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The Protein Components and Mechanism of Eukaryotic Okazaki Fragment Maturation

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ABSTRACT: An initiator RNA (iRNA) is required to prime cellular DNA synthesis. The structure of double-stranded DNA allows the synthesis of one strand to be continuous but the other must be generated discontinuously. Frequent priming of the discontinuous strand results in the formation of many small segments, designated Okazaki fragments. These short pieces need to be processed and joined to form an intact DNA strand. Our knowledge of the mechanism of iRNA removal is still evolving. Early reconstituted systems suggesting that the removal of iRNA requires sequential action of RNase H and flap endonuclease 1 (FEN1) led to the RNase H/FEN1 model. However, genetic analyses implied that Dna2p, an essential helicase/nuclease, is required. Subsequent biochemical studies suggested sequential action of RPA, Dna2p, and FEN1 for iRNA removal, leading to the second model, the Dna2p/RPA/FEN1 model. Studies of strand-displacement synthesis by polymerase δ indicated that in a reconstituted system, FEN1 could act as soon as short flaps are created, giving rise to a third model, the FEN1-only model. Each of the three pathways is supported by different genetic and biochemical results. Properties of the major protein components in this process will be discussed, and the validity of each model as a true representation of Okazaki fragment processing will be critically evaluated in this review.

KEYWORDS: DNA replication, FEN1, Dna2, RNase H, initiator RNA and DNA, Okazaki fragment processing

ABBREVIATIONS: FEN1, flap endonuclease 1; **PCNA**, proliferating cell nuclear antigen; **RPA**, replication protein A; **RFC**, replication factor C; **RTH1**, Rad Two Homologue; **pol α**, DNA polymerase α/primase complex; **pol δ**, DNA polymerase δ; **iRNA**, initiator RNA; **iDNA**, initiator DNA; **SV40**, simian virus 40; **DSBR**, double strand break repair; **NHEJ**, nonhomologous end joining; **BLM**, Bloom's syndrome protein; **WRN**, Werner's syndrome protein; **SGA**, systematic genetic analysis; **Sir**, silence information regulator; **AF2**, accessory factor 2; **cca/exo**, circle closing activity; **MF1**, maturation factor 1; **sgs**, slow growth suppressor; **Tag**, Tantigen.

INTRODUCTION

The unique antiparallel structure of DNA and the 5′-3′ polymerization activity of DNA polymerases left a puzzling mystery for biologists who were studying the mechanism of DNA replication. Since the replication fork moves in one direction, one daughter strand can be synthesized continuously, designated the leading strand, while the other (the lagging strand) must be generated discontinuously (Kornberg and Baker, 1992). A major breakthrough came in 1966 when Okazaki and his colleagues observed that newly synthesized DNA

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appeared as short pieces that were subsequently joined into continuous chains by pulse-chase experiments in prokaryotic cells (Okazaki *et al.*, 1968; Sakabe and Okazaki, 1966). This provided the first evidence for discontinuous DNA synthesis, and the short pieces of DNA have been designated Okazaki fragments in honor of their discoverer. Even though the cellular organizations of prokaryotic and eukaryotic cells are very different, the synthesis and joining of Okazaki fragments were found to be properties of all cells (Brewer, 1975; Brewer *et al.*, 1974; Flory, 1977; Friedman, 1974; Hershey *et al.*, 1973; Hershey and Taylor, 1974; Hunter *et al.*, 1977; Kaufmann *et al.*, 1978; Lynch *et al.*, 1972; Okazaki *et al.*, 1968; Perlman and Huberman, 1977; Sakabe and Okazaki, 1966).

Many protein components of the mammalian replication fork were identified through analysis of Simian virus 40 (SV40) replication *in vitro* (Bambara *et al.*, 1997; Murakami and Hurwitz, 1993; Waga *et al.*, 1994; Waga and Stillman, 1998). Although coordinated by a single viral protein, large T antigen (Tag), replication of SV40 otherwise relies on cellular components. Reconstitution reactions *in vitro* were used to understand individual reactions, revealing much of our current knowledge of the elongation steps in DNA replication (Eki *et al.*, 1992; Ishimi *et al.*, 1988; Stillman, 1989; Tsurimoto and Stillman, 1991a, 1991b; Waga *et al.*, 1994; Waga and Stillman, 1994; Weinberg *et al.*, 1990). Stillman and coworkers presented a model of SV40 DNA replication in which Tag, the eukaryotic single-stranded DNA-binding protein called replication protein A (RPA), and DNA topoisomerases unwind the replication origin (Tsurimoto *et al.*, 1990). DNA polymerase α/primase complex (pol α) is recruited to this structure and initiates synthesis on all strands by incorporation of an initiator RNA (iRNA) followed by a short stretch of DNA (iDNA) (Arezi and Kuchta, 2000; Bullock *et al.*, 1991; Murakami *et al.*, 1992). The ATP-dependent replication factor C (RFC) displaces the pol α by competing for RPA binding sites, triggering the loading and binding of the elongation complex, proliferating cell nuclear antigen (PCNA) and DNA polymerase δ (pol δ) (Tsurimoto *et al.*, 1990; Tsurimoto and Stillman, 1991b; Yuzhakov *et al.*, 1999). PCNA is a toroidal homotrimer that acts as a sliding clamp to increase the processivity of pol δ (Burgers, 1991; Krishna *et al.*, 1994; Lee and Hurwitz, 1990). As a result, a highly processive polymerization complex is assembled that performs leading-strand synthesis. The "polymerase switching" step just described constitutes the transition from the priming mode to the extension mode of DNA synthesis (Maga *et al.*, 2000; Matsumoto *et al.*, 1990; Mossi *et al.*, 2000; Tsurimoto *et al.*, 1990; Tsurimoto and Stillman, 1991b). Pol α synthesizes frequent primers to make segments of nascent lagging-strand DNA (Nethanel *et al.*, 1992). Surprisingly, the complete polymerase switching process occurs on each Okazaki fragment even though they are very short (Waga and Stillman, 1994).

