flaps, respectively. The template itself is 51 nt in length. The upstream primer is 26 nt in length, containing a 25 nt region complementary to the template, leaving 1 nt 3' flap. These substrates were designed to allow measurement of cleavage by FEN1. Additionally, an RNA-DNA substrate was created utilizing the above procedure. However, for the RNA-DNA substrate, the 32 nt long downstream primer contained 12 nt of RNA in the 5' region. Annealing resulted in a substrate with a 6 nt 5' RNA flap and 6 nt of RNA annealed to the template. Specific substrates used in each figure are indicated in the legends and pictured above the figures. The location of the 3' radiolabel on the downstream primer is indicated by an asterisk in the respective figures.

Cleavage Assays– For the fixed flap cleavage reactions, one or more of the proteins,

FEN1, Pif1, Dna2, or RPA, mixed together in reaction buffer containing 30 mM HEPES, 40 mM KCl, 4 mM $MgCl_2$, 0.01% NP-40, 0.5% inositol, 0.1 mg/ml bovine serum albumin, 1 mM dithiothreitol, 0.5 mM ATP, and 25% glycerol. To start the reactions, 5 fmol of 3' radiolabeled substrate was added for a total volume of 20 µl. Reactions were run for 10 min at 37 ºC. Reactions were stopped by adding 20 µl of 2x termination dye (90% formamide (v/v), 10 mM EDTA, and 0.01% bromphenol blue, and xylene cyanole), followed by heating at 95 ºC for 5 min. Products were separated by electrophoresis on a 22.5%/7 M urea polyacrylamide gel for 1 h and 40 min at 70 W. The gel was placed on filter paper and vacuum dried on a gel drier (Bio-Rad). Dried gels were exposed to a phosphor screen, which was scanned using a GE Healthcare PhosphorImager and analyzed with ImageQuant version 1.2 software.

Electrophoretic Mobility Shift Assay (EMSA)– Binding efficiency of Pif1, RPA, or Dna2 to a substrate with a flap of either 2, 6, or 30 nt was assessed using EMSA. For clearer visualization, the 2 nt flap substrate was 5' labeled in this experiment. Five fmol of substrate was incubated with increasing concentrations of the specific protein at either 50 or 500 fmol and incubated for 10 mins at 37 ºC in the same reaction buffer as described above, but lacking ATP and $MgCl_2$. The reactions were loaded on pre-run, non-denaturing 12% polyacrylamide gels in 1 X TBE. Gels were subjected to electrophoresis for 1.5 hours at a constant 250V.

Helicase Assay– To measure the helicase activity of Pif1 or Dna2, or the strand melting activity of RPA, the respective protein was added to a reaction containing a flap substrate of either 2, 6, or 30 nt in the reaction buffer utilized for our cleavage

reactions. For clearer visualization, the 2 nt flap substrate was 5' labeled in this experiment. Five fmol of substrate was incubated with concentrations of protein of 50 or 500 fmol and incubated for 10 min at 37 ºC. The reactions were terminated using a helicase dye consisting of 30% glycerol, 50 mM EDTA, 0.9% SDS, 0.125% bromophenol, and 0.125% xylene cyanole. The reactions were loaded on pre-run, non-denaturing 12% polyacrylamide gels in 1 X TBE. Gels were subjected to electrophoresis for 1.5 hours at a constant 250V.

Binding Assay– Purified scFEN1 (1 ng) and 1 ng of a purified two-nuclease protein (scDna2, scPif1 or scRPA) were allowed to bind together in a coupling buffer consisting of 25 mm HEPES (pH7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol for 2h at 4 °C (in a 1:1 ratio). When three proteins were bound to FEN1, 2 ng of the competing proteins were added first and allowed to bind for 2 hours. Then, 1 ng of the antibody-specific protein was added and incubated with the mixture for an additional 2 hours. Antibody to either Dna2, prepared as previously described (62), Pif1 (SCBT, sc-48377) or RPA70 (kindly provided by Dr. Marc Wold) or control IgG (SCBT sc-2027) were prebound to protein A-agarose for 1 h at room temperature and washed twice with phosphate-buffered saline. The bound proteins were then added to the washed protein A-agarose-antibody complex and incubated overnight at 4 °C with end-over mixing. The following day, the proteins were released from the protein A-agarose using elution buffer. The immunoprecipitates were separated on precast 4-20% gels (Bio-Rad). Western blot analysis was performed with anti-FEN1 polyclonal antibody

(Abcam ab17993). Cross reactivity of mouse monoclonal Pif1 antibody with purified scPif1 was tested and confirmed before the immunoprecipitation.

The amount of each protein used in each experiment is given in the appropriate figure legend. All enzyme assays were repeated at least in triplicate with a representative gel shown in each figure.

Results

Dna2 and RPA have been implicated in the two-nuclease pathway, important for the proper cleavage of long flaps formed during eukaryotic Okazaki fragment processing. Previous work has also shown that Pif1 helicase promotes the long flap pathway. Although the two-nuclease and FEN1-only pathways are generally represented as occurring separately from one another, we considered the possibility that the pathways have evolved to be interactive. Specifically, we set out to examine whether the components of the two-nuclease pathway influence the efficiency of the one-nuclease pathway.

Substrate Design– For our experiments, we created three substrates with flap lengths of 2, 6, or 30 nt. The 2 nt flap represents an intermediate formed during the beginning of strand displacement synthesis, which we would expect to have no affinity for Dna2, RPA or Pif1. The 6 nt flap represents an intermediate considered at the borderline for detectable binding by the three two-nuclease pathway proteins. The 30 nt flap serves as a positive control to which all three proteins should be able to

bind with maximum affinity. FEN1 is able to bind and cleave all of these flaps. By utilizing these three different lengths, we have been able to distinguish the influence of each protein on flap cleavage by FEN1 and whether that influence depends on binding of each protein to the flap.

Since our experimental protocol utilized fixed flap substrates, and because Dna2 and Pif1 are helicases, the potential existed that the helicase functions of these proteins would disrupt the substrates. The concern was that the helicases would partially displace the downstream primer, creating a gap. This would alter the double flap configuration favored by FEN1, decreasing FEN1 cleavage activity, and masking any stimulatory effects. This is not an issue *in vivo*, since elongation of the upstream primer by pol δ would continuously renew the favored double flap structure. To ensure we did not experience interference, we chose a buffer system in which the helicase functions would cause minimal disruption of our substrates.

Effect of Dna2 on FEN1 Cleavage– Dna2 was found to stimulate FEN1 cleavage on long flaps. It has been proposed that the ability of Dna2 to cleave long 5' flaps, terminating in a minimum flap length of about 6 nt, promotes FEN1 to cleave the remainder of the flap more efficiently. The action of Dna2 would remove regions of secondary structure in the flap that might interfere with tracking of FEN1. Moreover, Dna2 would also reduce the flap length so that RPA could not bind to interfere with FEN1 tracking. However, we were interested in determining whether Dna2 is able to directly stimulate FEN1 on the short flaps cleaved in the one-nuclease pathway.

To measure whether Dna2 influences FEN1 activity, we first performed a cleavage assay. For this experiment, amounts of Dna2 ranging from 0 fmol to 50 fmol were added to reactions containing FEN1 and varying flap length substrates. We observed a dose dependent stimulation of FEN1 cleavage by Dna2 (Fig. 1.1A). Interestingly, in addition to measuring stimulation on the 30 nt flap substrate, to which Dna2 is expected to bind avidly, we also measured significant stimulation on the 6 and 2 nt flaps, implying that the ability of Dna2 to stimulate FEN1 is independent of Dna2 binding to the substrate. After adding 50 fmol of Dna2 to the reaction in the presence of ATP, we measured a stimulation of FEN1 cleavage of 12.5-fold on the 2 nt flap substrate, 44.2-fold on the 6 nt flap substrate, and 15.9-fold on the 30 nt flap substrate.

Additionally, this experiment was performed in the absence of ATP. This was done to determine whether the helicase function of Dna2 is responsible for its ability to stimulate FEN1. The outcome was similar, showing that Dna2 is able to stimulate FEN1 cleavage even in the absence of helicase function.

To confirm the expected binding characteristics of Dna2 to the different length flap substrates, we performed an electrophoretic mobility shift assay (EMSA) utilizing all three substrates (Fig. 1.1B). For these EMSA measurements, we used protein levels of 50 and 500 fmol. Whereas 50 fmol of Dna2 represents the highest amount used in our cleavage assays, to observe high efficiency binding of the protein for an EMSA, a 10-fold increase in protein (i.e. 500 fmol) was needed. In the EMSA reactions, we utilize a buffer lacking both ATP and Mg^{2+}, to prevent unwinding or, in

the case of Dna2, cleavage. At 50 fmol of Dna2, we detected no binding to the 2 and 6 nt flap substrates, and small amount of Dna2 bound to the 30 nt flap substrate. At 500 fmol, there was little binding to the 2 nt flap substrate. More binding was observed on the 6 nt flap substrate, with the most binding occurring on the 30 nt flap substrate in the presence of 500 fmol of Dna2. This is consistent with the binding properties expected from Dna2.

Finally, we performed a helicase assay utilizing the nuclease-deficient mutant of Dna2. While the absence of ATP negated the helicase activity of Dna2 in the cleavage assay, the helicase assay was performed in conditions that allowed for maximal helicase activity by the nuclease-deficient Dna2 mutant. Although Dna2 did not show stable binding to the short flap substrates in our EMSA study, we envisioned that there could be transient binding of Dna2 to these substrates, allowing for some helicase activity. However, at 50 fmol of Dna2, the maximum amount used in our cleavage assay, we saw only minimal unwinding, and only on the 30 nt flap (Fig. 1.2). Even at 500 fmol of Dna2, unwinding of the downstream primer could only be observed on the 30 nt flap, suggesting that robust helicase activity by Dna2 requires a longer single-stranded DNA region for it to track and unwind the substrate. This confirms that while Dna2 can stimulate FEN1 cleavage on short flaps, it cannot perform its helicase or endonuclease functions.

Effect of Pif1 on FEN1 Cleavage– Pif1 has previously been shown to promote utilization of the two-nuclease pathway in reconstitution reactions. Because it

appears to be a two-nuclease pathway component, we assessed the ability of Pif1 to stimulate FEN1 on short flaps.

As with Dna2, we first performed a cleavage assay, now utilizing FEN1 and Pif1. Varying amounts of Pif1, ranging from 0 to 50 fmol were added to reactions containing FEN1. Results revealed a dose dependent stimulation of FEN1 cleavage by Pif1 (Fig. 1.3A). As with Dna2, stimulation of FEN1occurred not only for cleavage of the 30 nt flap substrate but also with the 6 and 2 nt flap substrates. This implies that the ability of Pif1 to stimulate FEN1 is not dependent on Pif1 binding to the flap. Addition of 50 fmol of Pif1 to the reaction resulted in a stimulation of FEN1 cleavage by 16.6-fold on the 2 nt flap substrate, 10.5-fold on the 6 nt flap substrate, and 9.4-fold on the 30 nt flap substrate. A similar experiment was also performed in the absence of ATP to determine whether the helicase function of Pif1 is in any way responsible for its ability to stimulate FEN1. We observed that, like Dna2, Pif1 is able to stimulate FEN1 cleavage even in the absence of ATP. A Pif1 helicase-deficient mutant was also utilized to further confirm that the stimulation of FEN1 by Pif1 does not require its helicase function (data not shown).

We expected that Pif1 would exhibit weak binding to a 2 or 6 nt flap but would bind avidly to a 30 nt flap. To confirm, we performed an EMSA utilizing all three substrates in the presence of Pif1 (Fig. 1.3B). We observed a very low level of binding on the 2 nt flap, even at 500 fmol of protein. As expected, the amount of binding increased on the 6 nt flap, although the amount of Pif1 bound was still low. On the 30 nt flap, binding was evident even at 50 fmol of Pif1 and even more

efficient at 500 fmol of Pif1. This result is consistent with our expectation that Pif1 would bind poorly to the short flap substrates, if at all, but readily to the 30 nt flap substrate.

In order to determine how the helicase of Pif1 is functioning on the different length flaps utilized in our previous assays, we performed a helicase assay. In addition to characterizing the helicase ability of Pif1, we also sought to confirm that Pif1 was not disrupting our substrates by removing the downstream primer. At 50 fmol of Pif1, the maximum amount used in our cleavage assay, we saw minimal unwinding of the downstream primer and only on the 30 nt flap (Fig. 1.2). This level of Pif1 was chosen intentionally with the expectation that it would not disrupt the substrate. At the higher concentration of 500 fmol, however, Pif1 displayed helicase activity, most effective on the 30 nt flap substrate, with some unwinding on the 6nt flap. This confirms what we expected: there is no helicase activity of Pif1 directly on the 2 nt flap, minimal activity on the 6 nt flap, and Pif1 is able to freely interact with the 30 nt flap. This pattern of unwinding is consistent with our hypothesis that Pif1, on short flaps, is at first working to stimulate FEN1 cleavage. However, after a critical flap length is reached, Pif1 is able to bind to the flap, allowing its helicase function to act. This allows Pif1 to lengthen the flap, thereby biasing the processing of the flap toward the two-nuclease pathway.

Effect of RPA on FEN1 Cleavage– RPA is a key component of the Okazaki fragment maturation pathway, responsible for switching processing from the one-nuclease pathway to the two-nuclease pathway via binding of the flaps. To better understand

these actions of RPA, we measured regulation of FEN1 by RPA on the same substrates that revealed stimulation of FEN1 by Dna2 and Pif1.

Concentrations of RPA ranging from 0 fmol to 100 fmol were added to reactions containing FEN1. This resulted in a dose dependent stimulation of FEN1 cleavage by RPA (Fig. 1.4A). On the 2 and 6 nt flap substrates, to which RPA cannot stably bind, FEN1 cleavage was increased by 9.3- and 8.3-fold, respectively, at 100 fmol of RPA. For the 30 nt flap substrate, we included two additional, higher amounts of RPA, 200 and 300 fmol. This allowed us to measure the expected inhibition of FEN1 by RPA on long flaps. At lower levels, RPA was indeed able to stimulate FEN1 cleavage; there was a 2-fold stimulation of FEN1 cleavage at 100 fmol of RPA. However, at 300 fmol of RPA, at which we expected stable coating of the flap, FEN1 cleavage was inhibited to 25% of its basal level. As with our previous assays, this experiment was also performed in the absence of ATP. We observed a generally similar stimulation/inhibition pattern irrespective of inclusion of ATP, although inhibition of FEN1 on the 30 nt flap substrate occurred at lower concentrations in the absence of ATP. This could simply be a result of a lower ionic strength of our buffer without the ATP present.

To confirm expected binding characteristics of RPA, we performed an EMSA utilizing all three substrates in the presence of RPA (Fig. 1.4B). There was little binding of RPA on the 2nt and 6 nt flap at 50 fmol of protein, but there was substantial binding at 500 fmol. RPA is expected to exhibit a low-affinity binding mode on shorter flaps, but once bound it may encourage local melting until the flap is

long enough for more stable interaction. On the 30 nt flap, substantial binding was evident at 50 fmol of RPA, with a complete substrate shift at 500 fmol.

Finally, we performed a melting assay for RPA. Although RPA does not have helicase activity, high levels of RPA have been show to melt double stranded DNA. Therefore, we intended to confirm that RPA was not removing either our upstream or downstream primers. At 50 fmol of RPA we saw no removal of either primer and even at 500 fmol of RPA, removal of either primer from the template was not observed (Fig. 1.2).

RPA has previously been reported to stimulate FEN1 cleavage. In 2003, Chai *et al*. observed that on a 9 or 1 nt flap substrate, the presence of RPA would stimulate FEN1 cleavage (99). Biswas *et al.* also noticed a similar stimulation followed by inhibition of FEN1 as RPA is titrated onto a 16 nt flap substrate (100). The detailed mechanism of RPA stimulation of FEN1, and whether it involved RPA interaction with the substrate, was not explored. Based on our results, stimulation of FEN1 by RPA does not appear to derive from an interaction of RPA with the flap substrate, but rather a direct interaction with FEN1.

After observing stimulation of FEN1 cleavage by Dna2, Pif1, and RPA, we also performed several negative controls. We showed that boiled Dna2, Pif1, and RPA do not possess the ability to stimulate FEN1 cleavage (data not shown). Additionally, we noted that stimulation of FEN1 cleavage was species specific, as human Dna2 and RPA failed to stimulate *S. cerevisiae* FEN1 (data not shown).

Additionally, to confirm that the stimulation of FEN1 by our proteins did not result from a contaminant in our protein preparations, Dna2, Pif1, and RPA (each containing a 6x-histidine tag) were allowed to adsorb to a Ni-NTA agarose column. Following protein sequestration, the flow-through supernatant fraction was added to FEN1 and produced no stimulation of cleavage. Following elution of each protein from the nickel agarose column, it stimulated FEN1 cleavage (data not shown).

Effect of Dna2, Pif1, or RPA on FEN1 Cleavage of an RNA Flap– Flaps are created when pol δ displaces the primer laid down by pol α and then extended by pol δ, which would generate an RNA segment of 10-12 nt on the 5' end, followed by DNA. However, neither Dna2, Pif1, nor RPA are known to bind to single stranded RNA. Therefore, it was important to determine whether protein components of the two-nuclease pathway are capable of stimulating FEN1 on a flap with a 5' RNA segment.

For these experiments, we utilized a substrate containing a 6 nt long 5' flap composed entirely of RNA. At the base of the flap, there are 6 additional nucleotides of RNA annealed to the DNA template. This represents the 12 nt of RNA anticipated to be laid down by pol α. We then repeated the titrations of Pif1, Dna2, and RPA that we performed previously utilizing the DNA flap substrates.

In each case, we observed stimulation of FEN1 cleavage. There was approximately a 12-fold stimulation at 50 fmol of Dna2 (Fig. 1.5A) and approximately a 13-fold stimulation at 50 fmol of Pif1 (Fig. 1.5B). Moreover, at 50 fmol of RPA, FEN1 cleavage was stimulated 8-fold (Fig. 1.5C). These values are consistent with the level of stimulation observed with a DNA flap substrate. We

noted with the RNA substrate, FEN1 cleavage produced two bands. This is not surprising, as FEN1 sometimes will move 1 nt further into the downstream primer where it then can cleave a second time. Therefore, for analysis of these results, FEN1 cleavage was assessed as a combination of the two cleavage products.

Effect of the Combination of Dna2, Pif1, and RPA on FEN1 Cleavage– After observing the ability of Dna2, Pif1 and RPA to stimulate FEN1 individually, we further explored the combined effect all three proteins on FEN1 activity. One could imagine a situation in which each protein stimulates FEN1 in a different way, perhaps by inducing different conformational changes within FEN1. In that case, we would expect that adding different proteins would produce an additive stimulation of FEN1. Alternatively, if each protein component induced the same structural change in FEN1, then we would expect that there would be a maximal level of stimulation that one protein could impose, that could not be exceeded by addition of one or more additional stimulatory proteins.

First, we determined the amounts of either Dna2, RPA, or Pif1 necessary to maximally stimulate a very low level (0.2 fmol) of FEN1 on a 6 nt flap. The low level assured that additive stimulations could be readily detected. The maximum stimulatory amounts were determined to be 400 fmol of Dna2, 400 fmol of RPA, and 300 fmol of Pif1 (data not shown). Using these concentrations, we then added Dna2, RPA, and Pif1 to FEN1 in different combinations (Fig. 1.6). FEN1 controls of 10 fmol and 2 fmol were also included. The 10 fmol of FEN1 is used to demonstrate that FEN1 can cleave 100% of our substrate. 2 fmol (a 10-fold increase over the 0.2

fmol control) is used to more easily visualize a basal level of FEN1 cleavage. We observed that the highest stimulation of FEN1 cleavage by a single protein resulted from addition of Dna2 (42.9% cleavage). When adding all three proteins together, there was little difference in the amount of FEN1 cleavage compared to only adding Dna2 (46.8%). Combining two proteins had little effect on the maximal cleavage observed as well. Rather, although each protein stimulated to a somewhat different maximum, the various combinations of proteins stimulated to a maximum that was not substantially different from the stimulation level with Dna2 alone. These results demonstrate that the two-nuclease pathway proteins do not exhibit an additive stimulatory effect and that the proteins are each not stimulating FEN1 in a unique manner.

Protein Components of the Two-Nuclease Pathway are Present in a Complex with FEN1– Stimulation of FEN1 by each protein component of the two-nuclease pathway even when those proteins were not expected to interact with the substrate suggested direct interactions of Dna2, Pif1 and RPA with FEN1. Pif1 and Dna2 are known to genetically interact (90), and direct associations between hFEN1 and hDna2 and scDna2 and scRPA70 have also been shown (70,101). Binding assays were performed to clarify the direct interactions of scFEN1 with scDna2, scPif1 and scRPA *in vitro*. Purified recombinant yeast proteins were bound to FEN1 overnight and were immunoprecipitated the following day with antibodies to Dna2, Pif1 or RPA70. The immunoprecipitates were separated on a SDS-polyacrylamide gel, and Western blotting was performed using antibody against FEN1. The non-specific IgG

control showed no contamination with FEN1 (Fig. 1.7, *lane* 1). Lane 2 served as a positive control for FEN1. Direct interaction of Dna2 and FEN1 was detected and confirmed as previously reported (Fig. 1.7, *lane* 5). Pif1 (Fig. 1.7, *lane* 8) and RPA (Figure 1.7, *lane* 11) also associated with FEN1 *in vitro*. Lanes 4, 7, and 10 serve as controls to demonstrate that Dna2, Pif1, and RPA, respectively, are not contaminated with FEN1 and thus stimulation by these proteins is not a result of additional FEN1 being included with these proteins. Lanes 3, 6, and 9 are controls to show that the Dna2, Pif1, and RPA antibodies, respectively, do not bind FEN1.

Our results suggest that each protein component of the two-nuclease pathway is able to directly interact and stimulate FEN1 *in vitro*. When all the protein components of the two-nuclease pathway were simultaneously allowed to bind FEN1, then individually immunoprecipitated with antibodies specific for either Dna2, Pif1 or RPA and finally immunoblotted for FEN1, we still observed interaction between individual proteins and FEN1 (Fig. 1.8). However, the quantity of each protein bound to FEN1 was lower in the presence of the other proteins. Possibly, the presence of other proteins in a complex partially occluded the antibody interaction sites, decreasing the efficiency of immunoprecipitation. Alternatively, this could be an indication of partial or even complete overlap of protein binding sites, such that the proteins competed for binding FEN1. Additionally, interactions of the proteins among themselves might have altered the efficiency of their binding to FEN1. Our experiments are not able to distinguish among these possibilities. Results are

consistent with formation of a complex containing all of the proteins, but also with complexes that allow just one or two proteins at a time to bind FEN1.

Discussion

Current models of eukaryotic Okazaki fragment processing envision that it occurs by two enzymatic pathways. The one-nuclease pathway is proposed to convert the large majority of short flaps to nicks for ligation through the nuclease action of FEN1. The two-nuclease pathway is thought to process those flaps that escape FEN1 cleavage to become long. The actions of these pathways until now have been viewed as sequential and functionally independent. They also may be viewed as unidirectionally competitive, in the sense that the one-nuclease pathway reduces substrate available for two-nuclease processing. We demonstrate here that all of the unique protein components proposed to participate in the two-nuclease pathway, Dna2, Pif1, and RPA, stimulate the cleavage activity of FEN1 via direct interaction. This stimulation is ATP independent and occurs on substrates with flaps that are too short to bind these two-nuclease pathway proteins. Additionally, although Pif1 and RPA cannot bind to RNA, and Dna2 cannot cleave RNA, all three proteins are able to stimulate FEN1 on a flap substrate containing a 5' RNA region.

Relative use of the two pathways has been inferred from reconstitution of Okazaki fragment processing using oligonucleotide substrates and purified enzymes *in vitro* (72,94). In the reconstituted system, instead of using a preformed flap

substrate, pol δ actively creates a flap. Pol δ, complexed with PCNA, extends an upstream primer into a downstream primer, generating a flap by strand displacement synthesis, the mechanism of flap creation in Okazaki fragment processing. In the presence of FEN1, the large majority of cleavage products were less than eight nt long (72). These observations suggest that the natural one-nuclease pathway is highly efficient, and that the two-nuclease pathway has evolved to catch a small percentage of the flaps that are missed by effective FEN1 cleavage. While it is currently not very clear why certain flaps escape FEN1 cleavage, one can imagine the deleterious effects on the genome stability if the system were not equipped with a fail-safe mechanism in the form of the two-nuclease pathway. FEN1 haplo-insufficiency has been linked to genomic instability and rapid progression to cancer (102,103). Furthermore, post-translational modification of FEN1 by either phosphorylation or acetylation inhibits its cleavage function (104-106). Uncoupling of FEN1 from the replisome or post-translational modification of the protein might drive the system to utilize the two-nuclease pathway.

Assuming that the replication machinery has evolved to maximize genome stability, it makes sense to favor processing of flaps by FEN1 while they are short, since problems caused by longer flap intermediates are easily envisioned. Long flaps can fold back onto themselves, forming secondary structures anticipated to inhibit processing. Formation of secondary structures not only inhibits the one-nuclease pathway, but these structures are also refractory to action by protein components of the two-nuclease pathway thereby creating an undesirable, unresolved structure. The

long flaps can also promote harmful recombination events that would disrupt the continuity of the DNA sequence. Additionally, it has been shown that repeated cleavage of short flaps by FEN1 promotes more effective strand displacement synthesis by pol δ (22,42). Thus, quick, efficient cleavage of short flaps would help lagging strand processing to the continuous final product occur more quickly, avoiding a series of long-lived breaks in the chromosome that can also promote recombination.

Furthermore, the requirement for removal of the RNA portion of the mixed primer made by pol α highlights the need for efficiency of the one-nuclease pathway. Pif1 cannot bind to an ssRNA flap (84) and RPA shows approximately a thousand-fold lower binding affinity to ssRNA as compared to ssDNA (64,107). Moreover, while Dna2 is able to track over an RNA flap, it cannot cleave (24,63). FEN1, however, is similarly active on RNA or DNA flaps (40). Therefore the RNA portion of the flap must be either fully displaced or displaced and cleaved before the two-nuclease pathway is even an option. Again, this suggests that the replication system has evolved in a way that favors FEN1 cleavage by allowing what would otherwise be inactive members of the two-nuclease pathway to stimulate FEN1 cleavage even when they themselves cannot bind or cleave.

With further displacement, the region of DNA beyond the RNA begins to become single stranded. FEN1 cleavage can now generate a nick that serves as a substrate for the ligation that completes fragment processing. Our results indicate that while the DNA flap is short, neither Pif1 nor Dna2 can exhibit helicase activity,

but can exert a stimulatory effect on FEN1. Only after additional DNA flap displacement, and only in the infrequent cases in which the flaps escape FEN1 cleavage, would the flaps achieve lengths of approximately 20 nt. At this point RPA would bind to inhibit FEN1, Pif1 would accelerate displacement, and these flaps would require a shift to the two-nuclease pathway.

To further characterize the manner in which Pif1, Dna2, and RPA stimulate FEN1, we evaluated whether these proteins could each stimulate FEN1 in an additive manner or whether the presence of any one of the three proteins was capable of driving FEN1 to maximum activity. When using low levels of FEN1 and observing stimulation by each individual protein, we note that the addition of a second or third protein has little to no effect on the ability of FEN1 to cleave over the stimulation already conferred. The highest level of stimulation from an individual protein is from Dna2, causing an increase in cleavage from 0.6% to approximately 43%, a stimulation of approximately 71-fold. The addition of RPA and Pif1 to the reaction only had a modest effect, increasing cleavage to about 47%.

We also demonstrated that each of the unique proteins of the two-nuclease pathway has a direct binding affinity for FEN1, independent of the presence of a DNA substrate. However, our binding measurements could not establish unambiguously whether all three proteins can bind FEN1 simultaneously, or whether they have evolved for a process of sequential binding during the execution of the two-nuclease pathway. Since single interactions, or complexes involving combinations of two or all three proteins are all capable of maximum stimulation of FEN1, the effect

on FEN1 cleavage of short flaps should be similar whether the proteins are bound simultaneously or sequentially.

Previous work has demonstrated that another protein involved in Okazaki fragment processing, PCNA, is also able to influence FEN1 cleavage. PCNA is a central component of Okazaki fragment processing that interacts with pol δ, FEN1 and DNA ligase I. It is a processivity factor for the polymerase and a stimulator of FEN1 and the ligase. It is also thought to coordinate the sequential actions of these proteins. PCNA, and its similarly-structured DNA repair counterpart, the 9-1-1 checkpoint complex, both bind and stimulate FEN1. Stimulation by PCNA has been extensively investigated (28,108,109). To exert stimulation, PCNA must be loaded onto the double stranded part of the FEN1 substrate, specifically upstream of the flap. Evidence suggests that it captures the FEN1 as it slides to the base of the flap and stabilizes FEN1 interaction with its cleavage site. While PCNA may also induce a conformational change in FEN1 that increases activity, effects on tethering FEN1 to its cleavage site and induction of a more active conformation are not readily distinguished. PCNA and 9-1-1 are capable of stimulating FEN1 cleavage activity over 10-fold, similar to the stimulation factors conferred by the protein components of the two-nuclease pathway.

FEN1, like other enzymes, presumably has a stimulation maximum that cannot be exceeded. While, experiments analyzing FEN1 cleavage function on cognate substrates *in vitro* are done in the presence of either individual or a combination of stimulating proteins, in the cellular system, the modes of interaction

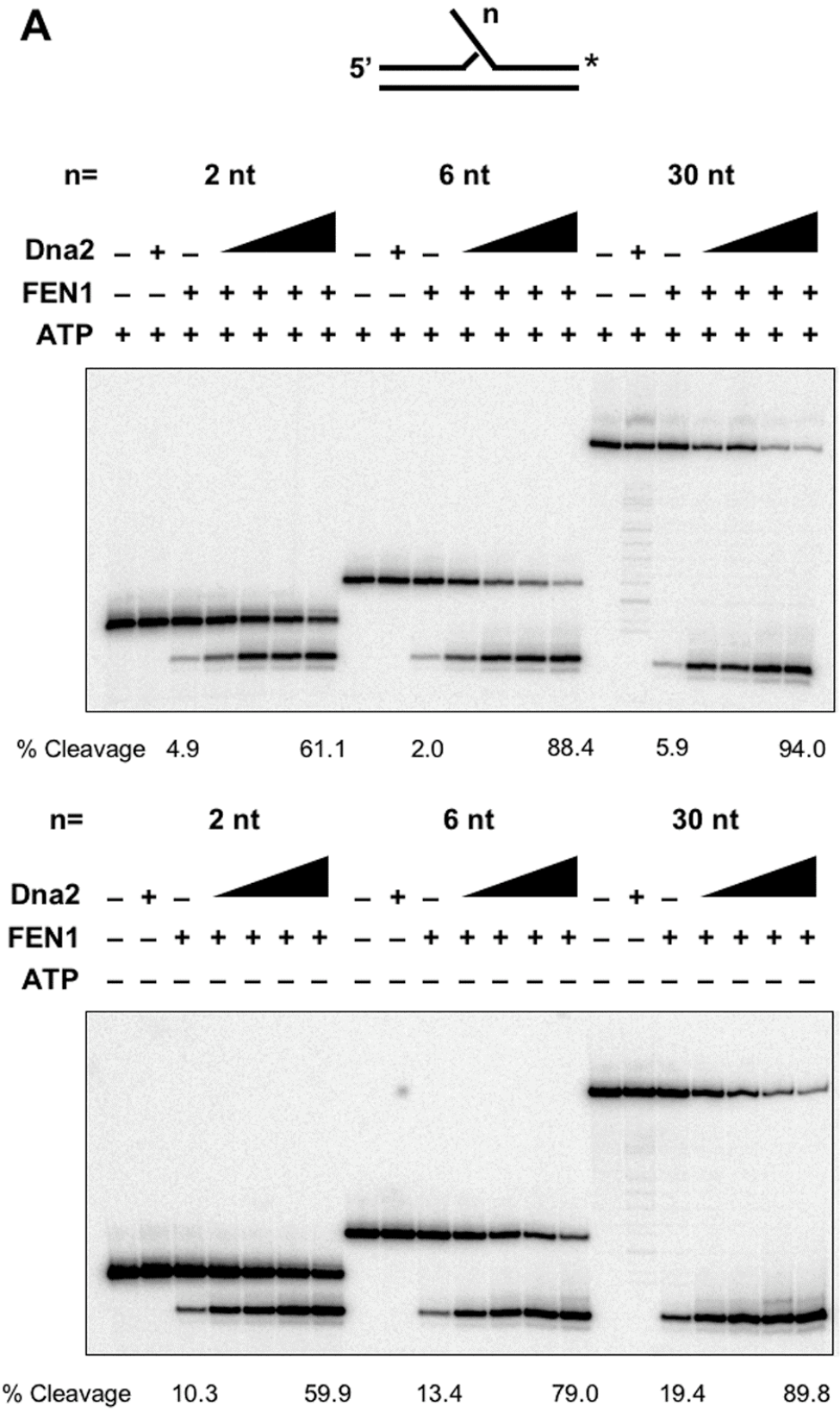
and stimulation might vary. For example, FEN1 interaction with PCNA might primarily provide for an increase in binding to the flap and interactions with Dna2, Pif1 and RPA may change the conformation of FEN1 in a manner that it can optimally function on the substrate.

Based on these observations and binding studies, we know that FEN1 is capable of complexing with multiple proteins, including Dna2, Pif1, RPA and PCNA. Moreover, it appears that only PCNA tethers the FEN1 to its cleavage site on a short flap substrate, as the two-nuclease proteins do not bind short flaps. However, the two-nuclease proteins are capable of interacting with FEN1 and each other, in a way that is likely to raise their local concentrations near flaps. When the flaps grow long, the two-nuclease components can develop additional productive interactions with the substrate. These interactions would help the two-nuclease components to be in proximity to their long-flap substrates as soon as they are created.

In summary, the protein components of the two-nuclease pathway for eukaryotic Okazaki fragment processing appear to interact directly with FEN1 in order to stimulate FEN1 cleavage of short flaps. We propose that these proteins form a complex with FEN1 and PCNA that involves either simultaneous or successive interactions. Moreover, if a flap becomes long enough to require the two-nuclease pathway, the presence of the complex assures that the two-nuclease proteins are available to carry out their functions.

Figure 1.1. Dna2 stimulates FEN1 cleavage of 2, 6, and 30 nt flap substrates.

A, cleavage by FEN1 (2 fmol) was assayed on a 2, 6, and 30 nt flap substrate in the presence or absence of ATP and varying amounts of Dna2 (5, 15, 25, or 50 fmol) as indicated in the figure and described under "Experimental Procedures." The substrate is depicted above the figure with the location of the radiolabel indicated by the *asterisk*. The length of the flap utilized is denoted above the corresponding lanes. *Plus* (+) indicates the presence and *minus* (–) the absence of the listed component. *B*, Dna2 binding activity (50 or 500 fmol) was measured on a 2, 6, and 30 nt flap substrate by non-denaturing PAGE.


A
n
5'
*
n=
2 nt
6 nt
30 nt
Dna2
FEN1
ATP
% Cleavage 4.9 61.1 2.0 88.4 5.9 94.0
n=
2 nt
6 nt
30 nt
Dna2
FEN1
ATP
% Cleavage 10.3 59.9 13.4 79.0 19.4 89.8

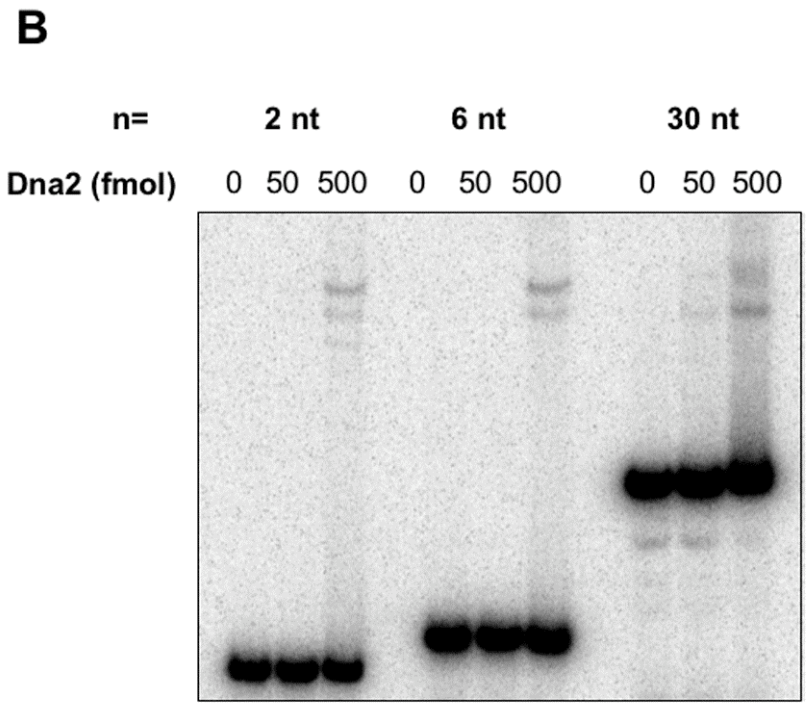

B
n=
2 nt
6 nt
30 nt
Dna2 (fmol)
0 50 500 0 50 500 0 50 500

Figure 1.2. Substrate unwinding by Pif1, Dna2, and RPA.

Unwinding of a 2, 6, and 30 nt substrate by Pif1, Dna2 E675A, or RPA was assessed by non-denaturing PAGE using 50 or 500 fmol of protein, as indicated in the figure and described under "Experimental Procedures." Substrate depictions and designations are as in Fig. 1.1.

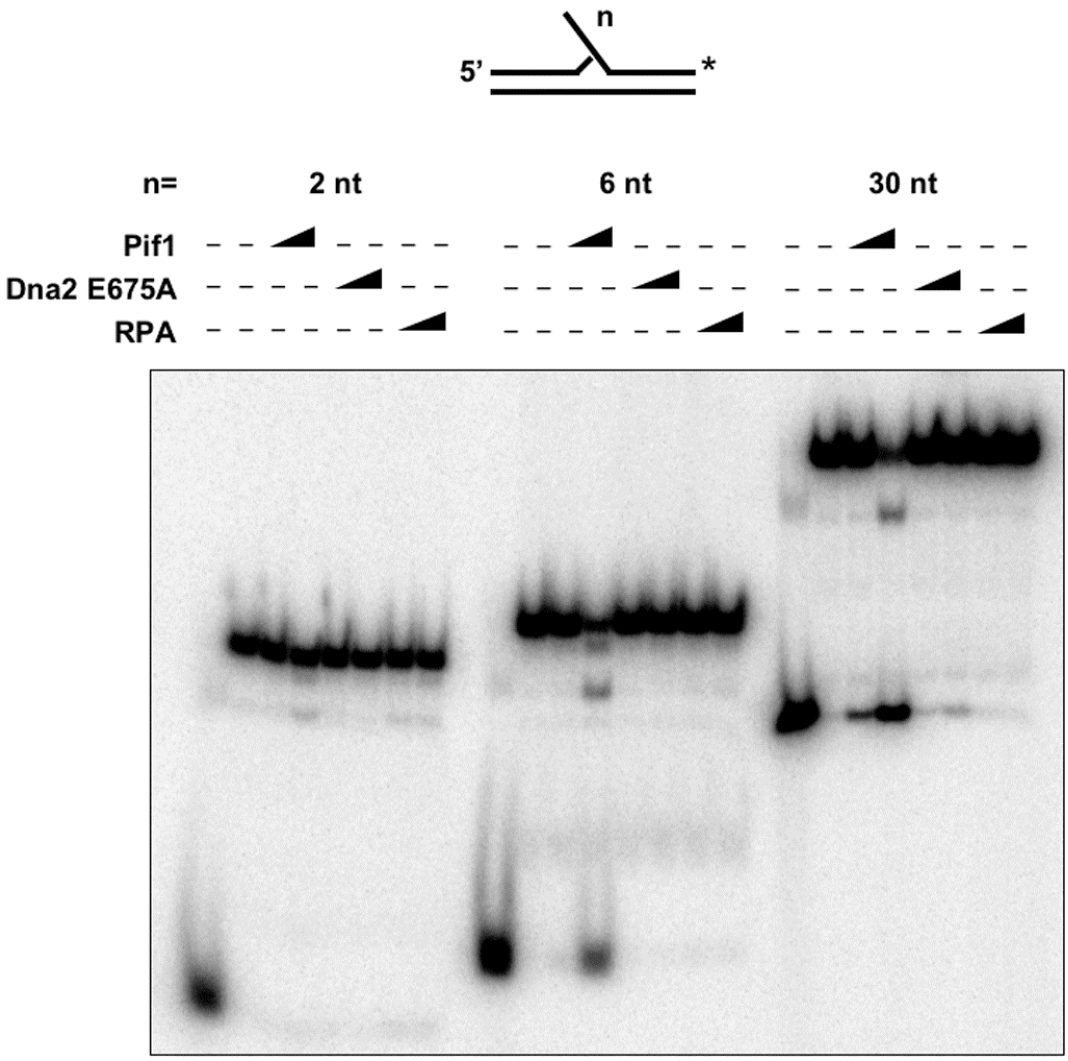
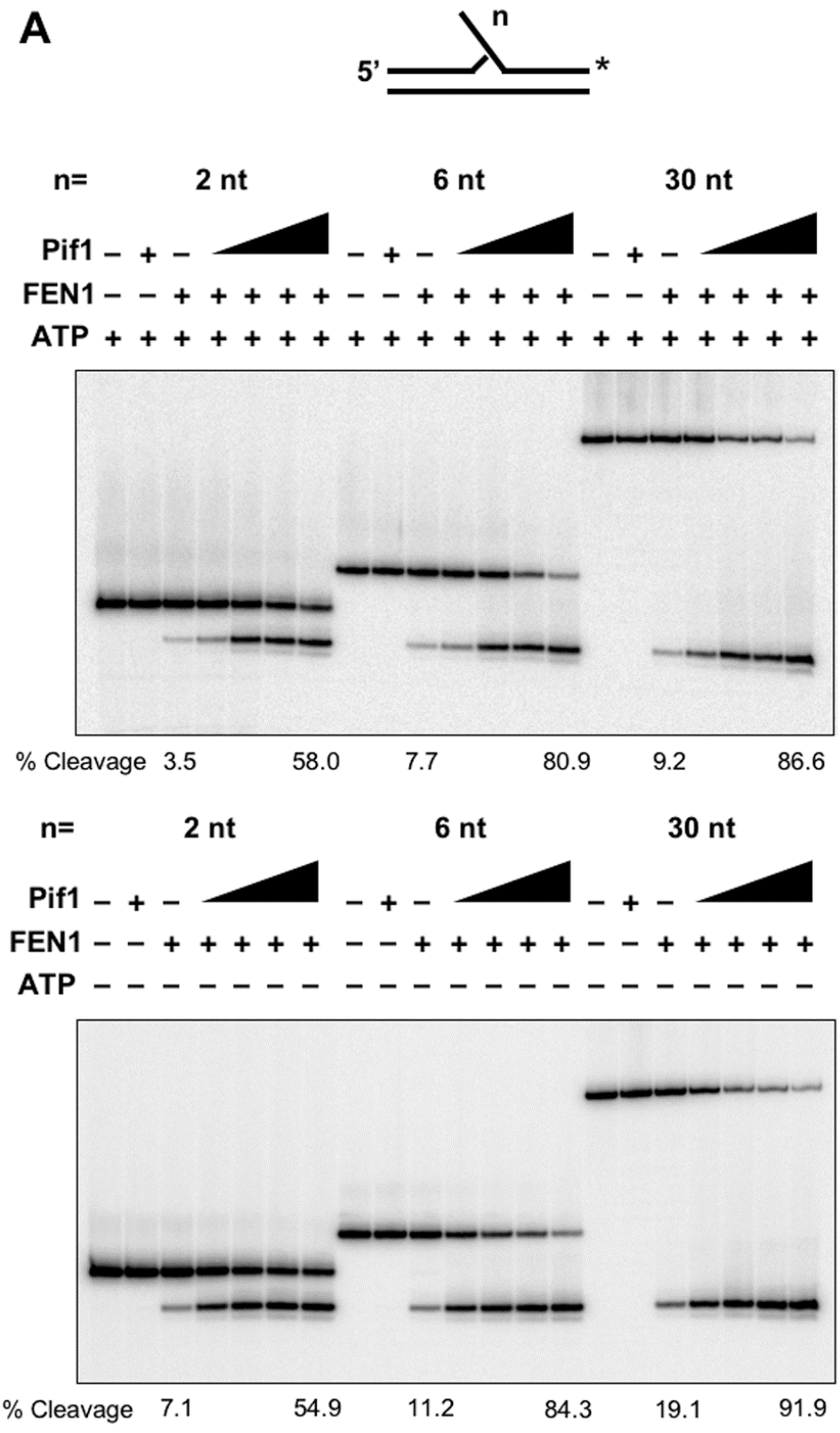

n
5'
*
n=
2 nt
6 nt
30 nt
Pif1
Dna2 E675A
RPA

Figure 1.3. Pif1 stimulates FEN1 cleavage of 2, 6, and 30 nt flap substrates.

A, cleavage by FEN1 (2 fmol) was assayed on a 2, 6, and 30 nt flap substrate in the presence or absence of ATP and varying amounts of Pif1 (5, 15, 25, or 50 fmol) as indicated in the figure and described under "Experimental Procedures." Substrate depictions and designations are as in Fig. 1.1. *B*, Pif1 binding activity (50 or 500 fmol) was measured on a 2, 6, and 30 nt flap substrate by non-denaturing PAGE.


A
n
5'
*
n=
2 nt
6 nt
30 nt
Pif1 – + – – + – – + –
FEN1 – – + + + + + – – + + + + + – – + + + + +
ATP +
% Cleavage 3.5 58.0 7.7 80.9 9.2 86.6
n=
2 nt
6 nt
30 nt
Pif1 – + – – + – – + –
FEN1 – – + + + + + – – + + + + + – – + + + + +
ATP –
% Cleavage 7.1 54.9 11.2 84.3 19.1 91.9

B

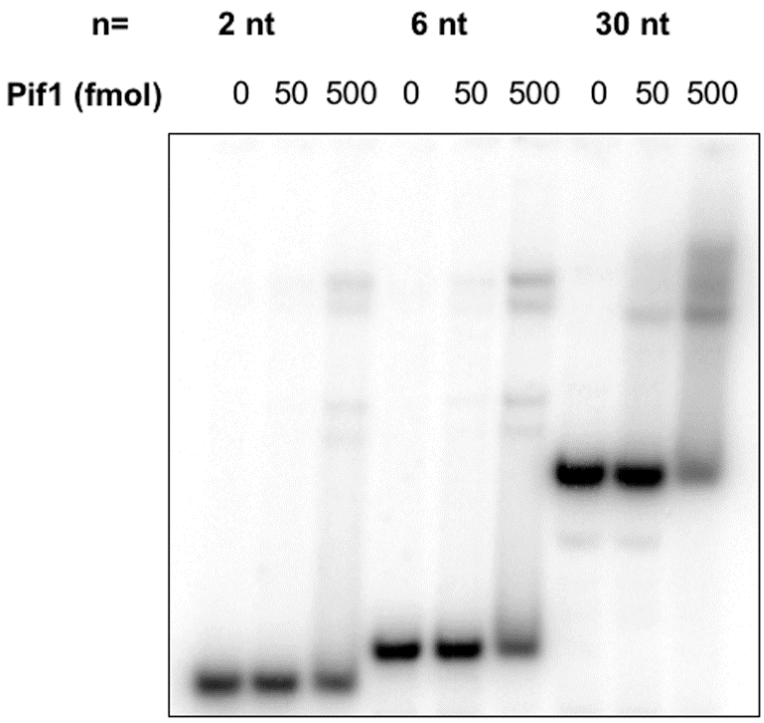
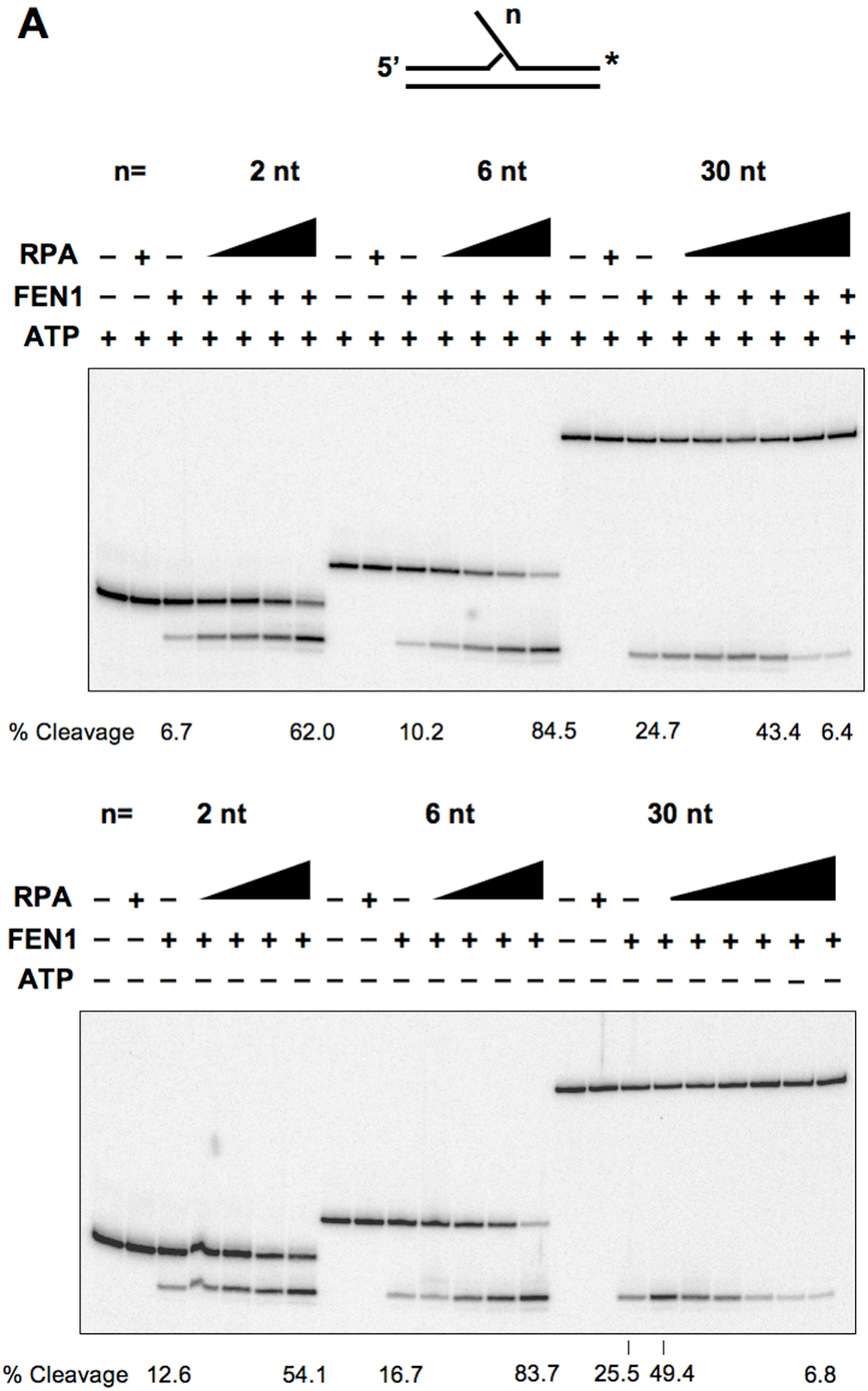


Figure 1.4. RPA stimulates FEN1 cleavage of 2, 6, and 30 nt flap substrates.