The replication fork machinery must carry out the synthesis and joining of Okazaki fragments. Each fragment is initiated by 8–12 nucleotides of RNA primer followed by approximately 100–150 nucleotides of DNA in SV40 and higher eukaryotic systems, including *Drosophila* (Anderson *et al.*, 1977; Arezi and Kuchta, 2000; Blumenthal and Clark, 1977; Burhans *et al.*, 1990; Murakami *et al.*, 1992; Nethanel *et al.*, 1988, 1992). The elongation rate of DNA synthesis has been estimated to be 40–60 nucleotides per second, which is consistent with the rate of average fork migration, 50 nucleotides per second (Ayyagari *et al.*, 2003; Raghuraman *et al.*, 2001). Under standard conditions, the estimated Okazaki fragment maturation rate (excluding the time for synthesis) for a 1.14 kb circular DNA is calculated to be 22–25 seconds. The maturation process of these fragments is a multienzymatic reaction even though the intermediates do not accumulate during synthesis *in vitro* (Ayyagari *et al.*, 2003). The initiator RNA and DNA (iRNA/DNA) generated by pol α/primase complex has to be removed to ensure the integrity of the genome (Bambara *et al.*, 1997; Hubscher *et al.*, 2002; Kool, 2002; Waga and Stillman, 1998). This 5′-end iDNA segment is about 20 nucleotides long (Arezi and Kuchta, 2000).

Since pol α lacks 3′-5′ exonuclease activity, it is more error prone than pol δ and pol ε. Thus, it has been proposed that processing of the 5′-termini of the nascent fragments must remove not only the RNA but also a small amount DNA in order to preserve fidelity of replication. The 3′-5′ exonuclease activity of p53 has been proposed to interact with pol α and increase its fidelity, although it is not clear whether this improvement is sufficient (Huang, 1998; Shevelev and Hubscher, 2002). Removal of this iRNA/DNA is thought to involve a 5′-displaced strand or flap intermediate and nucleases. A 5′-flap structure could form from strand-displacement synthesis by pol δ. This displaced flap, which contains the iRNA/DNA, will be cleaved in order to generate a nick for DNA ligase to complete the intact DNA strand (Bambara *et al.*, 1997; Waga and Stillman, 1998). The nuclease that is proposed to process this flap intermediate is called the flap endonuclease 1 (FEN1) (Bambara *et al.*, 1997; Lieber, 1997).

FEN1 was first isolated as a 5′-3′ exonuclease, designated the DNase IV, from rabbit bone marrow and lung (Lindahl, 1971; Lindahl *et al.*, 1969). Subsequently, analogous 5′-exonucleases were discovered in various systems, such as AF2 (accessory factor 2) from mouse lymphoblasts, factor pL from HeLa cells, cca/exo (circle closing activity) from mouse, MF1 (maturation factor 1) from human cells used in the SV40 system, and RTH1 (Rad2 homologue nuclease) from both *S. cerevisiae* and calf where RTH1 in *S. cerevisiae* was later recognized as the product of the *RAD27* gene (Goulian *et al.*, 1987, 1990; Ishimi *et al.*, 1988; Kenny *et al.*, 1988; Lindahl, 1970; Lindahl *et al.*, 1969; Murante *et al.*, 1996; Reagan *et al.*, 1995; Sommers *et al.*, 1995; Waga *et al.*, 1994). All of these nucleases have a molecular weight around 43 kDa and share similar enzymatic activities with the 5′-exonuclease domain of prokaryotic DNA polymerase I, which has been proposed to work with the polymerase domain for iRNA removal (Lyamichev *et al.*, 1993). FEN1 nucleases recognize nicked or gapped double-stranded DNA as a substrate and release mononucleotides from the 5′-terminus (Goulian *et al.*, 1987, 1990; Ishimi *et al.*, 1988; Lindahl, 1970; Lindahl *et al.*, 1969). These nucleases were suggested to be homologues based on their functions or sequences (Harrington and Lieber, 1994b; Ishimi *et al.*, 1988; Lieber, 1997).