A, cleavage by FEN1 (2 fmol) was assayed on a 2, 6, and 30 nt flap substrate in the presence or absence of ATP and varying amounts of RPA (15, 25, 50, or 100 fmol, with two additional reactions containing 200 and 300 fmol on the 30 nt flap substrate) as indicated in the figure and described under "Experimental Procedures." Substrate depictions and designations are as in Fig. 1.1. *B*, RPA binding activity (50 or 500 fmol) was measured on a 2, 6, and 30 nt flap substrate by non-denaturing PAGE.


A
n
5'
*
n=
2 nt
6 nt
30 nt
RPA
FEN1
ATP
% Cleavage 6.7 62.0 10.2 84.5 24.7 43.4 6.4
n=
2 nt
6 nt
30 nt
RPA
FEN1
ATP
% Cleavage 12.6 54.1 16.7 83.7 25.5 49.4 6.8

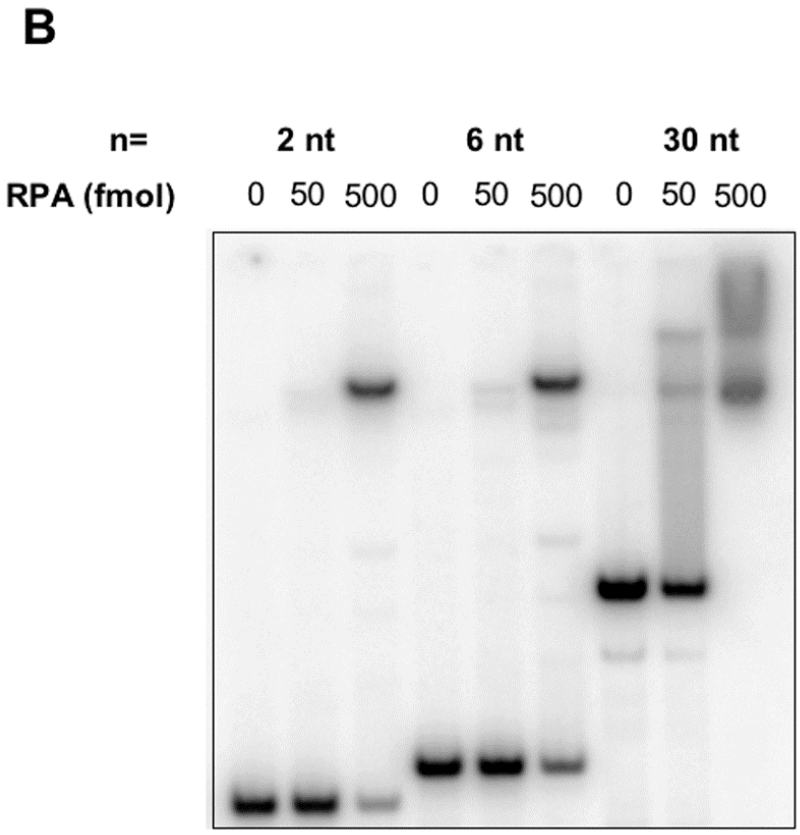
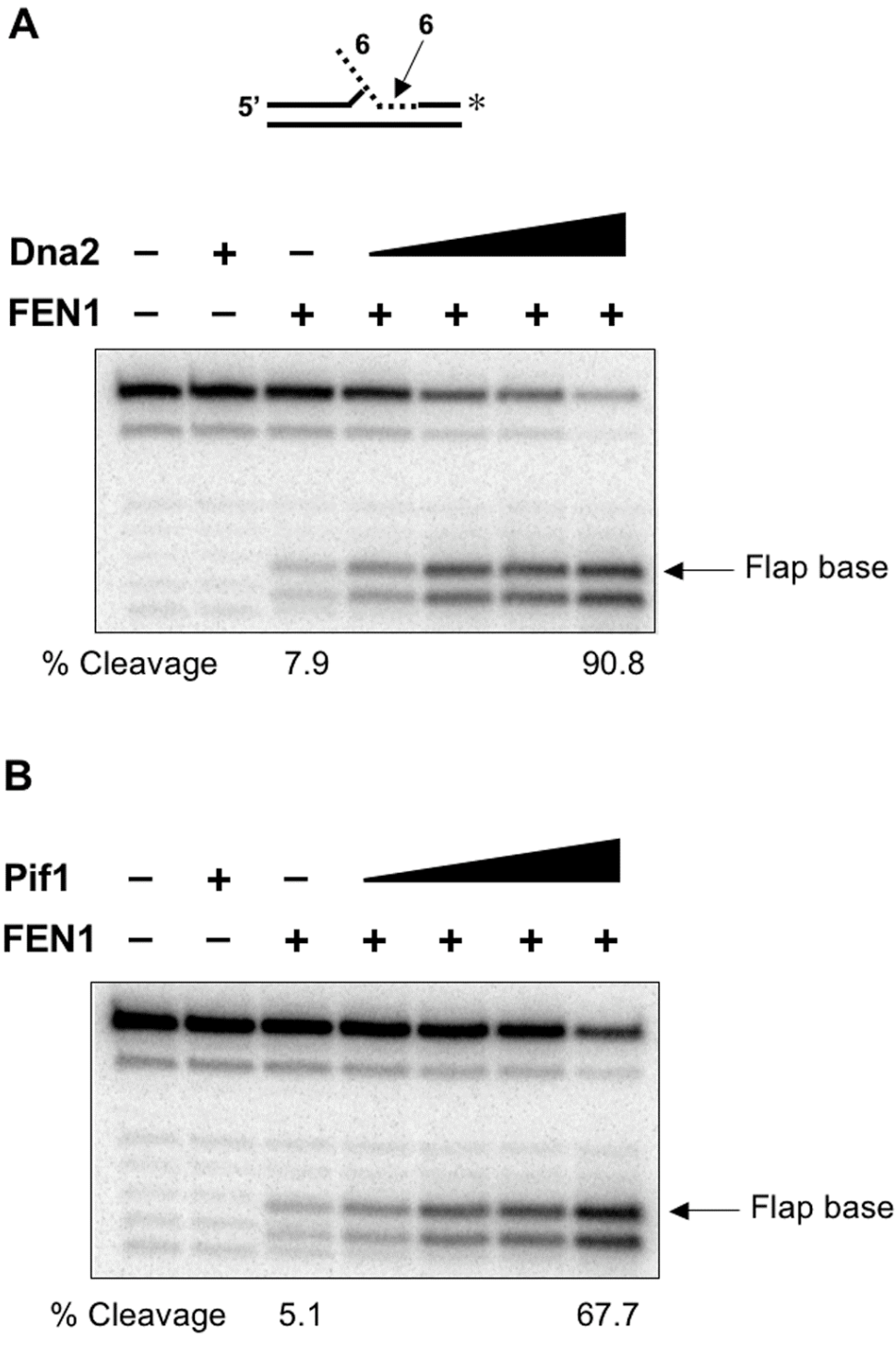

B
n=
2 nt
6 nt
30 nt
RPA (fmol)
0 50 500 0 50 500 0 50 500

Figure 1.5. Two-nuclease pathway proteins stimulate FEN1 on an RNA flap substrate.

A, cleavage by FEN1 (2 fmol) was assayed on a 6 nt RNA flap substrate in the presence of varying amounts of Dna2 (5, 15, 25, or 50 fmol) as indicated in the figure and described under "Experimental Procedures." The substrate is depicted above the figure with the location of the radiolabel indicated by the *asterisk*. The dotted line represents the RNA region of the substrate. *Plus* (+) indicates the presence and *minus* (–) the absence of the listed component. *B*, cleavage by FEN1 (2 fmol) on a 6 nt RNA flap substrate in the presence of varying amounts of Pif1 (5, 15, 25, or 50 fmol). *C*, cleavage by FEN1 (2 fmol) on a 6 nt RNA flap substrate in the presence of varying amounts of RPA (5, 15, 25, or 50 fmol).


A
6
6
5'
*
Dna2 – + –
FEN1 – – + + + + +
Flap base
% Cleavage 7.9 90.8
B
Pif1 – + –
FEN1 – – + + + + +
Flap base
% Cleavage 5.1 67.7

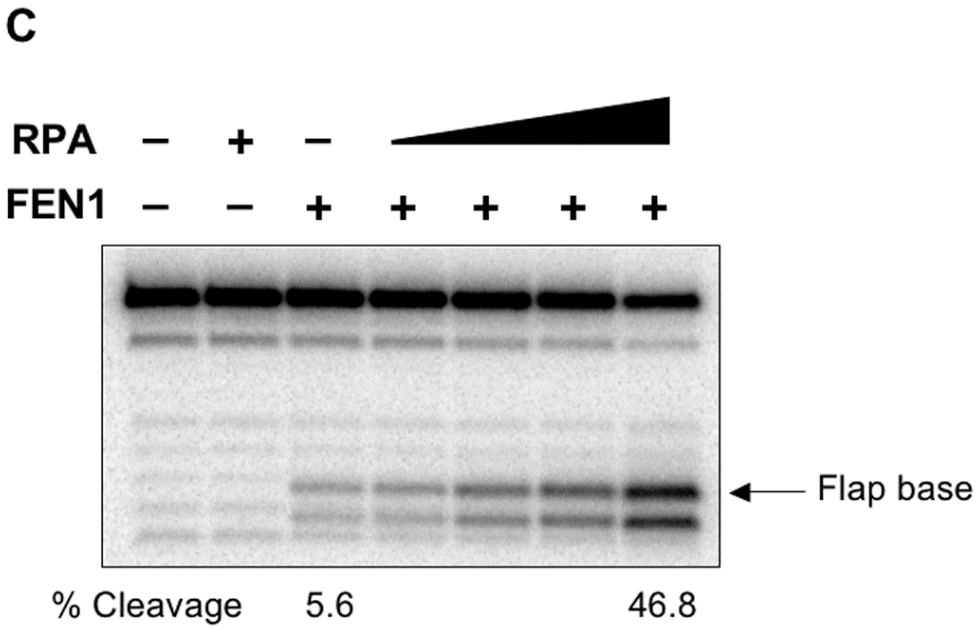

C
RPA – + –
FEN1 – – + + + + +
Flap base
% Cleavage 5.6 46.8

Figure 1.6. Individual proteins are able to maximize stimulation of cleavage by FEN1.

Cleavage by FEN1 (0.2 fmol) was assayed on a 6 nt flap substrate in the presence of combinations of Dna2 (400 fmol), RPA (400 fmol), and Pif1 (300 fmol) as indicated in the figure and described under "Experimental Procedures." Control reactions assessing cleavage at 2 and 10 fmol FEN1 are shown. Substrate depictions and designations are as in Fig. 1.1.

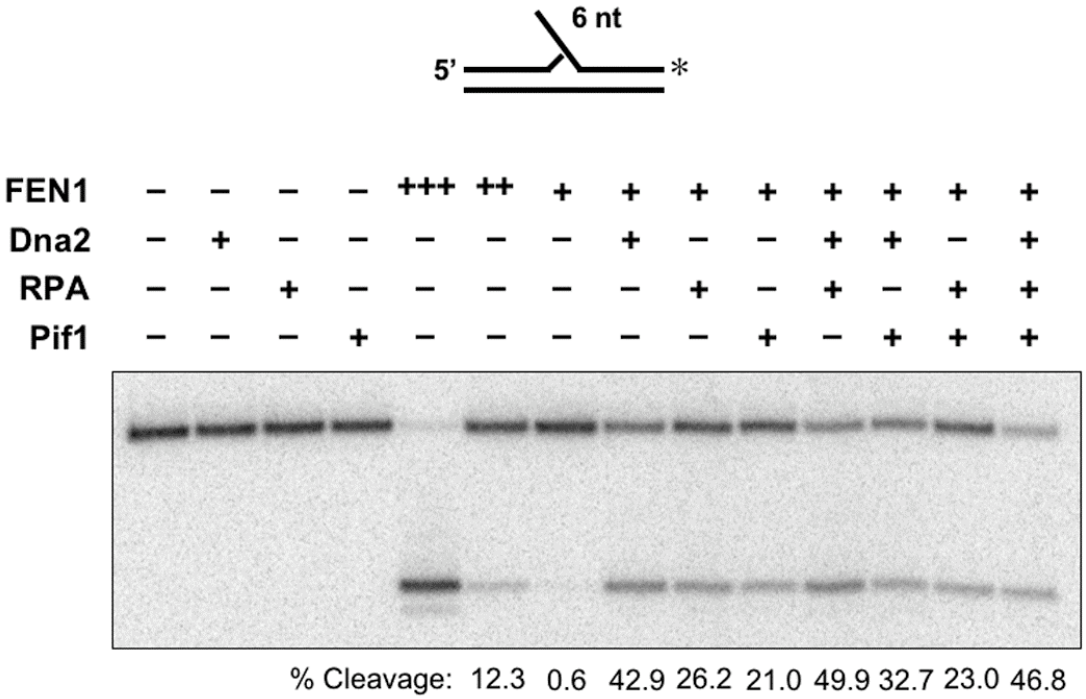

6 nt
5'
*
FEN1 – – – – +++ ++ + + + + + + + +
Dna2 – + – – – – – + – – + + – +
RPA – – + – – – – – + – + – + +
Pif1 – – – + – – – – – + – + + +
% Cleavage: 12.3 0.6 42.9 26.2 21.0 49.9 32.7 23.0 46.8

Figure 1.7. Dna2, Pif1, and RPA each directly bind FEN1.

Lane 1 is a non-specific IgG control. Lane 2 is a positive control for 200 pg purified FEN1. Lanes 3 (containing 1 ng purified FEN1), 4 (containing 1 ng purified Dna2) and 5 (containing 1 ng purified FEN1 and 1 ng purified Dna2) were immunoprecipitated with antibody to Dna2. Lanes 6 (containing 1 ng purified FEN1), 7 (containing 1 ng purified Pif1) and 8 (containing 1 ng purified FEN1 and 1 ng purified Pif1) were immunoprecipitated with antibody to Pif1. Lanes 9 (containing 1 ng purified FEN1), 10 (containing 1 ng purified RPA) and 11 (containing 1 ng purified FEN1 and 1 ng purified RPA) were immunoprecipitated with antibody to RPA. The immunoprecipitates were separated on a SDS-polyacrylamide gel, and Western blotting was performed using antibody against FEN1.

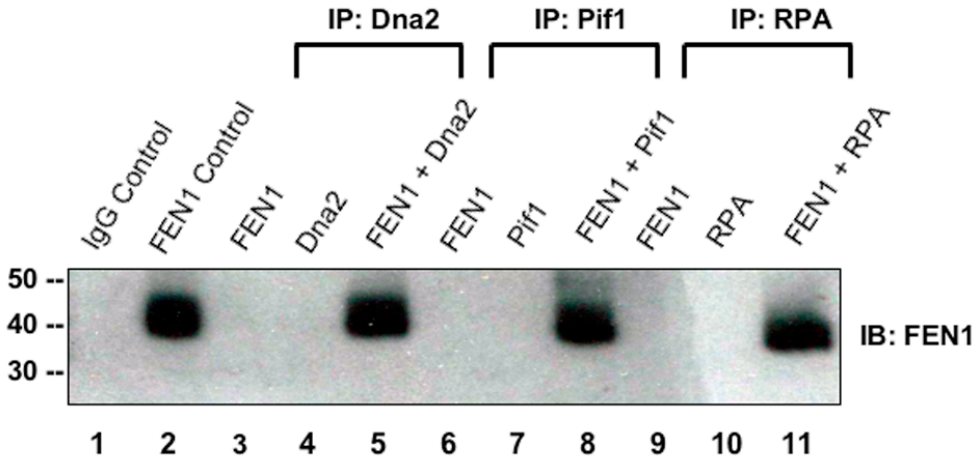
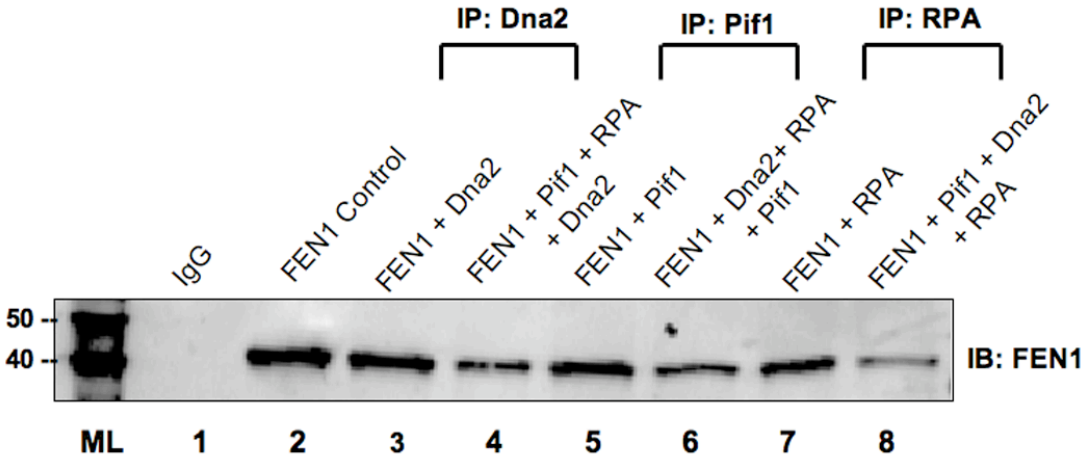

IP: Dna2
IP: Pif1
IP: RPA
IgG Control
FEN1 Control
FEN1
Dna2
FEN1 + Dna2
FEN1
Pif1
FEN1 + Pif1
FEN1
RPA
FEN1 + RPA
50 --
40 --
30 --
IB: FEN1
1 2 3 4 5 6 7 8 9 10 11

Figure 1.8. Binding of Dna2, Pif1, RPA, and FEN1.

Lane 1 is a non-specific IgG control. Lane 2 is a positive control for 200 pg purified FEN1. Lanes 3 (containing 1 ng purified FEN1 and 1 ng purified Dna2), and 4 (containing 2 ng purified Pif1, 2 ng purified RPA, 1 ng purified FEN1 and 1 ng purified Dna2) were immunoprecipitated with antibody to Dna2. Lanes 5 (containing 1 ng purified FEN1 and 1 ng purified Pif1), and 6 (containing 2 ng purified Dna2, 2 ng purified RPA, 1 ng purified FEN1 and 1 ng purified Pif1) were immunoprecipitated with antibody to Pif1. Lanes 7 (containing 1 ng purified FEN1 and 1 ng purified RPA), and 8 (containing 2 ng purified Dna2, 2 ng purified Pif1, with 1 ng purified FEN1 and 1 ng purified RPA) were immunoprecipitated with antibody to RPA. The immunoprecipitates were separated on a SDS-polyacrylamide gel, and Western blotting was performed using antibody against FEN1.


IP: Dna2
IP: Pif1
IP: RPA
IgG
FEN1 Control
FEN1 + Dna2
FEN1 + Pif1 + RPA + Dna2
FEN1 + Pif1
FEN1 + Dna2+ RPA + Pif1
FEN1 + RPA
FEN1 + Pif1 + Dna2 + RPA
50
40
IB: FEN1
ML
1
2
3
4
5
6
7
8

Chapter 2. Resolution of Fold-backs by Pif1 Restores Pol δ Synthesis

Abstract

During Eukayrotic lagging strand DNA synthesis, the presence of a fold-back region can be detrimental to replication. Flaps created by polymerase δ (pol δ) during strand displacement synthesis are cleaved by either flap endonuclease 1 (FEN1) or Dna2 nuclease, in conjunction with the single-stranded binding protein replication protein A (RPA). However, when a region of DNA that is self-complimentary becomes single-stranded it has the ability to fold back on itself. These fold-backs are resistant to cleavage by both FEN1 and Dna2. This in turn prevents the proper ligation of the Okazaki fragments that is necessary to create the final double-stranded product. However, results presented here demonstrate that the helicase Pif1 is able to restore the ability of pol δ to synthesize full-length products in regions containing self-complementary sequences by completely removing primers initiated by fold-back flaps. Additionally, these results indicate that the presence of a fold-back in the template slows pol δ synthesis, allowing fold-back flaps to be removed before being ligated into the lagging strand. Binding by RPA, however, is able to partially restore a normal rate of synthesis by pol δ.

Introduction

During eukaryotic DNA replication, the lagging strand is synthesized through the creation of Okazaki fragments, ~150 nucleotides (nt) in length (15). These fragments are initiated by a primer created by DNA polymerase α/primase (pol α), which synthesizes ~10 nt of RNA followed by ~20 nt of DNA (3). These primers are then lengthened by polymerase δ (pol δ). The interaction of pol δ to DNA is stabilized by the sliding clamp proliferating cell nuclear antigen (PCNA), which is loaded onto the temple DNA by replication factor C (RFC). Upon reaching the subsequent downstream Okazaki fragment, pol δ continues synthesis, displacing the 5' end into a single-stranded flap. This flap must be cleaved into a nick by a nuclease such that DNA ligase I (LigI) can ligate the fragments into a completed, continuous DNA strand (10,12).

There are two pathways through which Okazaki fragments are processed. In the first pathway, referred to as the one-nuclease or short flap pathway, flap endonuclease 1 (FEN1) is the only nuclease necessary for flap cleavage. In reconstitutions of the Okazaki fragment processing pathway, pol δ displaces mostly short flaps, ~1-6 nt in length, which are efficiently cleaved by FEN1 (21,22,34). Like pol δ, FEN1 binds to PCNA. PCNA stimulates both proteins and creates a complex that can tightly coordinate synthesis and cleavage (11). While this process allows for the cleavage of most flaps while they are short, reconstitutions have also shown that some flaps can escape early FEN1 cleavage to become long (42,72,94). Flaps that

reach ~25 nt in length can be stably bound by replication protein A (RPA), a single-stranded DNA binding protein (43). Once RPA is bound, FEN1 can no longer cleave (23), necessitating the use of a second nuclease.

The two-nuclease, or long flap, pathway utilizes Dna2 to cleave long, RPA-bound flaps (23). Dna2 possesses both a 5'-3' endonuclease activity and a 5'-3' helicase activity (24,52,54). Like FEN1, Dna2 is able to cleave a 5' flap structure (55). However, unlike FEN1, Dna2 cleaves multiple times and cannot cleave at the base of the flap, leaving behind a short flap of ~5-10 nt (56). Dna2 is not inhibited by RPA, and displaces RPA as it threads the flap (23,44). Although it does not cleave the base of the flap, the product Dna2 creates is too short to be bound by RPA, allowing FEN1 to complete cleavage, leaving behind only a nick to be sealed by LigI. Dna2 has been shown to be essential in *Saccharomyces cerevisiae* (51,52). It is likely that without Dna2, long RPA-bound flaps cannot be properly processed, leading to genomic instability and cell death.

Genetic evidence suggests that the influence of Pif1 may lead to flap lengthening. In *S. cerevisiae*, the deletion of *PIF1* rescues the lethality of *dna2Δ* (90,91), suggesting that in the absence of Pif1, flaps do not reach lengths that require cleavage by Dna2. Although biochemical studies from our group support this conclusion (72,94), the precise role Pif1 plays in Okazaki fragment processing remains unknown. Long flap creation is energetically wasteful, especially when short flaps are capable of being cleaved by FEN1 alone. However, if the presence of Pif1 is solely problematic for Okazaki fragment processing, it is likely that the cell would

have evolved to sequester Pif1 to only regions where it is beneficial, such as the telomere or mitochondria, where it regulates telomere growth and stabilizes mitochondrial DNA (78). As this is not the case, we believe that Pif1 must play a biologically important role at Okazaki fragments. One possibility is that the ability of Pif1 to unwind flaps is important at specific regions of the DNA, such as those containing self-complementary sequences.

Regions of DNA that are self-complementary have the potential to form fold-back flaps. These flaps are resistant to cleavage by both FEN1 and Dna2 (56). Dna2 or RPA can remove the structure of some fold-backs but only if the stability of the fold-back is weak: the helicase activity of Dna2 can be used to resolve weak fold-backs, and the strand melting activity of RPA can unwind some fold-back structures as well (44,61). However, neither protein is capable of unwinding fold-back flaps that contain a strong secondary structure, such as flaps that would be formed in areas containing triplet repeats. Interestingly, the *S. pombe* homolog of Pif1, Pfh1, has been shown to unwind the downstream primer of a fold-back flap substrate when there is a gap between the fold-back and the downstream-annealed region (82). We therefore considered that Pif1 might be able to completely remove primers that are initiated by fold-back flaps.

In this study, we investigated a potential biologically important role for Pif1 in Okazaki fragment processing. Utilizing a reconstitution system, we examined the effect of Pif1 on strand displacement synthesis through an Okazaki fragment initiated with a stable fold-back flap. Additionally, we investigated how the presence of a

fold-back in the template affected lagging strand synthesis. Our results provide evidence that Pif1 promotes removal of an entire fragment initiated by a fold-back flap from the template DNA. We also demonstrate that fold-backs in the template slow pol δ synthesis, providing Pif1 the opportunity to remove fold-back flaps before they are ligated into the final DNA product.

Experimental Procedures

Materials—Radioactive nucleotide [γ-^{32}P]ATP was obtained from PerkinElmer Life Sciences. Oligonucleotide primers were synthesized by Integrated DNA Technologies (Coralville, IA) or Midland Certified Reagents Company (Midland, TX). The primers and their sequences are listed in Table 1. Streptavidin and polynucleotide kinase were obtained from Roche Applied Science. Other reagents were the best grade commercially available.

Enzyme Expression and Purification—S. cerevisiae pol δ (92) and LigI (22) were over-expressed in *S. cerevisiae* and purified as previously described. *S. cerevisiae* PCNA (42), RFC (110), FEN1 (19), RPA (98), and Pif1 and helicase-deficient Pif1 K264A (72) were over-expressed in *E. coli* and purified as previously described. PCNA and FEN1 recombinant proteins had C-terminal His_6 tags. Pif1 recombinant protein had an N-terminal His_6 tag. *S. cerevisiae* Dna2 was over-expressed and purified from baculovirus High Five cells as previously described (52).

Oligonucleotide Substrates—Substrates composed of the oligonucleotides listed in Table 1 were designed to simulate intermediates of Okazaki fragment processing. The 5' ends of primers U_1, U_2, and I_1 were radiolabeled with [γ-^{32}P]ATP using polynucleotide kinase. The radiolabeled primers were separated by running the reactions on a 15%/7 M urea polyacrylamide gel. The radiolabeled products were then gel purified. To anneal substrates, component oligonucleotides were mixed in annealing buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol), heated at 95°C for 5 min, adjusted to 70°C, and slowly cooled to room temperature. When the upstream primer was labeled, the oligonucleotides were annealed at a 1:2:4 ratio of upstream primer to template to downstream primer. When the internal primer was labeled, the oligonucleotides were annealed at a 1:2:4:4 ratio of internal primer to template to upstream primer to downstream primer.

Two types of substrates were used in the following experiments. The first substrate type was designed to examine strand displacement synthesis through, and cleavage and ligation of, an Okazaki fragment initiated by a pre-created 18 nt fold-back flap. The substrate consisted of a 25 nt upstream primer (U_1), a 100 nt internal primer (I_1), and a 30 nt downstream primer (D_1) annealed to a 110 nt template (T_1), forming a 2 nt gap between the upstream and internal primers and a nick between the internal and downstream primers. The internal primer approximated a full-length Okazaki fragment, and the upstream and downstream primers represented the adjacent fragments. This substrate will be referred to as the internal 18 nt fold-back substrate.

The second type of substrate consisted of a set of three substrates, designed to examine synthesis through a fold-back in the template DNA. The first substrate of the set consisted of a 44 nt upstream primer (U_2) annealed to the 3' end of a 110 nt template (T_2). The template had an 18 nt fold-back 10 nt downstream of the upstream primer. The second substrate consisted of the same upstream primer (U_2) annealed to a template with only a 16 nt template fold-back downstream of the 3' end of the upstream primer (T_3). The number of GC pairs found in this fold-back was decreased in order to create a weaker secondary structure. These substrates will be referred to as the template fold-back substrates. A third, control substrate consisted of a 44 nt upstream primer (U_2) annealed to a 110 nt template (T_1), containing no downstream primer or significant secondary structure.

Strand-Displacement Synthesis Assays—Five fmol of radiolabeled biotinylated substrate were first incubated on ice with 500 fmol of streptavidin for 20 min. Streptavidin complexes with biotin on the substrate template ends, blocking the substrate ends and requiring that RFC load PCNA. For simplicity, the blocked ends are not depicted in the figures. Streptavidin-conjugated substrate was then incubated with various combinations and amounts of pol δ, PCNA, RFC, FEN1, Dna2, RPA, LigI, and Pif1 for 10 min at 30°C in 20 μl of reconstitution buffer (50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 25 μg/ml bovine serum albumin, 50 μM dNTPs, 1 mM ATP, 4 mM $MgCl_2$, and 75 mM NaCl). Reactions were stopped with 20 μl of 2X termination dye (90% formamide (v/v), 10 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanole), followed by heating for 5 min at 95°C. Reaction products

were separated by electrophoresis on a 22.5%/7 M urea polyacrylamide gel for 1 hour and 30 min at 80 W. The gel was dried and exposed to a phosphor screen, which was scanned with a GE Healthcare PhosphorImager and analyzed using ImageQuant version 1.2 software.

For the kinetic experiment shown in Fig. 2.4, the reactions were initiated in a total of 120 μl of reconstitution buffer and at given time points (0, 0.5, 1, 2.5, 5, and 10 minutes), a 20 μl sample was removed from each reaction, added to 20 μl of 2X termination dye, and heated for 5 min at 95°C. Products were then separated by electrophoresis and analyzed as described above.

Cleavage Assays—For the strand displacement-coupled cleavage assay shown in Fig. 2.1, reactions were run and analyzed as described above.

Helicase Assays—Five fmol of radiolabeled substrate were incubated with either Pif1 or pol δ, PCNA, and RFC with increasing amounts of Pif1 for 10 min at 30°C in 20 μl of reaction buffer. Reactions were stopped by adding 4 μl of 6X helicase dye (50 mM EDTA, 0.9% SDS, 30% glycerol, 0.125% bromophenol blue, and 0.125% xylene cyanole). Reaction samples were loaded onto a 12% native polyacrylamide gel, and products were separated by electrophoresis for 2 hours at 250 V. The gel was dried, scanned, and analyzed as described above.

The amount of each protein used in each experiment is given in the corresponding figure legend. All experiments were performed at least in triplicate, and a representative gel is shown in the corresponding figure.

Results

Previous work from our group has investigated the ability of Pif1 to remove secondary structure from a flap that has folded back on itself, to determine whether Pif1 could restore proper flap cleavage. However, results suggest that Pif1 simply cannot restore the ability to cleave a flap that contains a strong fold-back (111). Still, another mechanism for Pif1 resolution of fold-back flaps remains: Pif1 may be able to completely remove fold-back flaps in order to restore proper pol δ synthesis. It has been previously shown that the *S. pombe* homolog of Pif1, Pfh1, unwinds the downstream primer of flaps initiated by a fold-back, as long as there is a gap on which Pfh1 can load (82). The authors suggested that the Pif1-family of helicases could remove entire Okazaki fragments initiated by fold-back flaps. This would allow pol δ to resynthesize through the region of removed DNA without the need to displace a flap, eliminating the disruptive effects of the original fold-back flap. This previous study, however, used substrates that were too short to accurately simulate full-length Okazaki fragments. Additionally, they did not perform reconstitution experiments. Therefore, we wished to use our reconstitution system to examine whether Pif1 can in fact remove an entire fold-back-initiated Okazaki fragment.

To accomplish this, we designed a substrate containing an internal fold-back flap that simulates an Okazaki fragment initiated by a fold-back and flanked by two adjacent fragments. The upstream primer was 25 nt. A 2 nt gap was left in between the upstream and internal primer to allow pol δ a region in which to begin synthesis.

The internal primer was 100 nt containing a region of 42 nt that creates a 18 nt fold-back, leaving 58 nt to anneal to the template. The downstream primer was 30 nt. There was no gap, only a nick between the internal and downstream primer. We annealed the three oligonucleotides to a 110 nt template. The 5' end of the downstream primer contained a phosphate to allow ligation to the extended upstream primer. The 3' end of the internal primer contained a 3' phosphate group to prevent ligation to the downstream primer. The downstream primer also had a 5 nt 3' overhang to allow us to distinguish between a full-length synthesis product and a ligation product. When the upstream primer is labeled, the full-length synthesis product was predicted to be 110 nt and the ligation product 115 nt. When the internal primer is labeled, the full length product was predicted to be 125 nt and the ligation product 130 nt.

Internal Fold-back Flaps are Resistant to Cleavage by FEN1 and Dna2– We began by verifying that neither FEN1 nor Dna2 could cleave the fold-back in the internal fold-back substrate (Fig. 2.1). The internal primer was labeled at the 5' end to allow us to visualize cleavage products. Neither FEN1 nor Dna2 alone were sufficient to cleave the flap (*lanes 2 and 3*). The combination of FEN1, Dna2, and RPA also did not permit cleavage (*lane 4*). Pol δ, PCNA, and RFC were then added to observe cleavage of the fold-back in an actively synthesizing system. We again observed no cleavage by FEN1, Dna2, or the combination of FEN1, Dna2, and RPA (*lanes 6-8*). Interestingly, in the presence of FEN1, we observed some extension of the internal primer by pol δ to the end of the template (*lanes 6 and 8*). This suggests that the 3'

exonuclease of pol δ activated some of the ends of the internal primer by cleaving the 3' phosphate. Pol δ then extended those internal primers, and FEN1 was necessary to stimulate strand displacement through the downstream primer. However, the crucial observation is that the fold-back was immune to cleavage under all conditions, demonstrating that an Okazaki fragment initiated by a fold-back is resistant to processing by both flap-processing pathways.

Pif1 Removes an Okazaki Fragment Initiated by a Fold-back– We proceeded to investigate whether Pif1 could remove the entire internal fold-back primer in order to restore pol δ synthesis through the fold-back region. For these experiments, we 5' labeled the upstream primer in order to examine synthesis and ligation by pol δ (Fig. 2.2A, B). Pol δ alone strand-displaced a short distance into the internal primer (*lane 3*). When PCNA and RFC were present, strand displacement was stimulated, as expected (*lane 4*). A small amount of an 85 nt product was observed. This represents synthesis through the entire internal primer and pausing at the 5' end of the downstream primer. Interestingly, addition of LigI slightly stimulated synthesis up to the pause point (*lane 5*). Addition of FEN1 stimulated even more synthesis to the pause point (*lane 6*). As FEN1 does not cleave the fold-back flap (Fig. 2.1), stimulation by FEN1 must have been cleavage-independent. Neither Dna2 (*lane 7*) nor RPA (*lane 8*) stimulated synthesis. Combinations of FEN1, Dna2, and RPA did not stimulate synthesis beyond what was observed with FEN1 alone (*lanes 9-12*). Importantly, no synthesis beyond the pause point or ligation of the extended upstream primer to the downstream primer was observed under these conditions. However,

when Pif1 was added in the absence of FEN1, Dna2, and RPA, synthesis up to the pause point was stimulated, and full-length synthesis and ligation products appeared (*lanes 13-15*). Addition of RPA with Pif1 did not further improve stimulation of synthesis or ligation (*lanes 16-18*). When all proteins were added to the reaction, synthesis and ligation were stimulated to the highest amount observed (*lanes 19-21*). This suggests that Pif1 binds the internal primer, possibly at a gap created by pol δ strand displacement. Pif1 then unwound the entire internal primer, allowing pol δ to synthesize through the fold-back region. When pol δ reached the downstream primer, we observed that either LigI sealed the nick between the extended upstream primer and downstream primer, or pol δ continued strand displacement through the downstream primer. Pif1 thus allowed a fold-back flap, resistant to cleavage by either FEN1 or Dna2, to be processed by removal of the entire Okazaki fragment.

This experiment was repeated utilizing a helicase-deficient mutant of Pif1, which was unable to stimulate the formation of full-length products (data not shown). This demonstrates that the helicase activity of Pif1 facilitates the removal of and synthesis through the fold-back flap. To further demonstrate this point, a helicase assay was performed using the same fold-back substrate as in Figure 2.2A. While pol δ, PCNA, and RFC alone were unable to remove the fold-back, increasing amounts of Pif1 promoted fold-back flap removal (Fig. 2.3), further suggesting the importance of Pif1 for proper removal of fold-back flaps. Notably, Pif1 is able to unwind the internal primer without the assistance of pol δ. This suggests that DNA breathing

near the 5' end of the annealed region may present a sufficient opportunity for Pif1 to bind to the internal primer, independent of displacement by pol δ.

Template Secondary Structure Slows Pol δ Synthesis– To prevent ligation of the fold-back flap to the downstream primer in our reconstitution experiment, we placed a 3' phosphate on the fold-back primer. However, Okazaki fragments do not have phosphates at their 3' ends *in vivo*. Therefore, it is possible that a full-length fragment initiated by a fold-back flap could be ligated to the downstream fragment before removal by Pif1. If this occurred *in vivo,* the fold-back would be incorporated into the lagging strand and would be resistant to cleavage by any currently known DNA replication mechanisms. It would have to be handled by repair systems as a site of DNA damage.

However, if a fold-back sequence is present, it should not only create a fold-back in a newly synthesized Okazaki fragment flap but in the template as well. This template fold-back would potentially create an energetic barrier for pol δ synthesis. We therefore considered the possibility that Okazaki fragments containing a fold-back sequence might be extended at a slower rate than primers containing no secondary structure, which would result in ligation with the adjacent fragment being delayed.

To test this, we designed a template fold-back substrate, which consists of an upstream primer annealed to a template with an 18 nt template fold-back downstream of the 3' end of the upstream primer.

As a control, we used the standard-44 substrate, also lacking the downstream primer. The template in the control substrate has no significant secondary structure. We incubated each substrate with pol δ, PCNA, and RFC for increasing amounts of time (Fig. 2.4A). With the control substrate, pol δ synthesized some full-length product after only 30 seconds, with the amount of synthesis increasing over time (*lanes 1-6*). With the template fold-back substrate, synthesis was slower compared to the control substrate (*lanes 13-18*). Surprisingly, no full-length product was evident, even at the longest time. Synthesis reached a maximum at approximately 5 minutes and did not increase further after longer incubation. When utilizing this substrate, synthesis halts near the end of the fold-back sequence.

RPA has previously been shown to stimulate synthesis by pol δ through hairpin structures in the template, presumably by binding the template and melting those structures (112). We wanted to determine whether RPA would have this same effect on our template fold-back substrate and permit full-length synthesis by pol δ. On the control substrate, RPA stimulated synthesis slightly (Fig. 2.4A, *lanes 7-12*). On the template fold-back substrate, synthesis was stimulated, and there was some shift toward longer products (*lanes 19-24*). However, RPA was not able to promote full-length synthesis.

To further characterize the effect of a fold-back within the template, we utilized a second fold-back template. This substrate consists of an upstream primer annealed to a template with only a 16 nt template fold-back downstream of the 3' end of the upstream primer. Additionally, we decreased the amount of GC pairs found in

the fold-back in order to determine whether a weaker fold-back region would improve pol δ synthesis. Interestingly, when utilizing this substrate, we were able to observe full-length synthesis (Fig. 2.4B, *lanes 1-6*). We again saw a slight stimulation of synthesis by RPA (*lanes 7-12*).

The position at which synthesis halts is very important to the interpretation of these results. In the presence of the 18 nt fold-back, synthesis continued through the fold-back, allowing the fold-back flap to be made, but then slowed so the Okazaki fragment with the fold-back was not rapidly extended to a potential point of ligation. Some synthesis terminated within the hairpin, some near the end of the hairpin, and a small amount beyond the hairpin. The range of termination is broad, producing a variety of intermediate structures. When utilizing the 16 nt fold-back, a range of products were created throughout the fold-back region, with only a few products reaching full length. This would indicate that pol δ slowed as it synthesized through the fold-back region, resulting in a number of intermediates.

Upon strand displacement, some products are expected to form weak structure, susceptible to FEN1, while others would form more stable structure, allowing loading of Pif1 and displacement. The slowing of pol δ through the fold-back template would allow the stable fold-backs to be displaced by Pif1 before they can be joined to the continuous lagging strand.

Discussion

Previous reconstitution analyses of eukaryotic lagging strand DNA replication suggest that, *in vivo,* Pif1 helicase lengthens Okazaki flaps that escape immediate FEN1 cleavage (72). These long flaps are processed by the two-nuclease pathway, which requires Dna2 helicase/nuclease in addition to FEN1 (94). Although Pif1 has several known cellular roles, these results would appear to indicate that at Okazaki fragments, Pif1 only serves to create a requirement for Dna2. If Pif1 did not influence Okazaki fragment maturation, the multistep pathway involving Dna2 might not be required. It is unlikely that the fragment maturation process would have evolved this way if the only role of Pif1 were to promote the secondary pathway. Therefore, we hypothesized that Pif1 plays an important but previously unnoticed role at Okazaki fragments. In this study, we attempted to determine the nature of this role.

Some sequences, such as triplet repeats, have the potential to form stable secondary structures when they are displaced into flaps (fold-back flaps). Previous investigation of such flaps indicates that they are resistant to cleavage by FEN1 and Dna2, even in the presence of Pif1 and RPA. Helicase assays performed by Ryu and colleagues, though, suggest that the Pif1 homolog, Pfh1, is able to completely remove entire Okazaki fragments that are initiated by a fold-back (82). However, they did not test stimulation of FEN1, Dna2, or RPA, and their flaps did not simulate full-length Okazaki fragments. We felt that our reconstitution system was ideal to test this proposal. We designed a substrate that simulated a full-length Okazaki fragment with

a 5' fold-back flap and tested synthesis through the fragment in the presence of components from both flap-processing pathways (Fig. 2.2). We observed slight stimulation of synthesis by LigI alone and by FEN1, although FEN1 did not cleave. Additionally, there was no stimulation of synthesis by either Dna2 or RPA, and the combinations of FEN1, Dna2, and RPA did not stimulate synthesis above that observed with FEN1 alone.

This implies that the components of the two flap processing pathways are incapable of alleviating the block to synthesis presented by the fold-back flap. Therefore, if such a flap were to form *in vivo*, the fragment would not be cleaved and could cause genome instability through chromosome breaks or strand invasions. When Pif1 was present, we observed increased amounts of intermediate synthesis products and synthesis through the entire internal and downstream primers. In addition, we observed ligation of the extended upstream primer to the downstream primer. Both full-length synthesis and ligation imply successful complete displacement of the fold-back flap-initiated Okazaki fragment. To accomplish this, Pif1 likely bound the gap that would emerge as pol δ displaced a few nucleotides into the internal annealed region.

Removal of the fold-back primer would require Pif1 to unwind ~58 nt of DNA. This is almost twice the length of the previously measured processivity of Pif1, ~30 nt (83). It is possible that under our reaction conditions Pif1 has a higher processivity than has been measured. Alternatively, Pif1 may unwind the flap in a

cooperative fashion, in which one molecule unwinds a given distance, dissociates, and then another Pif1 molecule binds and continues unwinding.

RPA did not enhance stimulation of synthesis by Pif1; in fact, there was a slight decrease in the stimulation. It is possible that as Pif1 unwinds the internal primer, RPA binds the growing gap between the fold-back and the remaining annealed portion of the internal primer. If Pif1 unwinds long stretches via the cooperative mechanism described above, the RPA bound to the gap may prevent the second Pif1 molecule from binding. Specific interactions between RPA and Pif1 must be examined to fully understand this result.

The greatest stimulation of synthesis and ligation was observed when all proteins were present. This phenomenon was also observed when we examined processing of a long flap following Pif1 lengthening (94). The reason for this is not known. Numerous studies have shown that individual pairs of replication proteins interact with and stimulate each other. It is possible that when the replication system is reconstituted, these interactions combine to produce the most efficient synthesis and flap processing possible.

Overall, results suggest a unique and important role for Pif1 in Okazaki fragment processing. Namely, Pif1 fully displaces Okazaki fragments that form flap intermediates that are refractory to cleavage by either the FEN1-only or two-nuclease pathways. We were able to test this activity of Pif1 directly utilizing a helicase assay (Fig. 2.3). We observed that Pif1 is able to unwind the fold-back flap both on its own and in the presence of pol δ (*lanes 3 and 9*, respectively). We believe Pif1 is able to

unwind the fold-back on its own because of breathing of the annealed region, exposing a few nucleotides of single stranded DNA. Pif1 could bypass the fold-back and bind to this single-stranded region in order to unwind. In the presence of the replication complex, we observe that unwinding of the substrate decreases. It is likely that the replication complex occupies the region at the base of the flap. This would physically prevent Pif1 from binding to any single-stranded region that results from DNA breathing. Thus, Pif1 would have to wait until pol δ displaces an adequate amount of single-stranded DNA before it could bind to the flap. Importantly, the addition of Pif1 displaces fold-back flaps, which pol δ alone cannot accomplish (*lanes 4 and 9*).

A potential conceptual flaw, however, in this proposed role for Pif1 is the apparent likelihood that by the time Pif1 could displace the undesirable fold-back fragment, it would be joined to the adjacent downstream fragment. Once the fold-back fragment became part of the continuous lagging strand, no amount of displacement could remove it. Although our substrate was designed to prevent this from occurring, the possibility exists *in vivo*. However, we considered that the template sequence at a fold-back flap could also form secondary structure. Such a structure could potentially slow primer elongation.

When we tested the progress of pol δ-catalyzed primer elongation over a stable fold-back in the template, synthesis slowed as the fold-back was copied and terminated in a diffuse region within or beyond the fold-back (Fig. 2.4A, B). RPA stimulated synthesis, allowing a moderate shift toward longer products. As expected,

we see that the presence of a fold-back within the template slows pol δ synthesis. This would allow the upstream Okazaki fragment to be extended while pol δ is still in the process of synthesizing through the fold-back in the template. This is exactly the behavior that would allow Pif1 to effectively displace a fold-back fragment before it is ligated. Further studies on the importance of fold-back length and stability and the influence of Pif1 and other replication proteins will provide insight into how the polymerase-PCNA complex interacts with template fold-backs.

Based on these results, we propose an alternative Okazaki fragment processing pathway, specifically for fragments that form fold-back flaps (Fig. 2.5). As the template DNA is opened by a replicative helicase, it forms a fold-back. An Okazaki fragment initiated a short distance upstream of the template fold-back will be extended through the fold-back, but the pol δ-PCNA processive complex will be slowed by the presence of a fold-back in the template. Meanwhile, another molecule of pol δ will be extending the upstream Okazaki fragment. Elongation of the upstream fragment begins to displace the downstream fragment, and if the flap becomes long, a fold-back flap will form. The fold-back cannot be processed by either the FEN1-only or two-nuclease pathway. As pol δ displaces through the fold-back flap, a gap is created either in the loop or just past the stem, and Pif1 binds the gap. Pif1 then unwinds and removes the incomplete, cleavage-resistant fragment, allowing pol δ to continue extending the upstream primer through the gap. Upon reaching the next downstream fragment, standard flap processing and ligation will complete synthesis.

Slowing of pol δ during synthesis of the template fold-back might serve two important roles. First, a gap is left between the point of dissociation and the next downstream Okazaki fragment, so that the two fragments cannot be ligated. This prevents the fold-back from being incorporated into the Okazaki fragment before Pif1 has an opportunity to remove it. Second, pol δ dissociation from the template after synthesizing an incomplete fragment ensures Pif1 does not have to unwind a full-length Okazaki fragment but rather only a fraction of one.

Although it is energetically wasteful to synthesize a long DNA fragment only to have it displaced and presumably degraded, use of this pathway should be a rare event in DNA replication. There are likely to be very few flaps that form disruptive secondary structure *in vivo*. We have suggested that only a small fraction of flaps become long via Pif1 lengthening (94). However, other mechanisms are likely to produce long flaps. The histone acetylase p300 has been reported to acetylate FEN1, reducing its nuclease activity (105), and acetylate Dna2, greatly stimulating its nuclease and helicase activities (113). We have proposed that this is a regulatory process that leads to more flap displacement and use of the two-nuclease pathway in regions of actively transcribed chromatin (113). Nevertheless, only a small fraction of these flaps are likely to form in regions that allow for stable fold-backs. However, there are hundreds of thousands of Okazaki fragments processed each replication cycle in yeast (114) and many more in humans. If only a few flaps form secondary structure, then the cell must be prepared to deal with them to maintain genome

stability. Therefore the alternative pathway is likely a rarely used but critically important mechanism for genome maintenance.