The current view is that FEN1 is an evolutionarily conserved enzyme that performs a significant role in DNA replication (Harrington and Lieber, 1994a, 1994b; Kimura *et al.*, 2000; Qiu *et al.*, 2001; Shen *et al.*, 1997; Sommers *et al.*, 1995). In fully reconstituted systems, such as SV40 and mouse cells, FEN1 was required for the formation of intact DNA lagging strands. In the presence of polymerase, topoisomerase, RNase H, and DNA ligase, synthesis *in vitro* stops with many Okazaki fragments annealed to the parental DNA on the lagging strand. When FEN1 was included in the reactions, these segments were processed and joined. This indicates the need for FEN1 in the steps of lagging-strand synthesis where iRNA/DNA has to be removed prior to ligation (Goulian *et al.*, 1987, 1990; Ishimi *et al.*, 1988; Waga *et al.*, 1994).

FEN1 was later shown to be a member of a structure-specific endonuclease family with cleavage specificity for the base of a 5′ single-stranded flap (Harrington and Lieber, 1994a, 1995; Murante *et al.*, 1995). The unusual cleavage mechanism of FEN1 appears to involve a tracking process. FEN1 enters flap substrates at the 5′-end and moves to the base of the flap for cleavage (Murante *et al.*, 1995; Tom *et al.*, 2000). Structural studies of the protein identified a helical arch or loop domain, suggesting that tracking involves threading of the flap through the arch (Ceska *et al.*, 1996; Hosfield *et al.*, 1998b; Hwang *et al.*, 1998; Kim *et al.*, 1995). Later evaluation of the tracking mechanism showed that the flap does not thread through the protein (Barnes *et al.*, 1996; Bornarth *et al.*, 1999; Murante *et al.*, 1995), but the loop domain might still control movement of FEN1 on the flap. *In vitro* studies suggest that FEN1 is capable of removing iRNA/DNA by either its exonuclease or endonuclease activities (Huang *et al.*, 1994; Murante *et al.*, 1996; Rumbaugh *et al.*, 1997). However, the exonuclease is a much weaker activity and may depend on transient flap formation (Harrington and Lieber, 1994a; Lieber, 1997).

The flap created by displacement can equilibrate by branch migration to a variety of intermediates, of which only one is a highly preferred FEN1 substrate. It was recently found that *S. cerevisiae* FEN1 preferentially cleaves a double-flap structure that contains a one-nucleotide 3′-tail and a polynucleotide 5′-flap (Kao *et al.*, 2002). Similar specificity for double-flap substrates is also reported in the 5′-exonuclease domain of DNA polymerase I in prokarya, FEN1 homologues in Archaea, and FEN1 homologue in humans (Friedrich-Heineken *et al.*, 2003; Kaiser *et al.*, 1999; Xu *et al.*, 2000). Creation and analysis of FEN1 mutations in *S. cerevisiae* that could only utilize the double-flap substrate showed that it is an important replication intermediate (Kao *et al.*, 2002; Xie *et al.*, 2001).

Proliferating cell nuclear antigen (PCNA) has been demonstrated to interact with FEN1 and stimulates its activity 5- to 50-fold (Chen *et al.*, 1996; Li *et al.*, 1995; Tom *et al.*, 2000; Wu *et al.*, 1996). The product of FEN1 cleavage is a nicked substrate that will allow the subsequent ligation step by DNA ligase I and PCNA. PCNA also stimulates DNA ligase I about 5-fold after the two proteins form a functional complex (Tom *et al.*, 2001). These properties suggest that PCNA acts as the coordination platform to allow primer removal in a step-wise manner during the Okazaki fragment maturation pathway, and FEN1 is proposed to be a central component of this pathway in all organisms (Bambara *et al.*, 1997; Tom *et al.*, 2000, 2001; Waga and Stillman, 1998).

FEN1 was first cloned and characterized from mouse (Harrington and Lieber, 1994a). The homologue of the FEN1 gene in *S. cerevisiae* is called *RAD27* (also known as the *Rad Two Homologue* or *RTH1* and the open reading frame YKL510) (Johnson *et al.*, 1998; Reagan *et al.*,

1995; Sommers *et al.*, 1995), and the similar gene in *Schizosaccharomyces pombe* is *RAD13* (Lieber, 1997; Murray *et al.*, 1994). *RAD27* was originally identified by a genomic sequencing project in *S. cerevisiae* (Jacquier *et al.*, 1992). The *rad27*Δ deletion mutation causes the organism to be sensitive to UV radiation, and hence it utilizes the nomenclature of the *RAD* genes (Reagan *et al.*, 1995). *RAD27* is a homologue of *RAD2* in yeast, and along with *YEN1*, *EXO1*, and *DIN7*, it is a member of the Rad2 nuclease family (Johnson *et al.*, 1998; Sommers *et al.*, 1995). Studies with both Rad2p and Rad27p implied that both enzymes utilized branched DNA as their substrates (Harrington and Lieber, 1994b). However, genetic data indicated that *RAD2* did not complement *rad27*Δ in *S. cerevisiae*, and it is a much larger protein than FEN1 (Johnson *et al.*, 1998; Sommers *et al.*, 1995). Thus, *RAD27* encodes the true analogue to mammalian FEN1 (Harrington and Lieber, 1994b).