We cannot rule out the possibility that Pif1 plays other important roles at Okazaki fragments. Through its flap lengthening capacity, Pif1 may act as part of a correction mechanism for mutations introduced by pol α. The DNA patch synthesized and displaced by pol δ is estimated to be too short to remove the entire RNA/DNA primer synthesized by pol α (91). As mentioned previously, pol α is a low fidelity polymerase. All pol α-synthesized bases must be removed to ensure the best fidelity of DNA replication. Perhaps Pif1-promoted flap lengthening is required at locations of pol α mistakes. The long flap would include all mismatched nucleotides at displacement distances normally not achieved by pol δ. Additional promotion of flap displacement by acetylation of FEN1 and Dna2 should augment this effect, making complete removal of pol α errors even more certain in active chromatin (113). Our reconstitution system is well designed to address this issue. The downstream primer of the substrate could include a mismatch at variable distances from the 5' end, and incorporation of the mismatch into a final ligated product following strand displacement synthesis could be measured. We could then test the effect of Pif1 on this process. These and similar biochemical experiments will be crucial to further reveal the biological relevance of Pif1 in Okazaki fragment processing.

Table 2.1. Oligonucleotide sequences

Primer	Length (nt)	Sequence
Upstream (5′-3′)		
U_1	25	GTCCACCCGACGCCACCTCCTGCCT
U_2	44	GTCCACCCGACGCCACCTCCTGCCTTCAATGTGCTG GGATCCTA
Internal (5'-3')[a, b]		
I_1	100	**CGGTCGCGAGGCCTGCGC**TATTAT**GCGCAGGCCT CGCGACCG**AATGTGCTGGGATCCTACAACCAAGAC GAATTCCGGATACGACGGCCAGTGCCGACCG
Downstream (5′-3′)[c]		
D_1	30	*T*GCCAGCCTAAATTTCAATCCACCCTGACT
Template (3′-5′)[b,d]		
T_1	110	CAGGTGGGCTGCGGTGGAGGACGGAAGTTACACGA CCCTAGGATGTTGGTTCTGCTTAAGGCCTATGCTGC CGGTCACGGCTGGCACGGTCGGATTTAAAGTTAGG TGGG
T_2	110	CAGGTGGGCTGCGGTGGAGGACGGAAGTTACACGA CCCTAGGATGTTGGTTCTG**TCCAGAGCTCCGGACG AG**TATTAT**CTCGTCCGGAGCTCTGGA**TAAAGTTAG GTGGG
T_3	110	CAGGTGGGCTGCGGTGGAGGACGGAAGTTACACGA CCCTAGGATGTTGGTTCTGGAAG**TAGTCAACTGCT GCCA**TATTAT**TGGCAGCAGTTGACTA**TAAAGTTAG GTGGG

[a] Underline indicates a nucleotide with a 3' phosphate

[b] Boldface indicates fold-back region.

[c] Underline and italicized indicates a nucleotide with a 5' phosphate

[d] Templates T_1, T_2, and T_3 are biotinylated at both the 5′ and 3′ ends.

Figure 2.1. Internal fold-back flaps are resistant to cleavage by FEN1 and Dna2.

Cleavage by FEN1 (5 fmol) and Dna2 (50 fmol) was assayed on the internal 18 nt fold-back substrate (U_1:I_1:T_1:D_1) in the presence of various combinations of RPA (100 fmol), pol δ (23 fmol), PCNA (25 fmol), and RFC (25 fmol) as indicated in the figure and as described under "Experimental Procedures." The substrate is depicted above the figure with the location of the radiolabel indicated by the *asterisk*. *P* denotes the presence of a phosphate group. Lane 9 contains a radiolabeled 42 nt oligomer, the expected length of a FEN1 cleavage product. These experiments were performed by Dr. Jason Pike.

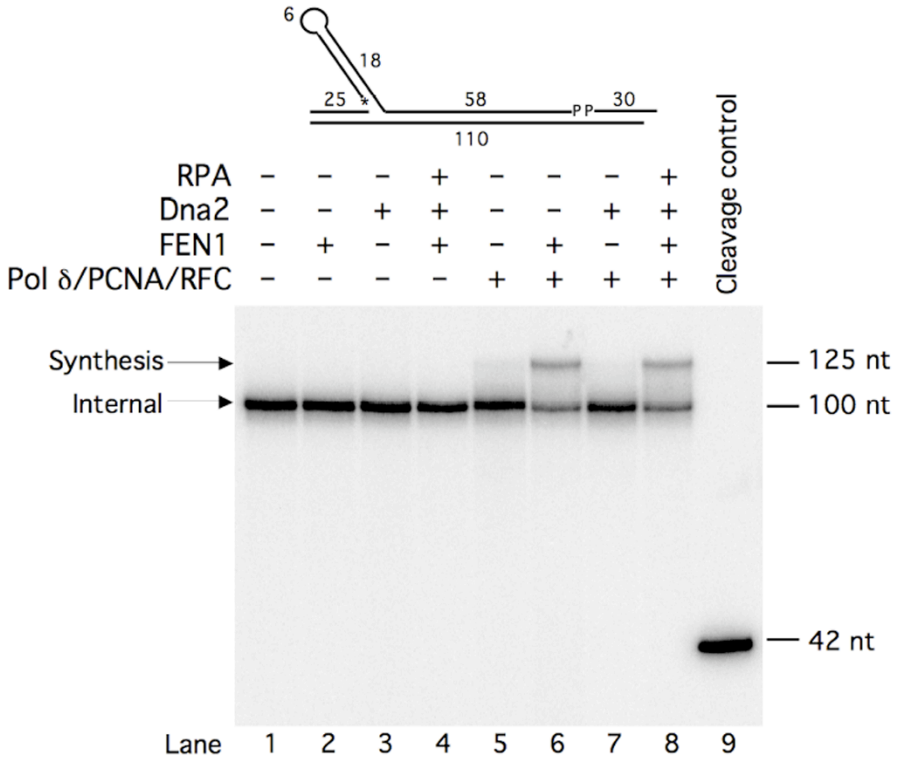

6
18
25
*
58
PP
30
110
RPA
Dna2
FEN1
Pol δ/PCNA/RFC
Cleavage control
Synthesis
Internal
125 nt
100 nt
42 nt
Lane
1
2
3
4
5
6
7
8
9

Figure 2.2. Pif1 removes an Okazaki fragment initiated by a fold-back.

A, Strand displacement synthesis by pol δ (23 fmol) and ligation by LigI (25 fmol) were assayed on the internal 18-nt fold-back substrate in the presence of various combinations of PCNA (25 fmol), RFC (25 fmol), FEN1 (5 fmol), Dna2 (50 fmol), RPA (100 fmol), and increasing amounts of Pif1 (50, 100, or 200 fmol) as indicated in the figure and as described under "Experimental Procedures." *B*, A magnification and overexposure of the boxed portion of A is shown. These experiments were performed by Dr. Jason Pike.

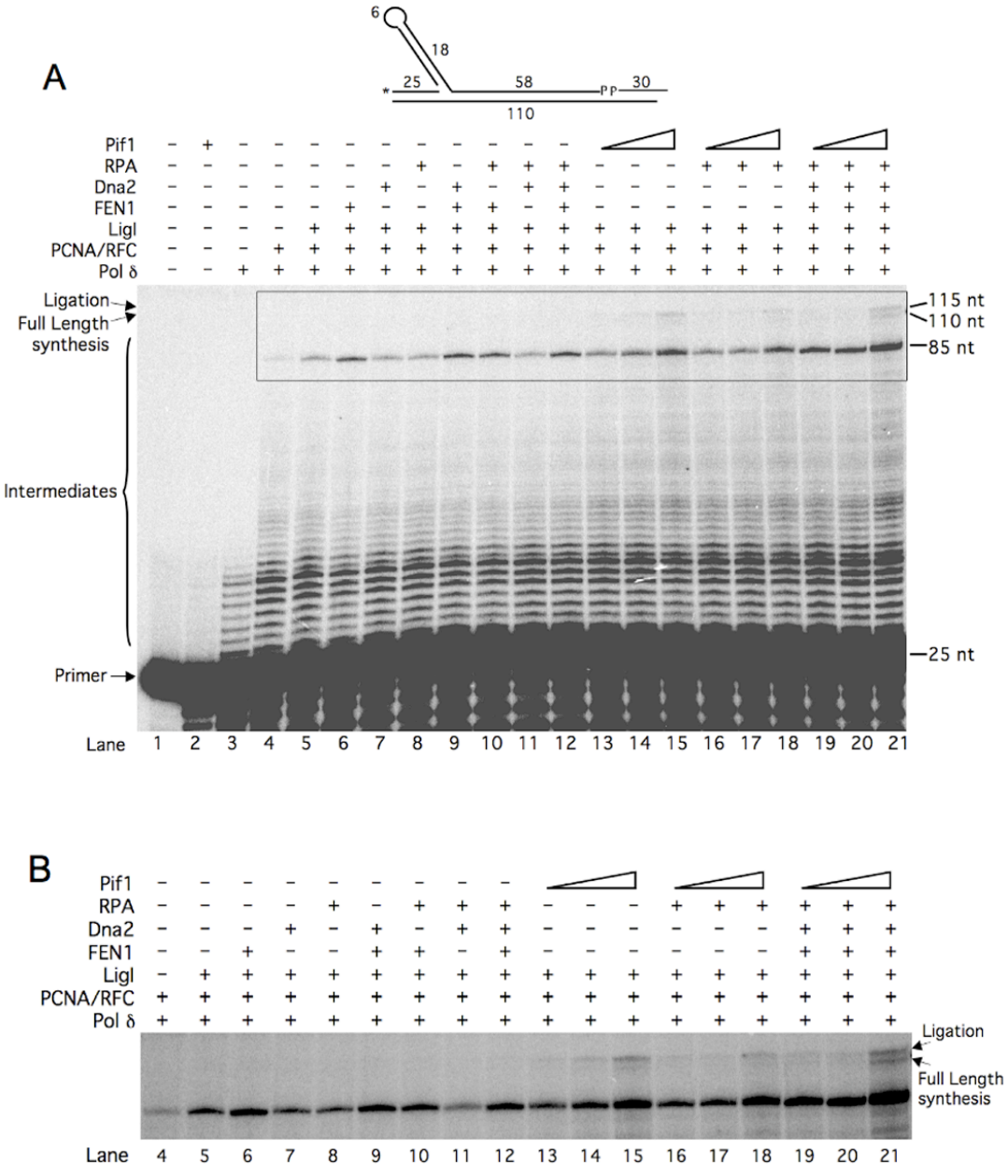

A
6
18
25
58
30
110
Pif1
RPA
Dna2
FEN1
Ligl
PCNA/RFC
Pol δ
Ligation
Full Length synthesis
Intermediates
Primer
115 nt
110 nt
85 nt
25 nt
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
B
Pif1
RPA
Dna2
FEN1
Ligl
PCNA/RFC
Pol δ
Ligation
Full Length synthesis
Lane 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Figure 2.3. Pif1 unwinds the internal fold-back primer from the template

Unwinding of the downstream primer by increasing amounts of Pif1 (50,100,200, 300 or 500 fmol) in the presence of pol δ (23 fmol), PCNA (250 fmol), and RFC (25 fmol) was assayed on the internal 18 nt fold-back substrate as described under Experimental Procedures. The control in Lane 2 contains 500 fmol of Pif1. dNTPs were included in the reaction buffer to allow for active synthesis. Substrate depictions and figure labels are as in Fig. 2.1. *Delta* (Δ) denotes boiled substrate.

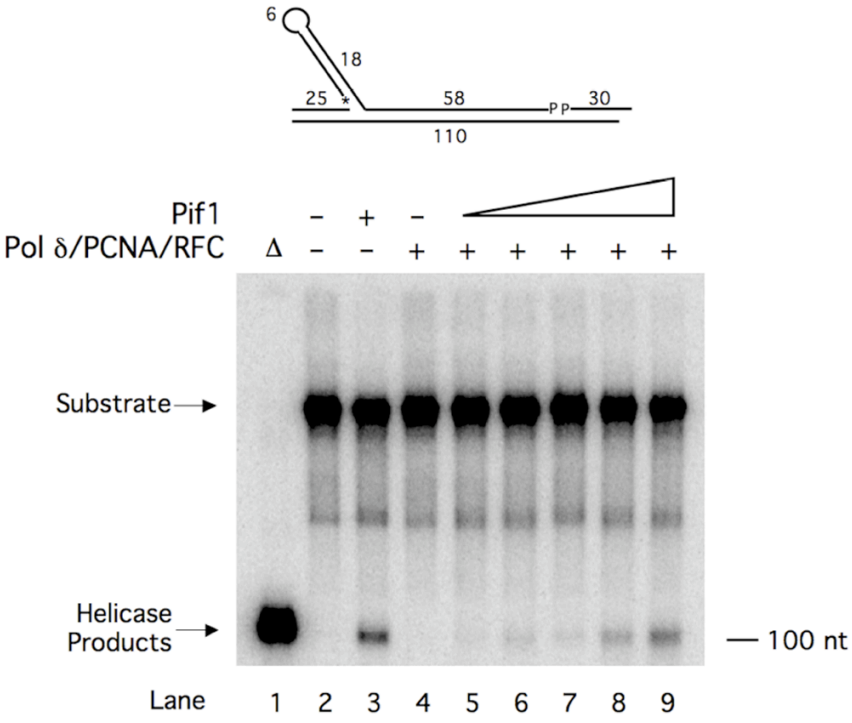
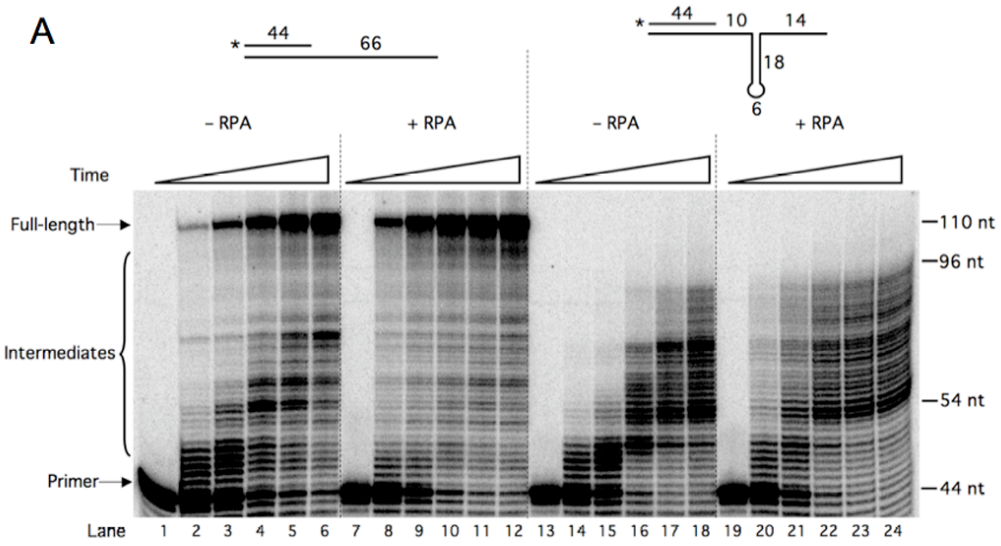

6
18
25
*
58
P P
30
110
Pif1
Pol δ/PCNA/RFC
Δ
Substrate
Helicase
Products
100 nt
Lane
1 2 3 4 5 6 7 8 9

Figure 2.4. Template secondary structure slows pol δ synthesis.

A, Synthesis by pol δ (23 fmol) was assayed on a standard substrate with a 44 nt upstream primer and lacking the downstream primer (U_2:T_1) (*lanes 1- 12*) and the 18 nt template fold-back substrate (U_2:T_2) (*lanes 13-24*) in the presence of PCNA (25 fmol) and RFC (25 fmol) and in the absence or presence of RPA (200 fmol) for increasing amounts of time (0, 0.5, 1, 2.5, 5, and 10 minutes) as indicated in the figure and as described under Experimental Procedures. Substrate depictions and figure labels are as in Fig. 2.1. *B*, Synthesis by pol δ (23 fmol) on the 16 nt template fold-back substrate (U_2:T_3) in the presence of PCNA (25 fmol) and RFC (25 fmol) and in the absence or presence of RPA (200 fmol) for increasing amounts of time (0, 0.5, 1, 2.5, 5, and 10 minutes). The experiment in Figure 2.4A was performed by Dr. Jason Pike.


A
*
44
66
*
44
10
14
18
6
– RPA
+ RPA
– RPA
+ RPA
Time
Full-length
Intermediates
Primer
110 nt
96 nt
54 nt
44 nt
Lane
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

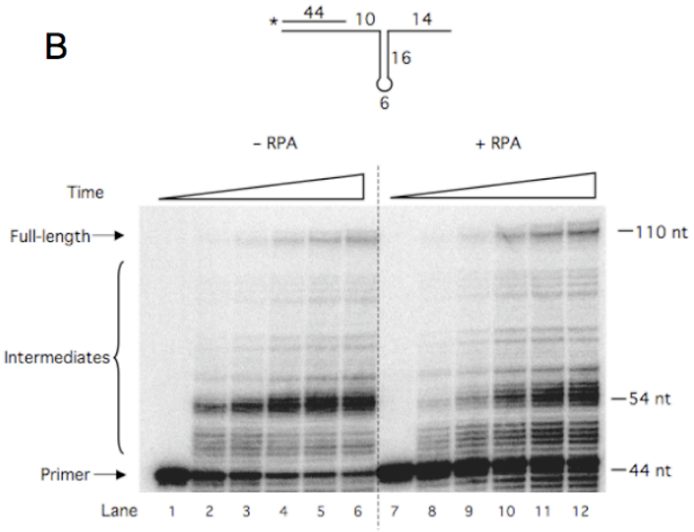
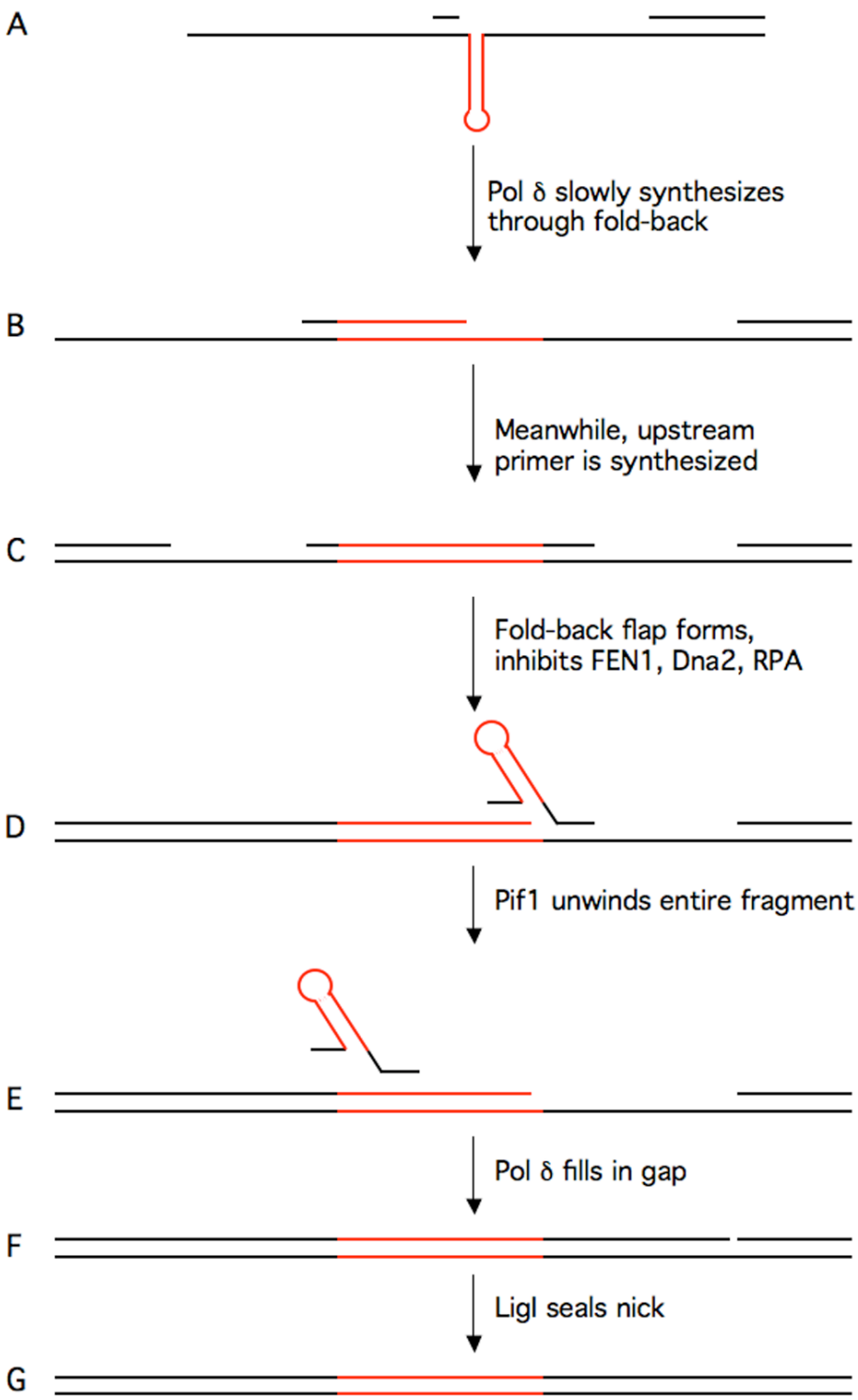

B
*
44
10
14
16
6
– RPA
+ RPA
Time
Full-length
Intermediates
Primer
110 nt
54 nt
44 nt
Lane
1 2 3 4 5 6 7 8 9 10 11 12

Figure 2.5. A model for an alternative Okazaki fragment processing pathway *in vivo*.

A, During DNA replication, at a sequence that has the potential to form secondary structure, a fold- back will form in the template DNA. The Okazaki fragment downstream of the fold-back is synthesized normally. A primer is laid down upstream of the template secondary structure. Note that the RNA portion of the primer is not depicted for simplicity. *B*, Pol δ will extend the primer through the secondary structure, but will dissociate near the end of the fold-back region. A gap between the extended primer and the downstream primer will remain. *C*, Meanwhile, the fragment upstream of the internal fragment will be synthesized normally. *D*, When it reaches the internal fragment, flap lengthening factors, such as Pif1 activity or acetylation of the nucleases, may create a long flap. The long flap will then fold back on itself. Neither the FEN1-only pathway nor the two-nuclease pathway can process the fold-back flap. *E*, Pif1 will bind a gap between the fold-back and the remaining downstream annealed region and, working with strand displacement synthesis from the upstream primer, unwind the entire internal Okazaki fragment. This synthesis will convert the fold-back region of the template to double strands that will no longer be capable of forming secondary structure. *F*, Pol δ will then continue synthesis through the gap until it reaches the downstream fragment. *G*, Conventional flap processing will produce a nick that LigI will then seal. The red lines represent segments with potential to form secondary structures.


A
Pol δ slowly synthesizes through fold-back
B
Meanwhile, upstream primer is synthesized
C
Fold-back flap forms, inhibits FEN1, Dna2, RPA
D
Pif1 unwinds entire fragment
E
Pol δ fills in gap
F
LigI seals nick
G

Chapter 3. Eukaryotes Employ Extended Strand Displacement Synthesis to Replace 5' End Region Mismatches in Okazaki Fragments

Abstract

In reconstitutions of eukaryotic Okazaki fragment processing, the large majority of RNA primers were removed by strand displacement of the 5' end of the downstream fragment into a short flap, cleavage by flap endonuclease 1 (FEN1) and ligation. *In vivo,* about 20 deoxynucleotides of initiating primer, beyond the RNA, are synthesized by the error-prone DNA polymerase α. Consequently, the 5' end regions of some fragments contain mismatches that require replacement by upstream fragment extension by the accurate DNA polymerase δ (pol δ). These mismatches would not all be corrected during short flap processing. We questioned whether processing proteins cooperate to remove and replace a greater length of strand from the 5' ends of fragments with mismatches. To assess correction, we developed a novel set of reconstitution substrates, each with a mismatch at some distance that, if corrected, creates a diagnostic EcoRI site. Reconstitutions revealed that a small fraction of flaps escape FEN1 cleavage to become longer. The Pif1 helicase further lengthens some of these long flaps before cleavage by FEN1. Pif1 did not respond to mismatches by promoting greater displacement. Only when generation of long flaps was augmented by reduction in FEN1 or low dNTP concentrations could a mismatch-dependent increase in displacement be observed. Introduction of a second mismatch near the 5' end of the downstream fragment did not improve loading or displacement by Pif1. In contrast, pol δ and FEN1 responded to mismatches both within several nucleotides of the 5' end of the downstream primer and up to 20 nt into the primer by

promoting farther strand displacement. These results suggest that Pif1 is influential in mismatch correction when growth conditions or regulation promote longer flaps, and reveal an inherent mechanism for mismatch correction by pol δ and FEN1.

Introduction

During eukaryotic DNA replication, the double-stranded DNA is unwound into its two single-stranded components that serve as templates for new DNA synthesis. One new DNA strand, the leading strand, is synthesized in a continuous manner by DNA polymerase ε and is lengthened in the same direction as the opening of the replication fork (1,2). The other strand, the lagging strand, grows in the opposite direction of the replication fork, and therefore must be made discontinuously, through the creation of short, ~150 nucleotide (nt) oligonucleotides segments known as Okazaki fragments (11,15).

Okazaki fragments are initiated by DNA polymerase α/primase (pol α), which synthesizes a primer consisting of 10 to 12 nt of RNA, followed by 10 to 20 nt of DNA (3). The primer is then lengthened by DNA polymerase δ (pol δ) (4). After pol δ adds 100- 200 nt to the primer to create the bulk of the fragment, it encounters the adjacent downstream Okazaki fragment. Additional strand displacement synthesis by pol δ creates a 5' flap, which must be cleaved by specific endonucleases. Cleavage of a flap removes the RNA and creates a nick that can then be sealed by DNA ligase I, completing lagging strand synthesis (10,11).

Cleavage of the 5' flap during strand displacement synthesis can occur through one of two pathways. In the short flap pathway, only one nuclease, flap endonuclease 1 (FEN1), is needed to cleave the 5' flap (21,22). FEN1 is able to recognize and bind the base of the flap, thread the single stranded flap from the 5'end to the base, and then cleave to produce a nick (25). However, reconstitution experiments have shown that some flaps can escape early FEN1 cleavage and become long (42). A flap which reaches 25-30 nt in length can be stably bound by replication protein A (RPA), the eukaryotic single stranded binding protein (43). Binding of RPA inhibits FEN1 cleavage (23,43), requiring the action of a second nuclease, Dna2. Dna2, an endonuclease which also possesses a 5'-3' helicase activity, can displace the RPA (44). Like FEN1, Dna2 binds to the flap base, but unlike FEN1, it does not cleave there. Instead Dna2 cuts periodically as it threads the flap, leaving a terminal flap that is 5-8 nt long (24). This flap is too short to be bound by RPA, leaving it free to be cleaved by FEN1 and ligated.

Pif1 is a 5'-3' helicase which has previously been shown to have an important role in telomere and mitochondrial DNA maintenance (73,84,88). Interestingly, Pif1 was shown to have a genetic interaction with Dna2, as deletion of *PIF1* rescues the lethality created by a Dna2 nuclease deficient mutant in yeast (90). Previous work from our research group has also suggested that Pif1 plays a role in the long flap pathway by stimulating the creation of longer flaps during Okazaki fragment processing (72).

Reconstitutions of Okazaki fragment processing *in vitro* suggest that FEN1 cleaves most flaps when they are only a few nucleotides long (72). Additionally, previous work from our group has shown that members of the long flap pathway (Dna2, RPA, and Pif1) are each able to stimulate FEN1 cleavage on short flaps (115). Taken together, these data would suggest that quick, efficient cleavage and ligation by FEN1 and DNA ligase is the preferred method for flap processing. This raises the question of why cells would maintain a long flap pathway and more generally what benefit there is in having pol δ displace farther into the downstream primer.

We hypothesized that longer displacement into the downstream fragment serves to correct mismatches present in the Okazaki fragment, specifically in the primer region synthesized by pol α. It is known that pol α, which lacks the ability to proofread, has a lower fidelity of synthesis than pol δ (6,116). Thus to maintain genome stability, it would be beneficial to allow pol δ to synthesize farther into the downstream primer. Mistakes in the primer created by pol α would then be corrected by pol δ. Because of its role in long flap creation, we anticipated that Pif1 would augment the ability of pol δ to correct mismatches in the downstream primer.

To test this hypothesis, we created a set of novel substrates designed to provide information on the distance of strand displacement synthesis before ligation. We used these substrates to assess the effects of different Okazaki fragment processing proteins, from *S. cerevisiae,* on strand displacement synthesis distance. We tested whether the interactions of mismatches in specific locations of the downstream primer with various combinations of processing proteins promoted

longer displacement in order to increase the likelihood that the mismatch would be properly repaired.

Experimental Procedures

Materials—The compound [γ-^{32}P]ATP was obtained from PerkinElmer Life Sciences. Oligonucleotide primers were synthesized by either Integrated DNA Technologies (Coralville, IA) or Midland Certified Reagents Company (Midland, TX). The primers and their sequences are listed in Table 1. Streptavidin, EcoRI, and polynucleotide kinase were obtained from Roche Applied Science. Other reagents were the best grade commercially available.

Enzyme Expression and Purification—*S. cerevisiae* pol δ (92) and LigI (22) were over-expressed in *S. cerevisiae* and purified as previously described. *S. cerevisiae* PCNA (42), RFC (110), FEN1 (19), Pif1 and Pif1 K264A (72) were over-expressed in *E. coli* and purified as previously described. *S. cerevisiae* Dna2 was over-expressed and purified from baculovirus High Five cells as previously described (52).

Oligonucleotide Substrates—Substrates were composed of the oligonucleotides listed in Table 1. Substrates were designed to observe pol δ synthesis, FEN1 cleavage, and EcoRI cleavage. To observe synthesis and EcoRI cleavage, the 5' end of U_1 was radiolabeled with [γ-^{32}P]ATP using polynucleotide kinase. The radiolabeled primers were separated by running the reactions on a 15%/7 M urea polyacrylamide gel. The radiolabeled products were then gel purified. To anneal substrates, component

oligonucleotides were mixed in annealing buffer (50 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM dithiothreitol), heated at 95°C for 5 min, then placed at 70°C and slowly cooled to room temperature. When the upstream primer was labeled, the oligonucleotides were annealed at a 1:4:8 ratio of upstream primer to template to downstream primer. When the downstream primer was labeled, the oligonucleotides were annealed at a 1:4:8 ratio of downstream primer to template to upstream primer.

Substrates utilized in these experiments were identical in structure but different in sequence. The first substrate consisted of a 44 nt upstream primer (U_1) and a 60 nt downstream primer (D_0) annealed to a 110-nt template (T_1), with a 6 nt gap between the upstream and downstream primers. This substrate has been used in previous reconstitution experiments (72,94) and will be referred to as the m0 substrate in the text, as it contains no mismatches. The second set of substrates consisted of the same 44 nt upstream primer (U_1) but different 60 nt downstream primers (D_5 containing a mismatch 5 nt from the 5' end of the downstream primer and D_{10} containing a mismatch 10 nt from the 5' end) annealed to the same 110-nt template (T_1), again leaving a 6-nt gap between the upstream and downstream primers. These primers are referred to as the m5 and m10 substrates, respectively.

Utilizing the EcoRI cleavage assay with a mismatch at 15 or 20 nucleotides into the downstream primer required altering the placement of the EcoRI site, in turn requiring a different template. This set of substrates utilized the same upstream primer (U_1) annealed to a different template (T_2). The downstream primers contained a mismatch at either 15 nt (D_{15}) from the 5' end of the downstream primer, termed the

m15 substrate, or 20 nucleotides (D_{20}), termed the m20 substrate. Both downstream primers were 60 nt, leaving a 6 nt gap between the upstream and downstream primer.

For substrates containing two mismatches, the same upstream primer and template used for the m15 substrate were utilized. A downstream primer containing a second mismatch 1, 2, 3, 4, or 5 nucleotides into the downstream primer was also anneal to the template. These substrates are referred to as the m15m1, m15m2, m15m3, m15m4, and m15m5 substrates, respectively.

Strand Displacement Synthesis Cleavage Assays—Five fmol of radiolabeled biotinylated substrate were incubated on ice with 500 fmol of streptavidin for 20 min. Streptavidin complexes with biotin on the substrate template ends, preventing PCNA from sliding off of the ends and requiring loading by RFC. For simplicity, the blocked ends of the substrates are not depicted in the figures. Streptavidin-conjugated substrate was then incubated with various combinations of pol δ, PCNA, RFC, LigI, and FEN1 and varying amounts of Pif1 or Dna2 for 20 min at 30°C in 20 μl of reconstitution buffer (50 mM Tris-HCl, pH 7.5, 2 mM dithiothreitol, 25 μg/ml bovine serum albumin, 50 μM dNTPs, 1 mM ATP, 4 mM $MgCl_2$, and 75 mM NaCl). Reactions were stopped with 20 μl of 2X termination dye (90% formamide (v/v), 10 mM EDTA, 0.01% bromophenol blue, and 0.01% xylene cyanole), followed by heating for 5 min at 95°C. Reaction products were separated by electrophoresis on a 22.5%/7 M urea polyacrylamide gel for 1 hour and 30 min at 80 W. The gel was dried and exposed to a phosphor screen overnight, which was then scanned with a GE Healthcare PhosphorImager and analyzed using ImageQuant version 1.2 software.

EcoRI Cleavage Assays—Reactions were run for 10 minutes as described above before the addition of 1 μL of either reaction buffer for controls or 4U/μL of EcoRI. Reactions were then run for an additional 10 minutes at 30°C. Reactions were stopped with 21 μL of 2X termination dye, followed by heating for 5 min at 95°C. Reaction products were separated by electrophoresis on a 22.5%/7 M urea polyacrylamide gel for 2 hour and 15 min at 80 W. Reactions were analyzed as described above.

The amount of each protein used in each experiment is given in the corresponding figure legend. All experiments were performed at least in triplicate, and a representative gel is shown in the corresponding figure.

Results

Previous analyses of FEN1 cleavage products generated during reconstitution of *S. cerevisiae* Okazaki fragment processing indicated that the majority of flaps were cleaved before they reached a length of six nt (72). Additionally, we found that protein components of the long flap pathway stimulate FEN1 cleavage, even on short flaps (115). These observations suggest that short flap processing is the strongly preferred method for removal of flaps created by pol δ strand displacement synthesis. However, by displacing a shorter distance into the downstream primer, fewer of the potential errors created by pol α are corrected by pol δ-directed synthesis. To explore this issue, we set out to better understand the factors that affect the distance of pol δ

strand displacement synthesis before ligation, especially investigating the influences of Pif1 helicase, a member of the long flap pathway with DNA-DNA and DNA-RNA separation activities as its apparent primary functions. We hypothesized that one role of Pif1 in fragment processing is to promote more displacement of downstream primers, so that mismatches they may contain can be corrected by synthesis from the upstream primer.

Beginning by examining the role of Pif1 in correctly matched fragment processing, we utilized a previously designed reconstitution system *in vitro*. The substrate consisted of a 44 nt upstream primer and a 60 nt downstream primer annealed to a 110 nt template. This left a 6 nt gap between the two primers through which pol δ could begin synthesis before encountering the downstream primer. The system also utilized RFC (25 fmol) and PCNA (25 fmol), to load and stabilize pol δ on the substrate, respectively. Each end of the template was biotinylated, allowing for conjugation with streptavidin. Protein-blocked termini prevented PCNA and pol δ from simply sliding off the ends of the substrate. FEN1 was also present in the reactions to cleave the flaps created by pol δ strand displacement synthesis. Depending on the product that was to be observed, ligase (25 fmol) may also have been added to the reactions.

Pif1 Helicase Function Moderately Increases Displacement of Long Flaps – We previously reported that Pif1 augments long flap formation (72), although it was unclear whether the effect was a consequence of helicase activity. To understand the basis of Pif1 influence on strand displacement, we tested here whether the effects of

Pif1 were dependent on helicase function. For the first part of the experiment, we added increasing amounts (50, 100, or 150 fmol) of Pif1 into reactions containing PCNA, RFC, pol δ (23 fmol) and FEN1 (5 fmol) (Fig. 3.1A, *lanes 4-6*). Labeling the downstream primer at its 5' end and then fractionating FEN1 cleavage products allowed us to observe the length of flap created before the first FEN1 cleavage. While FEN1 cleavage alone produced nearly all short products (*lane 3*), as more Pif1 was added to the reaction, we could clearly see the formation of longer products (*lanes 4-6*). We then performed the identical experiment with Pif1 K264A, a helicase deficient mutant (*lanes 7-9*) (86). No increase in long products occurred with the mutant, confirming the role of the helicase function in augmenting long flap formation. To ensure that the Pif1 mutant protein was properly folded, we confirmed that it was still able to stimulate FEN1 cleavage in the same manner as the wild type protein (data not shown).

A Substrate that Assesses Length of Displacement – Because Pif1 influences flap displacement, we questioned whether it increased the amount of the downstream primer removed before ligation. Although the reconstitution substrate was designed to reveal the efficiency of ligation, it did not allow measurement of the distance of displacement before ligation. To obtain this distance parameter, we constructed multiple substrates each with a defective EcoRI cleavage site placed at different locations in the downstream primer region. The sites contained a mismatch, making them refractory to EcoRI. Upstream primer extension through the sequence copies the template, replacing the mismatch and converting the site to a sequence sensitive to

EcoRI. Measuring cleavage efficiency on substrates with the site at different locations reveals the size of the patch replaced by pol δ. Experiments with this substrate include control reactions using the substrate with just an upstream primer extended by pol δ. Positive or negative versions of these controls have or lack EcoRI, and represent complete or zero cleavage, respectively.

Pif1 Has Little Effect on Displacement of Mismatched Primers - Initially, we tested substrates with EcoRI-mismatches at either 5, 10, 15 or 20 nt from the 5' end of the downstream primer (designated m5, m10, m15 and m20, respectively).

We have included an example gel of an experiment with the m15 substrate (Fig. 3.1B). We observed from the controls that, indeed, when DNA was correctly synthesized from the template, it produced a functional EcoRI cleavage site (*lane 3*). We also saw that on a substrate with a downstream primer, only a small percentage of products were corrected at or beyond 15 nt into the downstream primer (*lane 10*). Moreover, Pif1 had little effect on increasing the amount of corrected product and only at the highest concentration (200 fmol).

Two distinct features are consistently visible in the EcoRI cleavage system gels: the first is a doublet band at the position of the full-length product. Based on our synthesis control, we conclude that the lower band represents a product that was extended nearly all the way to the end of the template. We believe it is one nucleotide short because the streptavidin protein conjugated at the end of the template blocked complete synthesis, preventing incorporation of the last nucleotide. The upper band, then, represents a ligation of the two primers and is full length, as its

creation did not involve synthesis to the end of the template. The second observation is that the EcoRI cleavage products were a group of lengths varying by about 5 nt. This evidently occured because there was active pol δ in the assay at the time EcoRI was added. As cleavage by EcoRI leaves a 4 nt overhang in the template, pol δ could have added up to 4 nt, creating a small range of products.

Pif1 Increases Replacement Patch Length on Substrates Where Long Flaps Are Made – We considered that Pif1 may only influence displacement of flaps that escape FEN1 cleavage and become long enough to be bound by the helicase. As suggested by results in Figure 3.1A, only a very small percentage of Okazaki fragments in the conventional reconstitution system was processed by the long flap pathway. To examine the influence of Pif1 on this class of intermediates, the reconstitution systems containing EcoRI sites were tested with reduced FEN1 (2 fmol of FEN1 to 5 fmol of substrate).

Under these conditions, pol δ was readily able to synthesize a sufficient distance to correct a mismatch present 5 nt in from the 5' end of the downstream primer (Fig. 3.2A). The presence of Pif1 had no effect on correction of these mismatches, as pol δ synthesized to this point very efficiently. The same was true when the mismatch was placed 10 nt into the downstream primer, as pol δ was also highly efficient at correcting the mismatch (Fig. 3.2B). However, when the mismatch was placed 15 nt into the downstream primer, we detected only a modest amount of correction by pol δ in the absence of Pif1, of approximately 34.5% (Fig. 3.2C). Percent correction here is defined as the amount of product that is cleaved by EcoRI

divided by the total amount of cleaved product and full-length product created. As Pif1 was titrated into the reactions, there was a steady increase in EcoRI cleavage, with 44.7% cleaved at 200 fmol of Pif1. There was also a significant increase in EcoRI cleavage when the mismatch was placed 20 nt into the downstream primer, with correction increasing from 24.8% without Pif1 to 36.2% in the presence of 200 fmol of Pif1 (Fig. 3.2D).

These results suggest that pol δ is frequently able to displace ~10 nt into the downstream primer in order to correct a mismatch. However, pol δ is much less effective at displacing greater lengths. With the aid of Pif1, however, pol δ is able to displace farther, allowing it to correct errors 15 or 20 nt into the downstream primer. We expected that the helicase activity of Pif1 promoted mismatch correction by aiding pol δ displacement of the downstream primer. To confirm this, we repeated our experiment with the m15 substrate, this time with a helicase deficient mutant of Pif1. As expected, unlike the wild-type Pif1, the helicase deficient mutant of Pif1 was not able to increase mismatch correction by pol δ (Fig. 3.2E).

Idling by pol δ Allows for Long Flap Creation by Pif1- We have observed that decreasing the level of FEN1 present in the reconstitution system allowed Pif1 to increase the frequency of pol δ synthesis past 15 nt into the downstream primer. To determine whether any other likely cellular conditions would allow Pif1 to encourage longer patch synthesis by pol δ, we considered the condition of slow DNA replication in the presence of diminished concentrations of dNTPs. This causes pol δ to idle, a process in which it repeatedly displaces a small flap of 2-3 nt on the downstream

primer, then degrades nucleotides from the primer terminus with its 3' exonuclease function.

Initial experiments with dNTP concentration decreased by 75% (from 50 to 12.5 μM) in our reconstitution buffer caused a strong pol δ pause point at the downstream primer (data not shown), indicative of an idling pol δ. We chose the m15 substrate to assess the length of displacement before ligation.

When Pif1 was titrated into the reactions, there was an increase in EcoRI cleavage products, indicating that in the presence of Pif1, pol δ is more likely to synthesize 15 nt or more into the downstream primer (Fig. 3.3A). We also compared the activity of Pif1 to that of Dna2 nuclease/helicase as a control. We chose Dna2, a long flap pathway component, as it also possesses helicase activity and could potentially aid in unwinding the downstream primer. However, Dna2 was unable to cause pol δ to synthesize further into the downstream primer (Fig. 3B).

Low FEN1 and Low dNTP Concentrations Allows Pif1 to Strongly Promote Strand Displacement- We additionally questioned how low concentrations of both FEN1 and dNTPs would affect displacement by Pif1 and pol δ into the downstream primer. The combined conditions should increase pol δ idling, as it has been shown that low levels of FEN1 can also contribute to pol δ idling (34). We anticipated that these conditions would represent a cellular environment most favorable for Pif1 to influence strand displacement. For these experiments we again utilized the m15 substrate and the EcoRI cleavage assay.

When both FEN1 and dNTPs were decreased, we observed a substantial increase, approximately 2.3 fold, in pol δ strand displacement in the presence of 200 fmol Pif1 (Fig. 3.4A). Once again, we did not observe an increase in correction in the presence of Dna2 (Fig. 3.4B). Without Pif1 (Fig. 3.4A), pol δ was largely ineffective at synthesis past the pause point. Under these conditions, even low levels of Pif1 had a major effect in promoting strand displacement synthesis. Pif1 greatly enhanced the density of many products in lengths ranging from synthesis to the pause point to the Eco RI cleavage site.

Pif1 Did Not Displace More DNA in Response to Mismatches Near the 5' End of the Downstream Primer – If Pif1 had evolved to promote mismatch correction, it would potentially promote longer patch synthesis by pol δ is the presence of a mismatch near the 5' end of the downstream primer. Such a mismatch would encourage breathing of the strands, creating transient flaps. The creation of a flap should promote loading of Pif1, and subsequent robust displacement activity even before the arrival of the polymerase. If so, a mismatch in the reconstituted system near the 5' end of the downstream primer would result in longer flap formation and longer patch replacement in the presence of Pif1.

For these experiments, we utilized a substrate with two mismatches, the first ranging from 1 to 5 nt from the 5' end of the downstream primer, the second 15 nt from the 5' end. The second mismatch served a diagnostic purpose, reporting EcoRI cleavage upon ligation after long patch synthesis. Our control was a substrate with just the diagnostic mismatch. We then compared how often synthesis proceeded past

this distant point when a second mismatch was introduced 1, 2, 3, 4, of 5 nucleotides into the downstream primer.

There was very little difference in the ability of Pif1 to stimulate the creation of long flaps in the presence or absence of a mismatch within the first 5 nucleotides of the downstream primer. Here we show an example gel for an experiment in which there was a mismatch 2 nt into the downstream primer (Fig. 3.5A). We conclude that this mismatch did not allow improved loading of Pif1 leading to more flap displacement. Mismatches at positions one through five gave similar results (data not shown). However, it is apparent from the results that the presence of the mismatch within the first five nucleotides has the potential to enhance the amount of synthesis through position 15 before ligation, irrespective of the presence of Pif1.

Pol δ and FEN1 Respond to a Mismatch with More Strand Displacement – Extending this observation, the next set of experiments reported on the influence of mismatches within the 5' end region on the length of the patch replaced before ligation. These experiments utilized the reconstitution system containing PCNA-linked pol δ and FEN1 but no Pif1. When we introduced a mismatch 1 nt into the downstream primer, the pause point moved one nt farther into the downstream primer (Fig. 3.5B). With a mismatch two or three nt into the downstream primer, the well defined pause point was lost, with synthesis proceeding farther into the downstream primer, and the increased density of extension intermediates four to ten nt in length. Synthesis through the 15 nt point and activation of the EcoRI site was increased by these mismatches. This effect disappeared once the mismatch was placed 4 or 5

nucleotides into the downstream primer. With the mismatches placed at these points, the mismatch no longer affected the band density at the pause point, nor was there an increase in the amount of long products.

Mismatches in the Downstream Primer Lead to Farther Synthesis- For our initial experiments, the mismatches located five or more nt into the downstream primer served as a diagnostic tool to reveal and quantify whether pol δ synthesized past a designated point before ligation. However, these results did not demonstrate whether the presence of a mismatch itself was sufficient to allow farther strand displacement by pol δ. It was previously shown in assays of FEN1 activity that a mismatch in the downstream primer, several nucleotides into the annealed region, could promote cleavage up to and including the mismatched site (117).

Here we tested whether a mismatch in the downstream primer could alter the pattern of pol δ synthesis and FEN1 cleavage in a reconstituted system. For these experiments the downstream primer was labeled at the 5' end. This allowed us to visualize the point at which FEN1 first cleaves, representing the first point at which ligation could occur. Additionally, we utilized a control substrate that contained no mismatches, designated the m0 substrate. By comparing the cleavage patterns of the substrates, we could determine the effect of the mismatch on displacement into the downstream primer.

With the substrate lacking a mismatch nearly all cleavage products were short, approximately 1-8 nt in length (Fig. 3.6A). Progressive addition of Pif1 augmented the production of short products, as expected since Pif1 was shown to stimulate FEN1

cleavage. Pif1 also promoted the appearance of some longer products, confirming its ability to create longer flaps. Introduction of a mismatch 5 nt into the downstream primer, shifted the distribution to longer products (Fig. 3.6A). Similarly, mismatches at positions 10, 15 and 20 all promoted a shift to longer cleavage products (Figs. 3.6A & B). Addition of Pif1 further shifted the product distribution, although that shift was modest in magnitude.

These results indicate that the reconstituted system, involving interaction of pol δ and FEN1, can detect a mismatch in a variety of locations and responds by removing nucleotides a farther distance into the downstream primer. The presence of Pif1 increases this distance, consistent with the previously observed ability of Pif1 to augment the population of flaps that escape FEN1 cleavage and become long before they are cleaved. It is not clear, however, whether Pif1 activity is increased by the mismatch, or whether the greater strand displacement activity of the polymerase creates a flap onto which Pif1 loads more readily.

Discussion

Current understanding of eukaryotic lagging strand DNA replication suggests that quick, efficient cleavage by FEN1 followed by ligation by DNA ligase I is the preferred method for processing flaps created during strand displacement synthesis. Since Okazaki fragments are only about 150 nt in length, this approach avoids degradation and resynthesis of a high percentage of the lagging strand. However,

another consideration is that the first 15-20 deoxynucleotides of each fragment are made by the low fidelity pol α and are apt to contain mismatches. As it lacks the proofreading ability of pol δ, pol α is more error prone, with pol δ having an error rate of ~ 10^{-7}, and pol α having an error rate of ~10^{-4} (6). Results presented here provide evidence that the enzymes of Okazaki fragment processing can detect mismatches and allow pol δ to displace a farther distance into the downstream fragment before ligation. This approach restricts long patch replacement to the subset of fragments with synthesis errors, maximizing both replication efficiency and mismatch correction. Results additionally suggest that the helicase Pif1 promotes long patch replacement irrespective of the presence of mismatches, and participates in the long flap pathway of fragment processing regulated by acetylation of replication proteins.

In earlier examinations of the properties of FEN1 we found the presence of a mismatched nucleotide as far in as 15 base pairs from the 5' end of a primer shifted the endonucleolytic cleavage pattern farther into the strand (117). We concluded that helical instability caused by the mismatch promoted tracking and cleavage by FEN1, resulting in a greater distance of downstream fragment degradation. As this greater extent of cleavage was more likely to reach and pass the site of the mismatch, we proposed that FEN1 had evolved to cleave farther on a strand with a mismatch in order to be more effective at mismatch correction. We termed this property "5' proofreading". In the current work, we expanded on this observation by asking

whether the other protein components of Okazaki fragment processing collaborate with FEN1 to sense and selectively remove and replace mismatched nucleotides.

To answer this question we reconstituted Okazaki fragment processing on a novel EcoRI substrate system. This system consisted of a series of two-primer substrates in which a mismatched EcoRI site was placed various distances from the 5' end of the downstream primer. Resynthesis by pol δ through the mismatch allowed diagnostic EcoRI cleavage. The proportion of ligated products that were synthesized through the site could then be determined. This is a very useful tool since the EcoRI site can be placed at any distance from the 5' end of the downstream primer, allowing for a precise determination of the extent of pol δ synthesis and mismatch removal.