Deletion of the *RAD27* gene in *S. cerevisiae* resulted in slow growth at 30°C and no growth at 37°C (Sommers *et al.*, 1995). The terminal phenotype is a large-budded cell with the nucleus at the narrow point, a phenotype shared by many DNA replication mutants, such as mutants of pol δ, pol ε, and DNA ligase (Barnes *et al.*, 1992; Culotti and Hartwell, 1971; Morrison *et al.*, 1990; Sitney *et al.*, 1989). The double deletion mutant *rad27*Δ/*rad52*Δ caused an additional decrease in the rate of growth at the permissive temperature, suggesting that the recombinational repair pathway partially compensates for the *rad27*Δ mutation (Sommers *et al.*, 1995). Furthermore, the rate of spontaneous mitotic intrachromosomal recombination was increased over 15-fold by the *rad27*Δ mutation. The *rad27*Δ strain also exhibited a 150-fold increase in canavanine resistance frequency (Xie *et al.*, 2001). Mutations in *E. coli* in the 5′-3′ exonuclease domain of DNA polymerase I and in DNA ligase result in hyperrecombination. This is the result of the persistence of nicks in the chromosome caused by a delay in the joining of Okazaki fragments (Konrad, 1977; Konrad and Lehman, 1974). The *rad2*Δ mutation had no effect on the phenotype of *rad27*Δ, indicating that the Rad2 nuclease does not serve as the backup for the FEN1 nuclease at permissive temperature (Johnson *et al.*, 1998; Sommers *et al.*, 1995). However, over-expression of the *EXO1* gene, which expresses exonuclease I, suppressed some but not all of the phenotypic defects of *rad27*Δ in yeasts, indicative the existence of back-up pathways (Johnson *et al.*, 1998; Parenteau and Wellinger, 1999; Tishkoff *et al.*, 1997).

Genetic data have also implied that FEN1 participates in many DNA metabolic pathways in the cell besides DNA replication. The mutant strains are highly sensitive to methyl methanesulfonate, suggesting defective base excision repair (Reagan *et al.*, 1995; Vallen and Cross, 1995). The null mutant also displays an 80-fold increase in dinucleotide repeat tract instability, similar to that of mismatch repair mutations (Johnson *et al.*, 1995; Sommers *et al.*, 1995), and it has been shown to exhibit synthetic lethality with many other mutations in various DNA reactions. Moreover, Systematic Genetic Analysis (SGA) suggested that *sgs1* and *rad27* are also synthetically lethal (Tong *et al.*, 2001). Sgs1p (slow growth suppressor) is the yeast homologue for Bloom's syndrome protein (BLM) and Werner's syndrome protein (WRN) in humans that belongs to the RecQ helicase family and possesses a 3′-5′ helicase activity (Gangloff *et al.*, 1994; Watt *et al.*, 1995). In addition, WRN has been reported to interact with FEN1 and stimulate its activity (Brosh *et al.*, 2001). Overall, these observations support biochemical models of the role of flaps and FEN1 in replication, repair, recombination, and sequence instability.

THE RNASE H AND FEN1 MODEL

Besides cleaving DNA flaps, FEN1 can also use its endonucleolytic activity to cleave within the RNA (Murante *et al.*, 1996). This would suggest that FEN1 is the only nuclease necessary for processing iRNA/DNA. However, early in the analysis of iRNA removal, evidence began to gather that RNase H plays an important role. RNase H enzymes were first characterized in prokaryotes (*E. coli*), and based on the order of discovery, they were designated RNase HI and RNase HII (Busen and Hausen, 1975). Studies with these two enzymes and sequence alignment suggested that RNase HII is the predominant activity in prokaryotes (Ohtani *et al.*, 1999). When RNase H activities were reported in eukaryotes (*S. cerevisiae*), sequence comparison demonstrated similarity between RNase HI in *E. coli* and a new RNase H in eukaryotes. This RNase H in eukaryotes was named RNase H1 (Itaya *et al.*, 1991). Subsequent analysis of the properties of RNase H1 indicated that this enzyme most resembles RNase HII in prokaryotes. This caused confusion in the nomenclature of all RNase H enzymes. A different nomenclature was proposed for clarification (Crouch *et al.*, 2001). In this document, we simply use RNase H to designate the predominant activity in eukaryotes.

Eukaryotes contain as many as four classes of RNase H (Frank *et al.*, 1998). The predominant RNase H in mammalian cells has a native molecular weight of ~70 kDa and degrades RNA/DNA hybrids (Eder *et al.*, 1993; Frank *et al.*, 1998; Rong and Carl, 1990). RNase H has been implicated in DNA replication because it is induced in dividing cells, and its activity correlates with DNA synthesis (Busen *et al.*, 1977). Early *in vitro* systems revealed RNase H as one of the components in lagging-strand synthesis (Goulian *et al.*, 1987, 1990; Ishimi *et al.*, 1988). The biochemical characteristics of RNase H specifically suggested a role in Okazaki fragment processing. The eukaryotic RNase H has the capacity to recognize and cleave preferentially at one nucleotide upstream of the RNA–DNA junction of an RNA primer and downstream DNA (Huang *et al.*, 1994; Murante *et al.*, 1998; Qiu *et al.*, 1999; Turchi *et al.*, 1994).

Reconstitution of iRNA removal using calf proteins *in vitro* suggested that the iRNA primer of an Okazaki fragment is removed by the sequential action of RNase H and FEN1 (Turchi *et al.*, 1994). This led to the proposal of the RNase H/FEN1 pathway. RNase H endonucleolytically cleaves the initiator RNA one nucleotide upstream of the RNA–DNA junction, while the upstream primer is being synthesized by the polymerase δ holoenzyme, leaving a single ribonucleotide at the 5′-end on the downstream primer. This remaining ribonucleotide is processed by the exonucleolytic activity of FEN1. The cleavage by FEN1 occurs prior to the completion of synthesis, and steps of synthesis and ligation are required to form an intact DNA strand.

Even though this exonuclease model leads to the desired product, it is not consistent with the preference of FEN1 for cleavage of 5′-flap structures (Harrington and Lieber, 1994a; Murante *et al.*, 1995). Exonucleolytic cleavage by FEN1 is a weak activity, and it is only observed *in vitro* when the nuclease is present in large excess (Murante *et al.*, 1995; Xie *et al.*, 2001). *In vivo*, there are many processing sites that need efficient cleavage. Thus, the endonucleolytic activity of FEN1 is likely to be the predominant reaction. Also, in addition to iRNA, the iDNA synthesized by the more error-prone pol α complex needs to be removed to best maintain genome integrity (Arezi and Kuchta, 2000; Kool, 2002). These considerations all support a model in which the endonuclease is the biologically relevant function of FEN1. In the current view of a pathway involving both RNase H and FEN1, RNase H could act on the RNA–DNA hybrid before displacement synthesis from the upstream primer. Polymerization might then strand-displace the last ribonucleotide and the short stretch of deoxyribonucleotides that were generated by pol α/primase complex into a flap intermediate. FEN1 and PCNA would then remove this flap by endonucleolytic cleavage to allow the subsequent ligation step by DNA ligase I and PCNA (Figure 1).

Why would two nucleases be needed? One possibility is that the displaced RNA of some Okazaki fragments folds back into a structure that effectively blocks the tracking or cleavage reaction of FEN1. Biochemical analyses showed that as foldbacks become larger, they become progressively more inhibitory to cleavage by FEN1 (Henricksen *et al.*, 2000). Interestingly, long foldbacks retained their capacity for inhibition even when they were at the end of a long flap, with a twenty or more nucleotide segment of single-stranded DNA separating the foldback and the cleavage site. This is additional evidence for a tracking step in the FEN1 reaction. An RNA/DNA hybrid foldback could be even more stable than an equivalent-length DNA foldback (Henricksen and Bambara, 1998). This implies that another nuclease may be needed to allow effective removal of iRNA/DNA in conjunction with FEN1, and this nuclease could be RNase H.

Another variation on the model is that RNase H acts in concert with the synthesis of the iRNA. Previous reports indicate that eukaryotic RNase H stimulates the activity of pol α for synthesizing DNA on single-stranded templates. *Drosophila* RNase H stimulates both the rate of DNA synthesis from DNA primers and also the DNA synthesis resulting from priming *de novo* by the primase activity (DiFrancesco and Lehman, 1985). Results with *Drosophila* and yeast RNase H revealed that the presence of RNase H shortened the extended primers (DiFrancesco and Lehman, 1985; Karwan *et al.*, 1983). More and shorter extended primers would imply that the nuclease promotes dissociation of the polymerase from an extending DNA terminus so that it can recycle to a position on the template to initiate a new primer. A calf RNase H had a similar effect on the homologous pol α (Hagemeier and Grosse, 1989). In addition, RNase H is one of the abundant proteins in the cell, and it copurifies with many replication proteins (Siegal *et al.*, 1992; Turchi *et al.*, 1994; Waga *et al.*, 1994). Together, these results suggest that RNase H acts early in the synthesis of Okazaki fragments rather than in concert with FEN1, which acts just before ligation in the maturation pathway.