In previous reconstitutions of fragment processing, we observed that the RNA primers were removed by a flap cleavage mechanism in which flaps were created by strand displacement synthesis directed by pol δ and then cleaved by FEN1 (42). We analyzed the effects of mismatches on this reaction in two ways. In the first, we introduced a mismatch in the first several nucleotides of the downstream primer and additionally placed an EcoRI diagnostic site at position 15. The presence of a mismatch in the first several nucleotides greatly altered the pause pattern when pol δ synthesis encountered the 5' end of the primer (Fig. 3.5B). Displacement was distributed over longer distances, and the cleavage at the EcoRI site was augmented. In the second, we used substrates with a 5' label on the downstream primer to quantify the sites of the first FEN1 cleavage. Here the 5' end region was correctly matched, and a single EcoRI mismatch was placed at position 5, 10, 15 or 20. The

mismatches at 5 or 10 greatly increased longer FEN1 cleavage products (Fig. 3.6A). Mismatches at 15 and 20 were much less effective (Fig. 3.6B). Pif1 had very little effect in either of these assays. Its greatest effect was to augment the quantity of the FEN1 products made in response to mismatches at positions 15 and 20. We conclude for the majority of intermediates containing mismatches, the combination of pol δ and FEN1 detects the mismatch and replaces a longer patch. Moreover, for the majority of mismatched intermediates, Pif1 does not significantly increase the length of patch replacement.

Is there a role for Pif1 in improving mismatch removal during Okazaki fragment processing? Our previous results showed that Pif1 increased the fraction of long flaps produced and cut by FEN1 in the conventional, correctly matched reconstitution system. We show here that this action of Pif1 is dependent on its helicase activity. Moreover, shifting the system toward processing by the long flap pathway in reactions that promoted pol δ idling and inefficient FEN1 cleavage revealed a significant effect of Pif1 on mismatch correction (Fig. 3.4A). A likely explanation for the specific effectiveness of Pif1 on long flap intermediates is that the Pif1 needs a minimum length of single stranded flap for efficient loading.

Although pol δ idling and inefficient FEN1 cleavage presented an opportunity for Pif1 to increase pol δ synthesis patch length, Dna2 did not exhibit the same ability (Fig. 3.4B). Although Dna2 possesses helicase activity, it is much weaker than Pif1, which would account for its inability to stimulate farther strand displacement synthesis. In fact, the presence of Dna2 impeded displacement farther into the

downstream primer. Although an increase in full-length ligation product was observed in the presence of Dna2, there was a decrease in correction of the m15 substrate. This is likely a result of the nuclease activity of Dna2: Dna2 can cleave flaps that escape early FEN1 cleavage, instead of lengthening them as Pif1 does. In this way, Dna2 cleavage would aid FEN1 cleavage, leading to more early ligation products than were observed in the absence of Dna2. As early ligation halts pol δ displacement into the downstream primer, it would lead to a decrease in the population of long displacement products.

Our results are suggestive that although Pif1 augments the production of long flaps, it does not work more effectively in response to a mismatch that potentially produces a transient flap long enough for Pif1 to bind. This is evident from results with mismatches near the 5' end of the downstream primer, which do not appear to promote loading of Pif1. Moreover, the effects of Pif1 on EcoRI sites are most evident at distances of 15 to 20 nt, mismatches unlikely to destabilize the 5' Pif1 site of loading. Instead, the role of Pif1 appears to be to generally promote long flap creation and processing, which incidentally corrects mismatches in the 15 to 20 nt range.

How then do the properties and role of Pif1 relate to the desirability of replacing a longer patch only on fragments containing a mismatch? We were able to shift processing to the long flap pathway by reducing the concentration of FEN1 and by promoting idling of pol δ. Recent observations suggest that the cell regulates the functions of DNA replication and repair proteins by post-translational modification.

A relevant modification to mismatch correction is acetylation. Acetylation of FEN1 decreases its activity. Acetylation of pol δ increases its strand displacement capacity, which would lead to the creation of more single stranded loading sites for Pif1. Acetylation of Dna2 promotes both nuclease and helicase activity. These changes are likely to promote the loading of Pif1.

The histone acetyl-transferase p300, which activates chromatin for transcription, can provoke all of these changes. This suggests that DNA replication is regulated in active chromatin to proceed by a long flap pathway. The role of Pif1 would then be to promote long flap creation and long patch replacement in every Okazaki fragment made by the modified proteins. This would not be efficient but would be effective at removing mismatches. It is possible that the cell is willing to sacrifice efficiency of DNA synthesis in active chromatin, in order to maximally protect the information contained in highly expressed genes.

In the future, the EcoRI system should be very useful to characterize, *in vitro*, how modification of these proteins effects pol δ synthesis and mismatch correction to determine how the cell is able to regulate replication fidelity. Of particular interest to such a study is whether mismatches within the pol α primers promote regulatory mechanisms that acetylate the flap processing proteins.

In summary, our results indicate that eukaryotic cells employ at least two methods to balance efficiency with fidelity during Okazaki fragment processing. An important goal is to remove and replace mismatches made by DNA polymerase α in the 5' end regions of some of the fragments. Pol δ and FEN1 can sense the presence

of a 5' end region mismatch and synthesize a longer patch. Pif1 does little to increase the patch length of pol δ synthesis. However, Okazaki fragment processing proteins are regulated by acetylation, which changes their properties to promote the longer flap pathway. Intermediates of the long flap pathway allow loading of Pif1. The Pif1 then employs helicase activity to encourage strand displacement, resulting in replacement of a longer patch. Although Pif1 does not appear to be more effective on fragments containing mismatches, its ability to generally lengthen the replacement patch should increase the effectiveness of mismatch correction.

Table 3.1. Oligonucleotide sequences

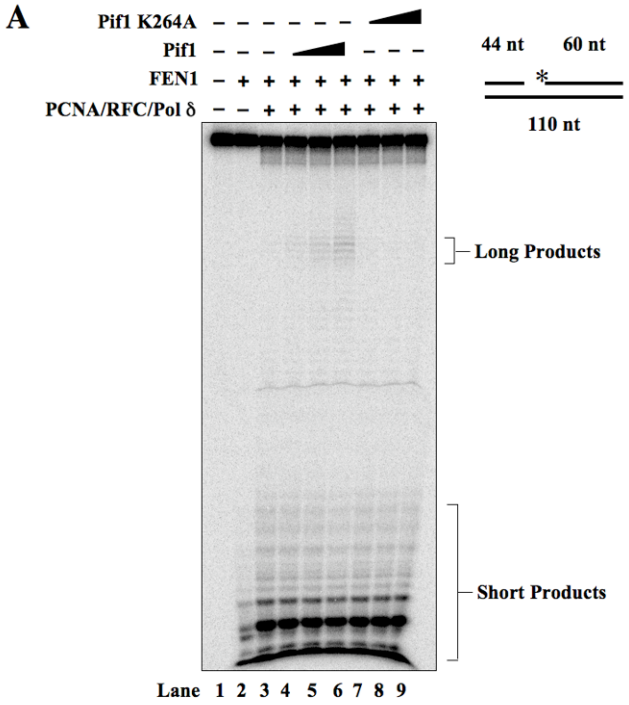
Primer	Length (nt)	Sequence
Upstream (5′-3′)		
U_1	44	GTCCACCCGACGCCACCTCCTGCCTTCAATGTGCT GGGATCCTA
Downstream (5′-3′)		
D_0	60	AGACGAATTCCGGATACGACGGCCAGTGCCGACC GTGCCAGCCTAAATTTCAATCCACCC
D_5	60	AGAC**U**AATTCCGGATACGACGGCCAGTGCCGACC GTGCCAGCCTAAATTTCAATCCACCC
D_{10}	60	AGACGAATT**U**CGGATACGACGGCCAGTGCCGACC GTGCCAGCCTAAATTTCAATCCACCC
D_{15}	60	AGACCGGATACGAC**U**AATTCGGCCAGTGCCGACC GTGCCAGCCTAAATTTCAATCCACCC
D_{20}	60	AGACCGGATACGACGAATT**U**GGCCAGTGCCGACC GTGCCAGCCTAAATTTCAATCCACCC
Template (3′-5′)[a]		
T_1	110	CAGGTGGGCTGCGGTGGAGGACGGAAGTTACACGA CCCTAGGATGTTGGTTCTGCTTAAGGCCTATGCTGC CGGTCACGGCTGGCACGGTCGGATTTAAAGTTAGG TGGG
T_2	110	CAGGTGGGCTGCGGTGGAGGACGGAAGTTACACGA CCCTAGGATGTTGGTTCTGGCCTATGCTGCTTAAGC CGGTCACGGCTGGCACGGTCGGATTTAAAGTTAGG TGGG

[a] Templates T_1, and T_2 are biotinylated at both the 5′ and 3′ ends.

Letters in bold indicate a mismatch

Figure 3.1. Pif1 helicase function moderately increases displacement of long flaps.

A, strand displacement synthesis by pol δ (23 fmol) was assayed on the m0 substrate in the presence of various combinations of PCNA (25 fmol), RFC (25 fmol), and FEN1 (20 fmol), and in increasing amounts (25, 50, or 100 fmol) of either Pif1 (lanes 4-6) or Pif1 K264A (lanes 7-9) as indicated in the figure and as described under "Experimental Procedures." The substrate is depicted above the figure with the location of the radiolabel indicated by the *asterisk*. *Plus* (+) indicates the presence and *minus* (–) the absence of the listed component. *B*, Strand displacement synthesis by pol δ (100 fmol) and EcoRI cleavage was assay on either the synthesis control substrate (lanes 2-3) or the m15 substrate (lanes 1 & 4-15) in the presence of PCNA (25 fmol), RFC (25 fmol), Ligase (25 fmol), and FEN1 (5 fmol) (Reaction Mix), and in increasing amounts of Pif1 (50, 100, 150, or 200 fmol).


A
Pif1 K264A
Pif1
FEN1
PCNA/RFC/Pol δ
44 nt
60 nt
110 nt
Long Products
Short Products
Lane 1 2 3 4 5 6 7 8 9

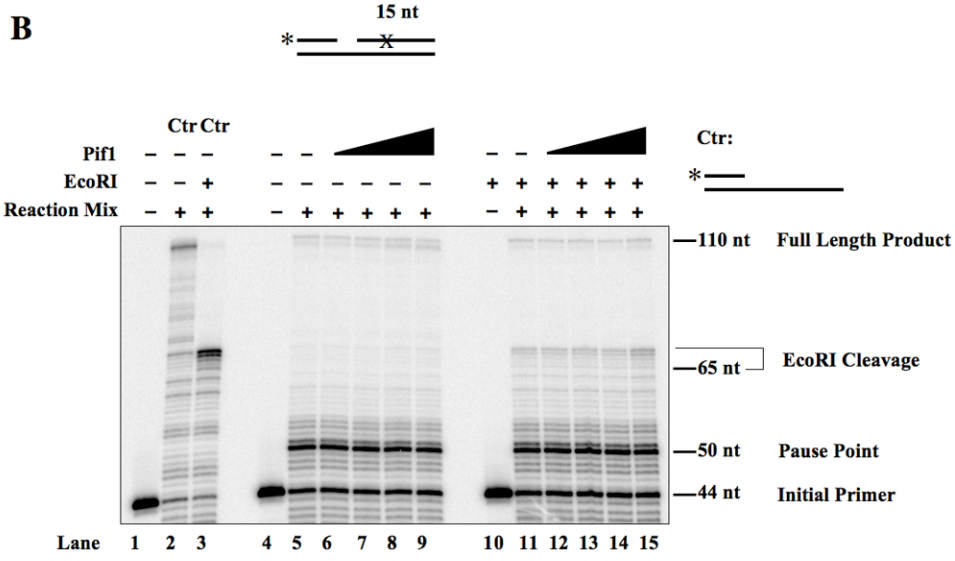
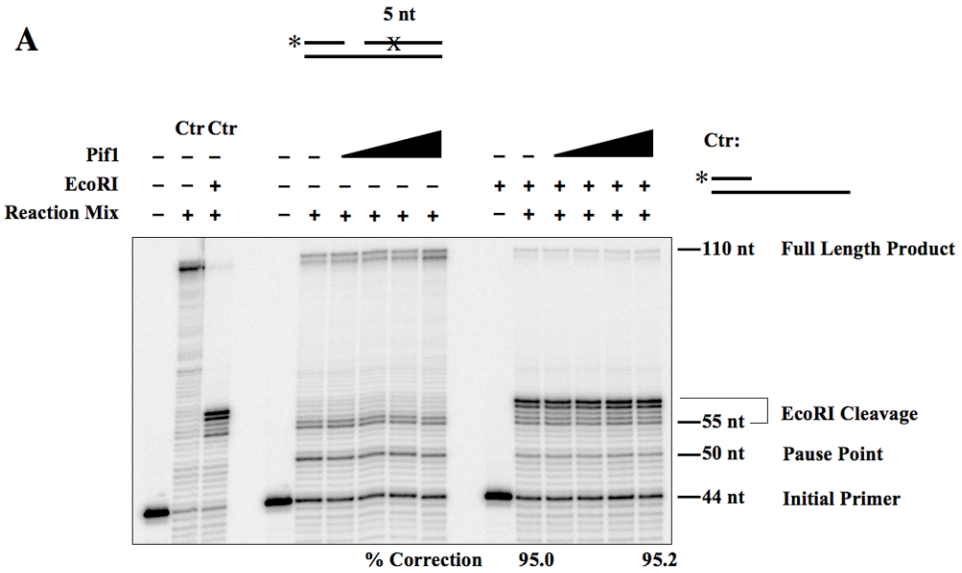
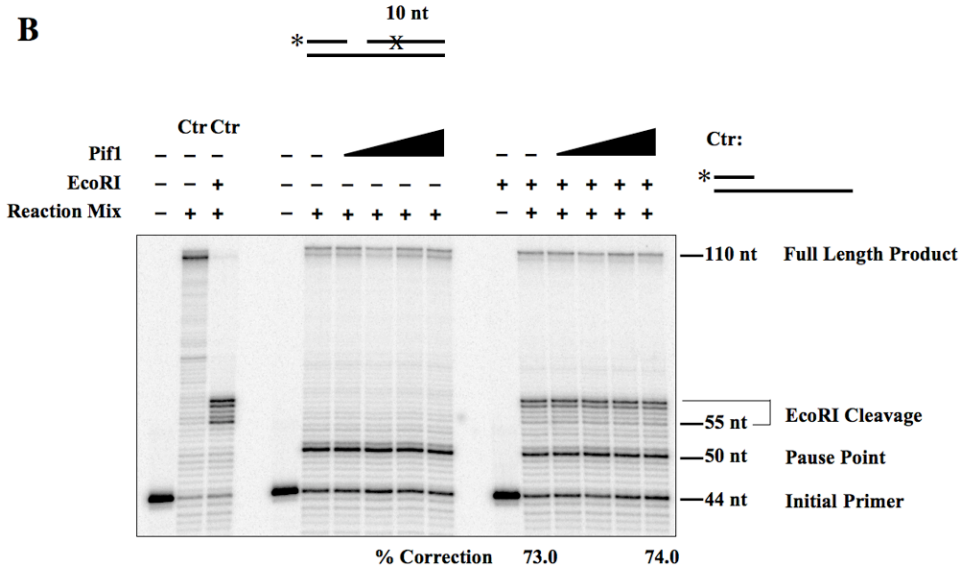
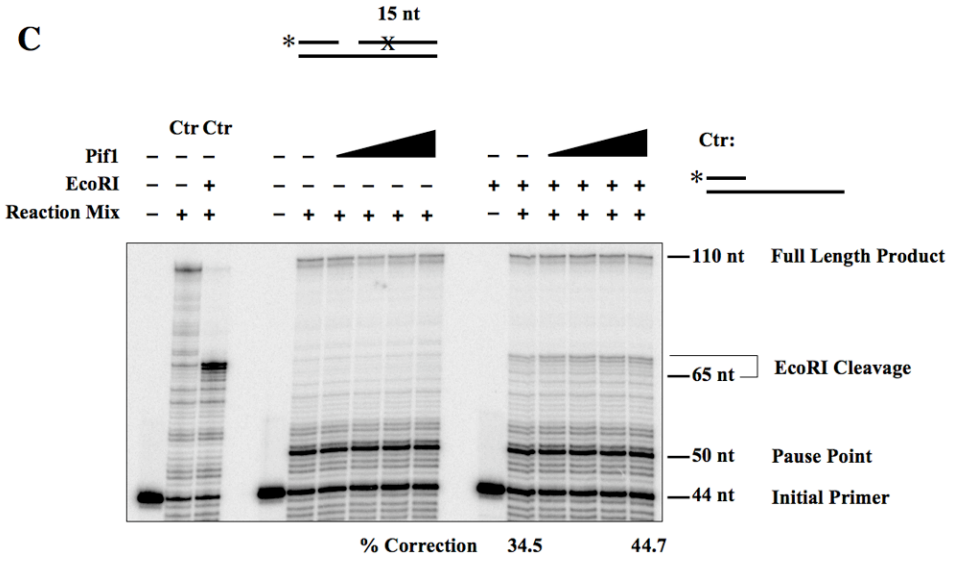

B
15 nt
Ctr Ctr
Pif1
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

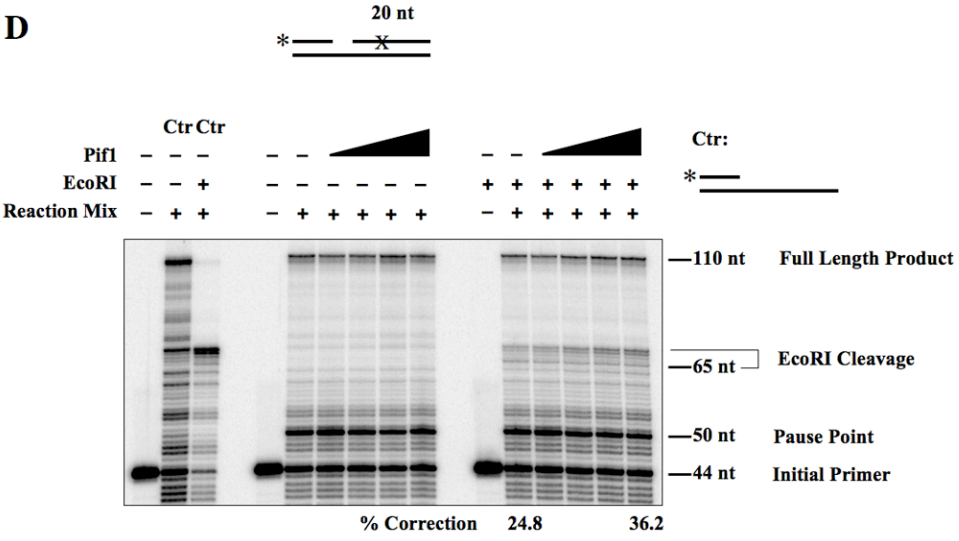
Figure 3.2. Pif1 increases replacement patch length on substrates where long flaps are made.

A, strand displacement synthesis by pol δ (100 fmol) and EcoRI cleavage was assay on either the synthesis control substrate or the m5 substrate in the presence of PCNA (25 fmol), RFC (25 fmol), LigI (25 fmol), and FEN1 (2 fmol) (Reaction Mix), and in increasing amounts of Pif1 (50, 100, 150, or 200 fmol), as indicated in the figure and described under "Experimental Procedures." Substrate depictions and designations are as in Fig. 3.1. *B*, the reaction was performed as in Fig. 3.2A, but utilizing the m10 substrate and synthesis control substrate. *C*, the reaction was performed as in Fig. 3.2A, but utilizing the m15 substrate and synthesis control substrate. *D*, the reaction was performed as in Fig. 3.2A, but utilizing the m20 substrate and synthesis control substrate. *E*, the reaction was performed as in Fig. 3.2A, but utilizing the m15 substrate and synthesis control substrate and utilizing increasing amounts of the helicase-deficient Pif1 K264A (50, 100, 150, or 200 fmol).


A
5 nt
Ctr Ctr
Pif1
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
55 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
95.0
95.2


B
10 nt
Ctr Ctr
Pif1
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
55 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
73.0
74.0


C
15 nt
Ctr Ctr
Pif1
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
34.5
44.7


D
20 nt
Ctr Ctr
Pif1
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
24.8
36.2

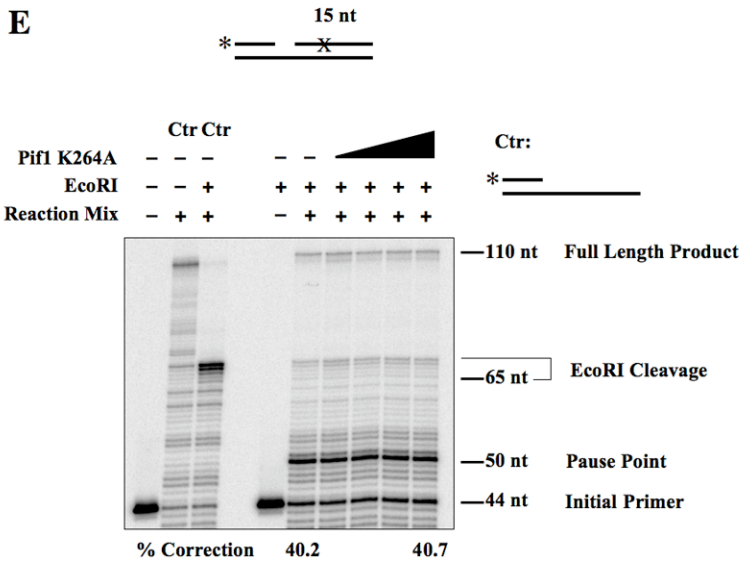
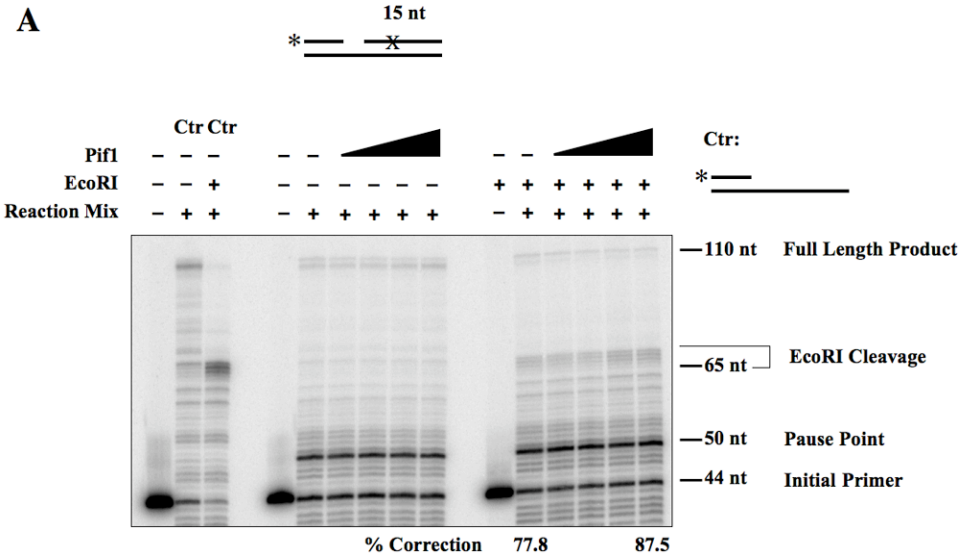

E
15 nt
Ctr Ctr
Pif1 K264A
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
40.2
40.7

Figure 3.3. Idling by pol δ allows for long flap creation by Pif1.

A, pol δ idling was simulated by decreasing dNTP concentrations from 50 μM to 12.5 μM. Strand displacement synthesis by pol δ (100 fmol) and EcoRI cleavage was assay on either the synthesis control substrate or the m15 substrate in the presence of PCNA (25 fmol), RFC (25 fmol), LigI (25 fmol), and FEN1 (5 fmol) (Reaction Mix), and in increasing amounts of Pif1 (50, 100, 150, or 200 fmol), as indicated in the figure and described under "Experimental Procedures." Substrate depictions and designations are as in Fig. 3.1. *B*, the reaction was performed as in Fig. 3.3A, but utilizing increasing amount of Dna2 (50, 100, 150, or 200 fmol).