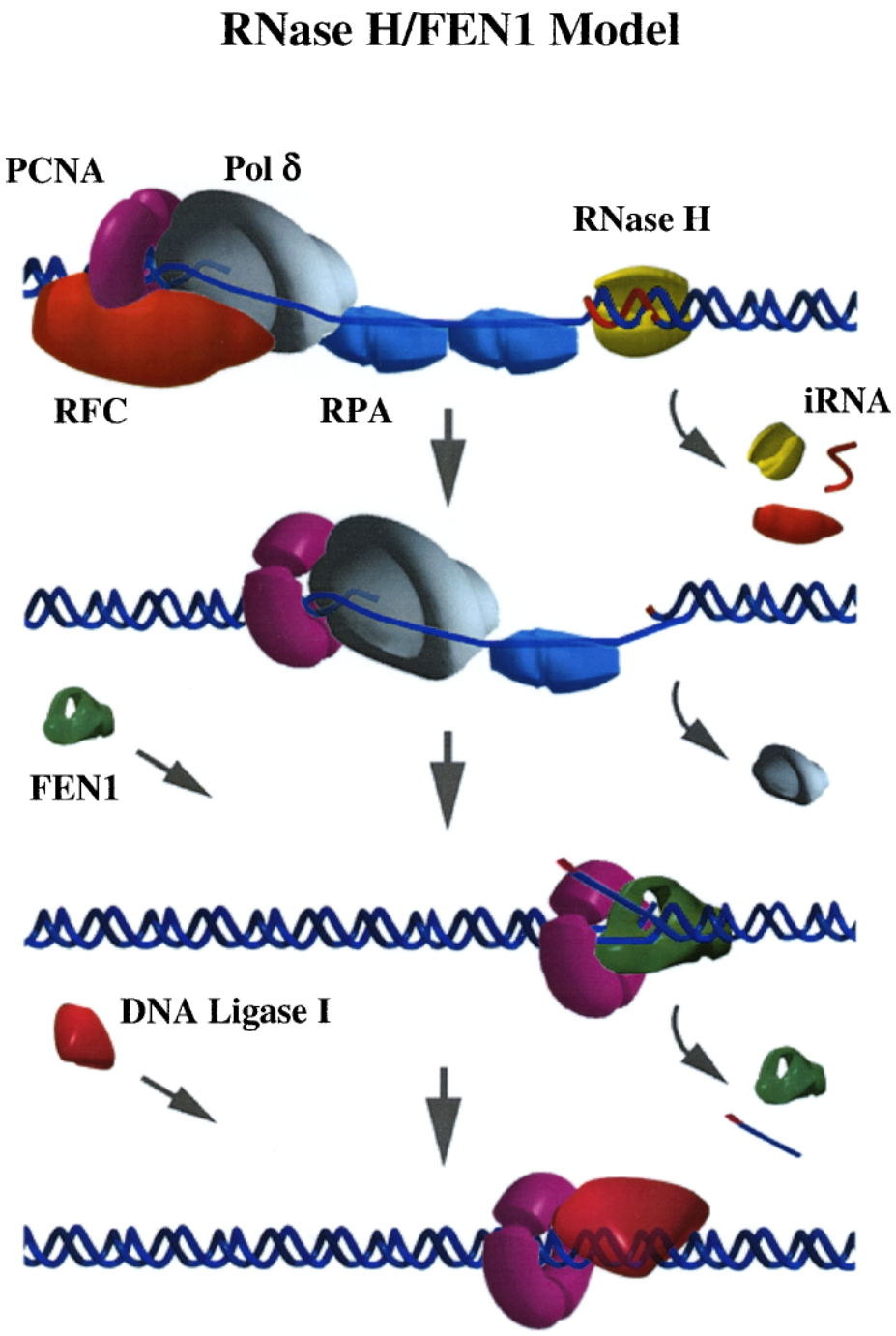

FIGURE 1. The RNase H/FEN1 model. RNase H acts early before the completion of the upstream segment synthesis. Cleavage by RNase H occurs at one nucleotide 5′ to the RNA–DNA junction. The elongation complex is composed of PCNA and pol δ, which carries out processive DNA synthesis and the strand-displacement reaction. The displaced flap containing the remaining ribonucleotide from the RNase H cleavage is processed by FEN1 and PCNA. The result of this cleavage is a nicked product that is sealed by the subsequent action of PCNA and DNA ligase I. (Figure appears in color online at www.crbmb.com)

The *S. cerevisiae* RNase H gene encodes a 35 kDa polypeptide accounting for 75% of cellular RNase H activity. While conservation of this nuclease throughout species attests to its importance, the null mutant does not have serious deficiencies (Frank *et al.*, 1998), suggesting the presence of a redundant or parallel pathway for RNase H activity in the cell. Such pathways could involve FEN1 or Exo1 (Qiu *et al.*, 1999; Tishkoff *et al.*, 1997).

Yeast genetic analyses also implied that both RNase H (RNH35p) and Rad27p cooperate in removing RNA primers during Okazaki fragment processing (Qiu *et al.*, 1999). While single deletion of either *RAD27* or *RNH35* is not lethal, the double-deletion mutation further impaired the growth defects of the *rad27*Δ mutant. Over-expression of RNase H in yeast partially rescued the severe growth defect of the double mutant (Qiu *et al.*, 1999). This would suggest a minor pathway that involves only RNase H, but not FEN1. In fact, biochemical analysis of yeast RNase H showed that large excess of the enzyme could allow the cleavage of the last ribonucleotide in the primer, but this reaction is very slow (Qiu *et al.*, 1999).

This is the evidence for the cooperation of the two nucleases in iRNA removal. While a model involving RNase H is satisfying in many ways, the fact that RNase H activity is not essential suggests that the primary means for initiator RNA removal does not use RNase H (Frank *et al.*, 1998). One of the alternatives is the recently proposed pathway involving Dna2p (Bae *et al.*, 2001).

THE DNA2 AND FEN1 MODEL

The *S. cerevisiae DNA2* gene was identified in a genetic screen for replication defects (Kuo *et al.*, 1983). *DNA2* was found to be an essential gene that encodes a 172 kDa protein (Budd *et al.*, 2000; Lee *et al.*, 2000). Cloning of the gene revealed 6 conserved helicase motifs, and it is now known that the enzyme is multifunctional with helicase, single-stranded DNA-dependent ATPase, and endonuclease activities. The helicase domain is located toward the C-terminus, while the nuclease domain is close to the N-terminus, and it belongs to the RecB class of helicase/nucleases with a RecB homology located in the middle of the protein (Bae *et al.*, 1998; Budd *et al.*, 2000). Several functional homologues of Dna2p have been identified in different species. These include *S. cerevisiae*, *S. pombe*, *Arabidopsis thaliana*, *Caenorhabditis elegans*, *Xenopus laevis* (Budd *et al.*, 2000; Gould *et al.*, 1998; Kang *et al.*, 2000; Lee *et al.*, 2000, 2003; Liu *et al.*, 2000). Recently, Dna2p homologues were reported and cloned in the hyperthermophilic bacterium (Archaea), *Pyrococcus horikoshii*, and in human (Eki *et al.*, 1996; Higashibata *et al.*, 2003; Imamura and Campbell, 2003). The human *DNA2* gene complemented the replication defect of yeast *dna2-1* mutants (Imamura and Campbell, 2003). This implies that the important role of Dna2p is conserved in both Archaea and Eukarya. Many genetic and physiological studies have been done with *DNA2*, but there is limited biochemical knowledge about its enzymatic activities and its role in DNA metabolism.

The temperature sensitive (ts) allele of *DNA2*, *dna2-1*, arrests in S phase (Budd and Campbell, 1995) and extracts from the mutant cells are deficient in replication of supercoiled plasmids *in vitro* (Braguglia *et al.*, 1998). In addition, a homozygous *dna2* deletion mutant in *C. elegans* exhibited many replication-related phenotypes, such as a reduced brood size, embryonic lethality, cell division delay, and temperature-dependent growth (Lee *et al.*, 2003). Further studies of *S. cerevisiae dna2* mutants implied a role for Dna2p in the late S phase during cell cycle progression, consistent with a role in DNA replication (Choe *et al.*, 2002; Fiorentino and Crabtree, 1997). An association of Dna2p with Okazaki fragment processing was first implied by Campbell and colleagues, with results showing that Dna2p interacts with the Rad27p (FEN1) both physically and genetically in *S. cerevisiae* (Budd and Campbell, 1997). Dna2p coimmunoprecipitates with Rad27p when both proteins are overproduced, indicative of a protein-protein interaction. Overexpression of Rad27p relieved the temperature sensitivity of the *dna2-1* mutant and suppressed the lethality of a *dna2*Δ mutant. Conversely, the overproduction of Dna2p also suppressed the temperature sensitivity of *rad27*Δ null cells. In addition, *rad27*Δ/*dna2-1* double mutants were synthetically lethal, suggesting that Rad27p and Dna2p share a functional interaction, and they may participate in the same pathway or at a similar stage of the replication process (Budd and Campbell, 1997; Liu *et al.*, 2000).