A
15 nt
Ctr Ctr
Pif1
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
77.8
87.5

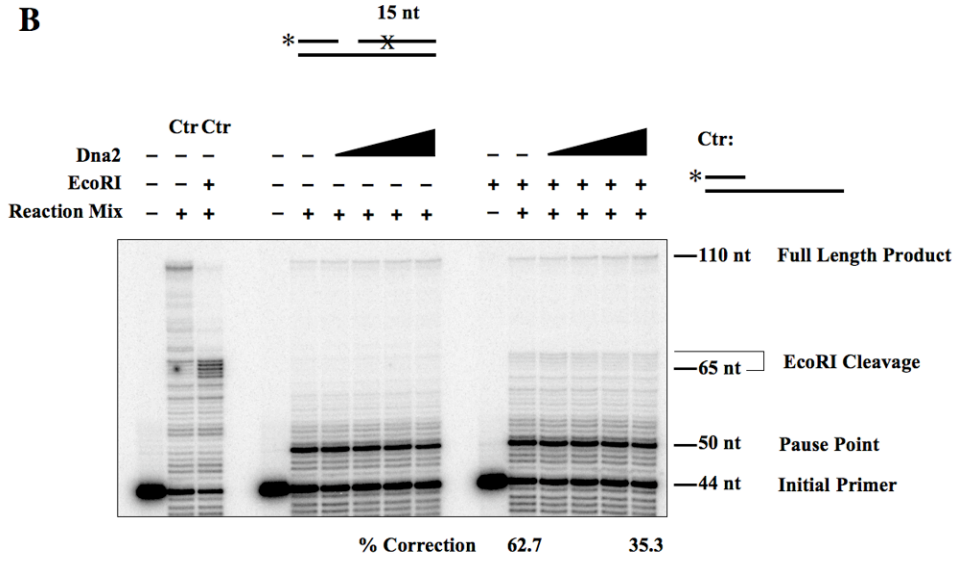
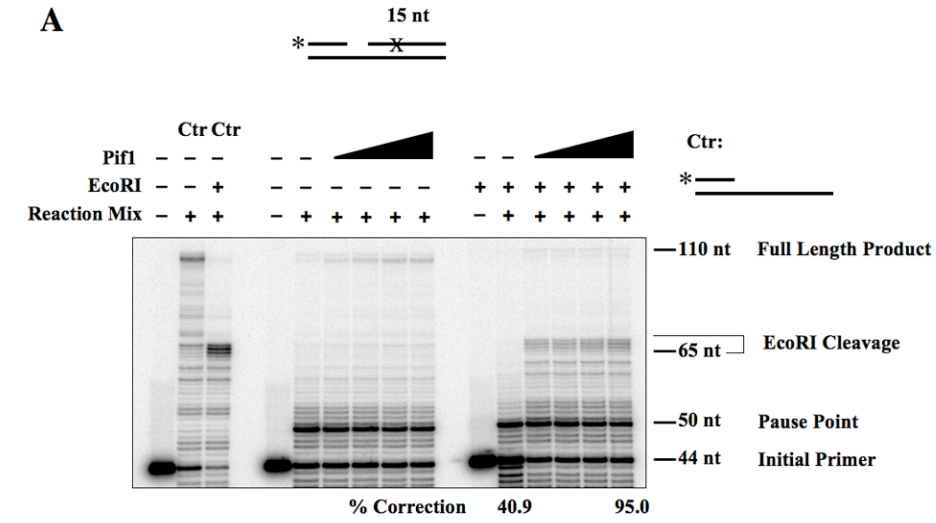

B
15 nt
Ctr Ctr
Dna2
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
62.7
35.3

Figure 3.4. Low FEN1 and low dNTP concentrations allow Pif1 to strongly promote strand displacement.

A, pol δ idling was simulated by decreasing dNTP concentrations from 50 μM to 12.5 μM. Strand displacement synthesis by pol δ (100 fmol) and EcoRI cleavage was assay on either the synthesis control substrate or the m15 substrate in the presence of PCNA (25 fmol), RFC (25 fmol), LigI (25 fmol), and FEN1 (2 fmol) (Reaction Mix), and in increasing amounts of Pif1 (50, 100, 150, or 200 fmol), as indicated in the figure and described under "Experimental Procedures." Substrate depictions and designations are as in Fig. 3.1. *B*, the reaction was performed as in Fig. 3.4A, but utilizing increasing amount of Dna2 (50, 100, 150, or 200 fmol).


A
15 nt
Ctr Ctr
Pif1
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
40.9
95.0

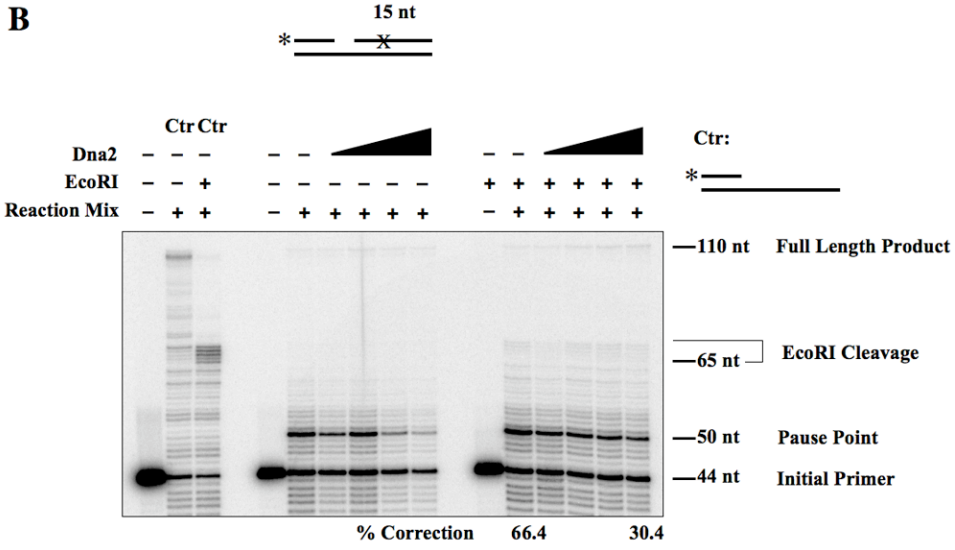
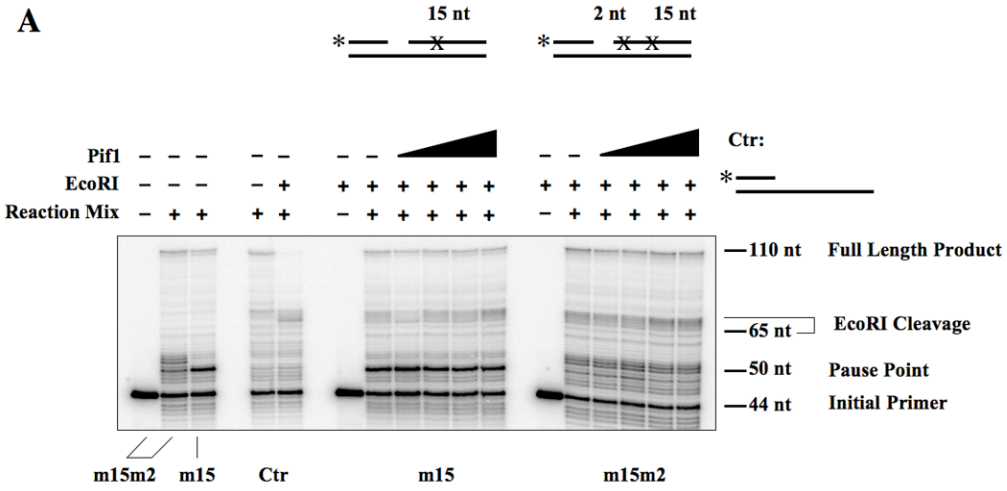

B
15 nt
Ctr Ctr
Dna2
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer
% Correction
66.4
30.4

Figure 3.5. Pol δ and FEN1 respond to a mismatch with more strand displacement.

A, strand displacement synthesis by pol δ (100 fmol) and EcoRI cleavage was assayed on the synthesis control substrate, m15, or m15m2 substrate in the presence of PCNA (25 fmol), RFC (25 fmol), LigI (25 fmol), and FEN1 (5 fmol) (Reaction Mix), and in increasing amounts of Pif1 (50, 100, 150, or 200 fmol) as indicated in the figure and described under "Experimental Procedures." Substrate depictions and designations are as in Fig. 3.1. *B*, strand displacement synthesis by pol δ (100 fmol) and EcoRI cleavage was assayed on either the synthesis control substrate, m15, m15m1, m15m2, m15m3, m15m4, or m15m5 substrate in the presence of PCNA (25 fmol), RFC (25 fmol), LigI (25 fmol), and FEN1 (5 fmol).


A
15 nt
2 nt
15 nt
Pif1
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
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44 nt
Initial Primer
m15m2
m15
Ctr
m15
m15m2

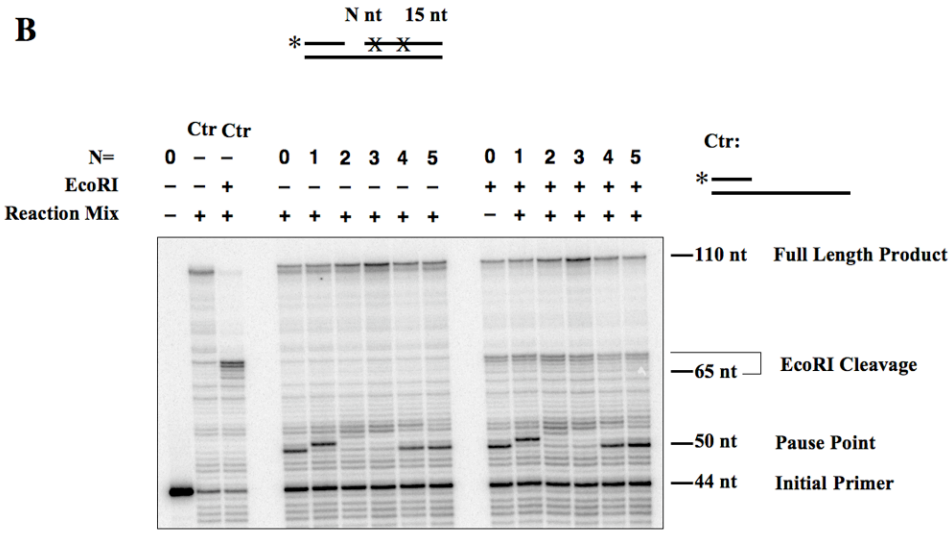
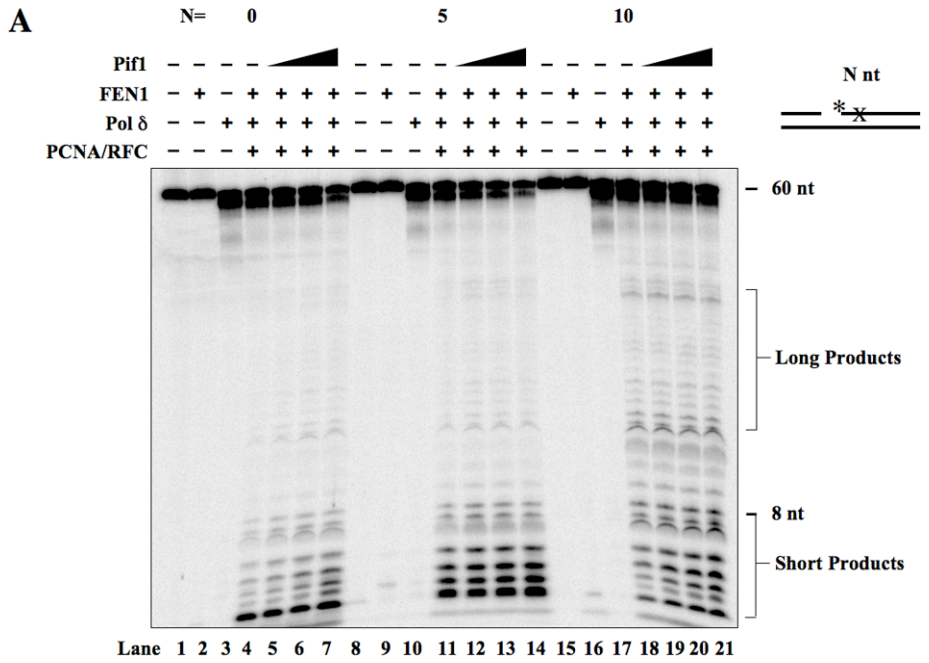
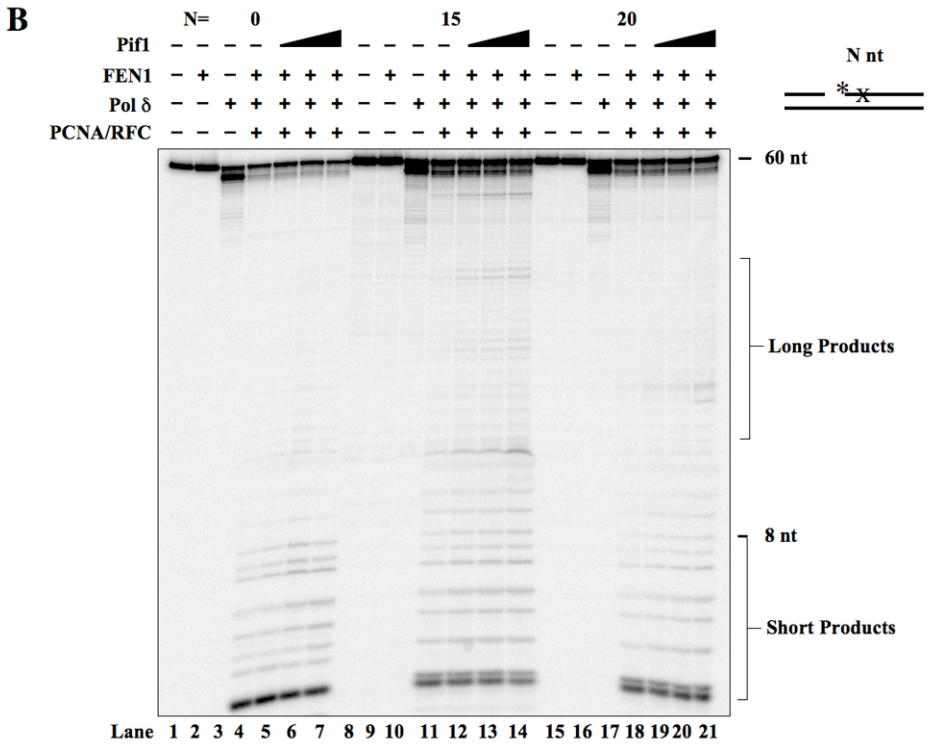

B
N nt
15 nt
Ctr Ctr
N=
0 1 2 3 4 5
0 1 2 3 4 5
EcoRI
Reaction Mix
Ctr:
110 nt
Full Length Product
65 nt
EcoRI Cleavage
50 nt
Pause Point
44 nt
Initial Primer

Figure 3.6. Mismatches in the downstream primer lead to farther synthesis.

A, cleavage by FEN1 (2 fmol) was assayed on the m0 substrate (*lanes 1-7*), the m5 substrate (*lanes 8-14*), or the m10 substrate (*lanes 15-21*) in the presence of various combinations of PCNA (25 fmol), RFC (25 fmol), and FEN1 (5 fmol), and in increasing amounts of Pif1 (50, 100, 150, or 200 fmol) as indicated in the figure and described under "Experimental Procedures." Substrate depictions and designations are as in Fig. 3.1. *B*, cleavage by FEN1 (2 fmol) was assayed on the standard m0 substrate (*lanes 1-7*), the m15 substrate (*lanes 8-14*), or the m20 substrate (*lanes 15-21*) in the presence of various combinations of PCNA (25 fmol), RFC (25 fmol), and FEN1 (5 fmol), and in increasing amounts of Pif1 (50, 100, 150, or 200 fmol).


A
N=
0
5
10
Pif1
FEN1
Pol δ
PCNA/RFC
N nt
60 nt
Long Products
8 nt
Short Products
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21


B
N=
0
15
20
Pif1
FEN1
Pol δ
PCNA/RFC
N nt
60 nt
Long Products
8 nt
Short Products
Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21

Conclusion

Accurate synthesis of both strands of DNA during DNA replication is of vital importance in all organisms as failure to do so can lead to mutations, disease, and death. Therefore, a thorough understanding of the mechanisms of DNA replication and repair is an important tool for combating diseases that arise from DNA damage. Due to its intricate nature, detailed study is required to fully elucidate the processes of lagging strand DNA replication. Of particular interest is understanding how the two pathways of flap processing (the long and short flap pathway) interact to ensure proper production of a high fidelity double-stranded DNA product.

The work presented here suggests that the short flap, or FEN1-only, pathway of Okazaki fragment processing is the predominant pathway through which flaps created by pol δ strand displacement synthesis are cleaved. Although the activities of the long flap processing proteins Dna2, Pif1, and RPA were previously well characterized, it was still unclear how they influenced the cleavage of short flaps. Results presented here demonstrate that each long flap processing protein is able to stimulate FEN1 cleavage of very short, short, and long flaps (Figs. 1.1, 1.3, 1.4). Additionally, although Pif1 and RPA cannot bind RNA, and Dna2 can bind but not cleave, each protein is still able to stimulate FEN1 cleavage of RNA flaps (Fig. 1.5). Each protein is also able to maximally stimulate FEN1, with no observable synergistic effect when utilizing different combinations of Dna2, Pif1, or RPA (Fig. 1.6). This suggests that these proteins do not stimulate FEN1 through unique mechanisms but rather being in complex with one or more of these proteins induces a

change in FEN1 that promotes cleavage. Co-immunoprecipitation of the long flap processing proteins reveals that each protein can directly interact with FEN1 in order to bring about the observed increase in FEN1 cleavage (Fig. 1.7). Further supporting the conclusion that the short flap pathway is the preferred method of flap processing, results from the reconstitution system *in vitro* reveal that a majority of the flaps that are created during strand displacement synthesis are cleaved while they are still short, ~6 nt or less (Fig. 3.1).

Even though cleavage of short flaps by FEN1 appears to be the preferred method for flap cleavage during Okazaki fragment processing, several alternative roles for long flap pathway members have been found. As previously mentioned, Dna2, Pif1, and RPA can each stimulate FEN1 activity on short flaps and RNA flaps. In this way, even when they themselves are unable to process a flap, members of the long flap pathway are still playing an important role in replication by ensuring quick, efficient FEN1 cleavage. Additional roles for these proteins include the ability of Pif1 to remove Okazaki fragment primers initiated by fold-back flaps that are resistant to cleavage by both Dna2 and FEN1 (Figs. 2.2, 2.3). Although the exact percent of replication products that can form fold-backs *in vivo* is not currently known, even if the occurrence of such flaps is low, it is still vital to ensure that these regions are properly replicated; the task of DNA replication in higher eukaryotic cells includes synthesis and joining of millions of Okazaki fragments, which must be accomplished at an efficiency level of 100% to avoid mutation. RPA also has an important role in replicating through regions that contain potential fold-back

sequences, as the presence of RPA aids pol δ in synthesizing through fold-backs in the template (Fig. 2.4). Finally, the presence of Pif1 aids in mismatch correction by pol δ by allowing synthesis farther into the downstream primer under conditions that favor long flap creation (Figs. 3.2, 3.3, 3.4). Through these mechanisms, members of the long flap pathway play an important role in maintaining both the efficiency and fidelity of lagging strand synthesis.

Although previously published genetic data as well as previous work with our reconstitution system clearly demonstrated that Pif1 promotes the creation of long flaps (72,90), a question remained: what is the benefit derived from having Pif1 create long flaps? If long flap creation were solely problematic for replication, the system would have evolved in such a way as to sequester Pif1 away from replicating DNA. In answer to this question, we have found that one positive role of flap lengthening by Pif1 is that it aids pol δ in the correction of mismatches found beyond 10 nt from the 5' end of the downstream primer (Fig. 3.2). Although we anticipated that Pif1 would encourage pol δ to displace farther into the downstream primer under normal reconstitution conditions, the effect of Pif1 on displacement is most noticeable when the reconstitution system is already biased towards long flap creation. It would seem, then, that the role of Pif1 is more important under conditions that already encourage long flap formation.

In vivo, long flap creation may be regulated by post-translational modification of the replication proteins, specifically through acetylation. It has been known for some time that acetylation of FEN1 inhibits its ability to cleave (105,106). More

recent work has shown that acetylation of Dna2 stimulates its helicase and nuclease activities (70). This suggests that acetylation of the flap processing proteins leads to an increase in utilization of the long flap pathway. This is an important finding because, in a broader sense, it implies that there is an intentional switching between the short and long flap pathways of Okazaki fragment processing. This is very different from previous views of the pathways in which it was thought that the two pathways compete with each other. Instead, it suggests that both pathways are necessary to the cells but each one is primarily utilized under different circumstances.

Such a view of the two pathways is consistent with our observations, as the process of lagging strand synthesis has clearly evolved to utilize two distinct pathways. If the long flap pathway were solely meant as a back up for those flaps that escape early FEN1 cleavage, it would not likely have developed the specific characteristics it now has. In essence, if one was to view the role of the long flap pathway as solely a back up mechanism, it raises a number of difficult questions. For example, why utilize a second nuclease? Clearly Dna2 is needed to remove RPA from long flaps in order to for the flaps to be cleaved. But if that were the only reason Dna2 is still utilized in flap processing, why would FEN1 not have evolved to remove RPA instead, eliminating the need for Dna2? The likely reason is because without two nucleases, there could not be regulation between a long and short flap pathway.

Therefore, further investigation into the mechanism of regulation between the two pathways would provide valuable information on how the replication system

has developed to balance efficiency and fidelity of replication. Even though FEN1 cleavage of short flaps is the preferred method of flap cleavage under the conditions we have investigated, and the long flap pathway seems to act mainly as a backup, in reality the long flap pathway could be of vital importance in mismatch correction or to fulfill some other valuable role in DNA replication. In order to investigate this possibility, it is important to determine what cellular conditions lead to increased utilization of the long flap pathway. Based on our findings *in vitro*, it would be particularly significant to determine whether acetylation of the replication proteins, which we believe is an important regulation mechanism, occurs in response to mismatches in the pol α primer.

Regardless of which protein is modified or the chemical nature of the modification, the EcoRI system is a valuable tool for investigating the effects of post-translational modification on the replication system. With the potential of post-translational modifications to alter very specifically the activities of the replication proteins, regulation by modification creates a plethora of routes for investigation. Utilizing the EcoRI system, we can determine how acetylation of pol δ affects its processivity and rate of synthesis. Similar experiments could be performed with PCNA, to determine whether post-translational modification improves its ability to load onto the template and bind pol δ, leading to a higher processivity of the complex. EMSAs would be a useful tool in determining whether a modified RFC is more efficient at loading PCNA onto the template. As pol δ synthesis halts after ligation of the two Okazaki fragments to each other, we could investigate how modification of

LigI affects ligation efficiency and how that relates to the distance of strand displacement synthesis.

Experiments utilizing acetylated Dna2, FEN1, RPA, and Pif1 would allow us to determine how post-translational modification of the flap processing proteins influences flap length, displacement into the downstream primer, mismatch correction, and rate of synthesis. It is already known that FEN1 activity is decreased and Dna2 activity is increased, likely leading to utilization of the long flap pathway. We would, therefore, anticipate an increase in RPA binding, in order to stabilize the increased population of long, single stranded flaps that are being formed. If regulation were used to create longer flaps, then we would expect to see an increase in Pif1 helicase activity as well. One might expect to see a decrease in the rate of synthesis if it required the arduous process of long flap creation. However, it is possible that the increased activity of some of the lagging strand synthesis proteins is able to compensate. The discovery of the importance of post-translational modifications on lagging strand synthesis proteins creates a number of unanswered questions, which in turn presents an abundance of opportunities for future investigation.

These future experiments will benefit greatly from the use of the EcoRI system. Although the original reconstitution system is useful for examining cleavage products or ligation products, flap length is not always an accurate indicator of synthesis, as multiple cleavages may occur or, in the presence of a helicase, flap lengthening could proceed ahead of synthesis. Similarly, ligation products are unable

to show the distance of synthesis, as the size of a ligation product will always be the same, regardless of how far into the downstream primer pol δ has synthesized. The EcoRI system, however, is able so show synthesis distance in ligated products accurate to the nucleotide. Placement of the EcoRI site in a specific region in conjunction with manipulation of the location of a mismatch allows for a highly informative determination of the length of pol δ strand displacement synthesis. Moreover, use of the EcoRI system is not limited solely to observing pol δ synthesis. It can also be a potent tool to investigate the efficacy and interactions of other repair proteins *in vitro*.

Utilizing the EcoRI system, we have demonstrated an intrinsic ability of pol δ and FEN1 to act together in order to repair mismatches found in the pol α primer (Figs. 3.5, 3.6). Our results indicate that the cooperative action of these two proteins readily repairs mismatches within the first 10 nt of the downstream primer. Cleavage assays also reveal that the presence of a mismatch farther into the downstream primer results in longer flap formation (Fig. 3.6). The presence of Pif1 causes a moderate increase in pol δ repair of these mismatches. Several questions still remain: what is the mechanism that allows farther displacement by pol δ in the presence of a mismatch? One possibility is that the presence of the mismatch destabilizes the double-stranded DNA, relieving some of the energetic barrier faced by pol δ as it undergoes strand displacement synthesis. In a similar vein, the presence of a mismatch could potentially relieve torsional stress associated with the process of unwinding a helical structure. Additionally, there is the question of why pol δ

struggles to displace farther than 10 nt into the downstream primer. Although the presence of a mismatch 15 nt into the downstream primer leads to a greater distance of displacement by pol δ, there is still a significant amount of products in which a mismatch 15 nt into the downstream primer is not corrected. We observed that Pif1 helps pol δ to reach these lengths under conditions that encourage long flap formation (Fig. 3.2C, D). Does this mean that the system has evolved such that pol δ will only synthesize these longer lengths if specifically regulated to do so, i.e. via the utilization of post-translational modification to promote the long flap pathway? Once again, studies *in vivo* of how the replication proteins are modified in the response to mismatches would be integral in answering these questions.

In summary, these findings demonstrate new, important roles for protein components of both the long and short flap pathways. Although data indicate quick efficient, cleavage by FEN1 is the predominant method for flap processing, the long flap proteins work in a number of ways to support this pathway and to handle the problems that arise when flaps escape early FEN1 cleavage. In this way, the system appears to have evolved in such a way to balance replication efficiency with fidelity. Future studies *in vivo* are likely to provide further insight into how these two pathways are regulated to ensure even tighter coordination of efficiency and fidelity.

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