Point mutations in the nuclease domain are lethal, while mutations in the helicase domain are lethal on rich carbon sources but can grow on media that support slow growth rates (Budd *et al.*, 2000; Formosa and Nittis, 1999; Lee *et al.*, 2000). This argues for the essential role of this enzyme in DNA metabolic pathways. Biochemical characterization has suggested that yeast Dna2p is a weak 5′-3′ helicase and a single-stranded DNA-specific endonuclease, which much prefers a free end (Bae *et al.*, 1998; Bae and Seo, 2000), even though a

circular DNA has been shown to be cleaved by Dna2p (Budd *et al.*, 2000). Dna2p could cleave the displaced 5′-flap made by pol δ indicating an overlap with FEN1 in its substrate specificity. However, replication protein A (RPA) was found to stimulate Dna2p while inhibiting the activity of FEN1 on a model substrate containing a 30-nucleotide 5′ RNA-DNA flap (Bae *et al.*, 2001). In a reconstituted reaction, presence of a 30-nucleotide flap and RPA produces a requirement for Dna2p in the maturation pathway. This is RPA-specific since SSB and Dna2p are unable to support the same reaction (Ayyagari *et al.*, 2003; Bae *et al.*, 2001). Additionally, the Dna2p nuclease activity was stimulated by the presence of RNA, although cleavage could occur only within the DNA region (Bae and Seo, 2000). Therefore, Dna2p could, in principle, eliminate the RNA primer synthesized by pol α in a single cleavage event. This has led to the proposal that FEN1 and Dna2p function sequentially in the presence of RPA and long flaps during Okazaki fragment processing (Bae *et al.*, 2001) (Figure 2).

After RFC triggers pol α/pol δ switching, it disengages from the replication machinery and the complex of pol δ and PCNA carries out processive synthesis (Podust *et al.*, 1998; Yuzhakov *et al.*, 1999). The PCNA-pol δ complex, upon encountering a downstream Okazaki fragment, performs strand-displacement synthesis generating a 5′-flap structure. RPA has been proposed to coat the flap and limit the displacement to about 30 nucleotides (Maga *et al.*, 2001). The resultant flap includes initiator RNA and some adjacent DNA, but the displacement does not continue far enough to dissociate the entire segment. Coating by RPA initially occurs at an 8-nucleotide length of single-stranded DNA (8-nucleotide binding mode), and then the binding region gradually stretches to a length of 30 nucleotides (30-nucleotide mode) (Iftode *et al.*, 1999; Wold, 1997). This binding mode of RPA is consistent with its proposed role in limiting the strand-displacement synthesis to a 30-nucleotide flap (Maga *et al.*, 2001). As soon as the RNA flap reaches 8 nucleotides in length, RPA binds to the flap. When the flap reaches 30 nucleotides, RPA converts to its stable binding mode, and this stops the pol δ complex from strand displacement and inhibits FEN1 cleavage. The helicase/nuclease Dna2 protein may then be recruited to bind (Bae *et al.*, 2001; Bae and Seo, 2000). The RPA coating of the flap was found to stimulate nuclease activity of Dna2p. The ternary complex among Dna2p, flap, and RPA is very stable, and will only dissociate upon catalysis by Dna2p. From this result, Seo and colleagues proposed the RPA-governed endonuclease switching model, in which RPA coordinates the sequential action of Dna2p and FEN1 (Bae *et al.*, 2001). Dna2p would cleave the coated flap beyond the RNA, leaving a 5–7 nucleotide flap from which RPA dissociates. The remaining flap will become a favorable substrate for FEN1, allowing subsequent cleavage by the FEN1–PCNA complex, generating the nicked substrate for ligation by DNA ligase I and PCNA (Tom *et al.*, 2000, 2001).

THE FEN1-ONLY MODEL

Recently, Burgers and colleagues utilized a system reconstituted from purified *S. cerevisiae* replication proteins to distinguish the roles of Dna2p and FEN1 nucleases in the maturation pathway. The authors employed two template systems: one was a circular template to measure the coupling of replication to Okazaki fragment processing, while the other was a biotinylated linear template allowing them to obtain and accurately analyze replication products. Their findings suggest that the average patch size of the strand-displacement synthesis by the pol δ-PCNA complex is 8–12 nucleotides in Okazaki fragment processing (Ayyagari *et al.*, 2003). This contrasts with the flap length estimated earlier with human proteins, which was about 30 nucleotides (Maga *et al.*, 2001). Knowledge of how much flap is displaced would suggest whether the Dna2p pathway occurs *in vivo*. If the flap length is long, RPA would be able to coat the flap, and a Dna2p step would be required to complete processing of the flap intermediates. However, if the flap is short, FEN1 would be predicted to efficiently process the flaps on its own.

Burgers and colleagues found that processing of the lagging strands during strand displacement synthesis is not Dna2p dependent, and FEN1 alone is sufficient for nicked product formation. Ligation occurs at nearly 100% efficiency in the absence of Dna2p. The strand-displacement synthesis is PCNA-dependent. FEN1 and pol δ alone carry out nick translation, which is also a PCNA-dependent process. The nick translation is not stimulated by Dna2p, even though all the reactions contain RPA. Dna2p does not alter the rate of nick translation even if the downstream primer has an iRNA. When FEN1 and pol δ act together for nick translation, only small oligomers are released (Ayyagari *et al.*, 2003). This shows that FEN1 cleaves frequently as the flap is created by the strand-displacement reaction, not allowing it to become long enough for RPA

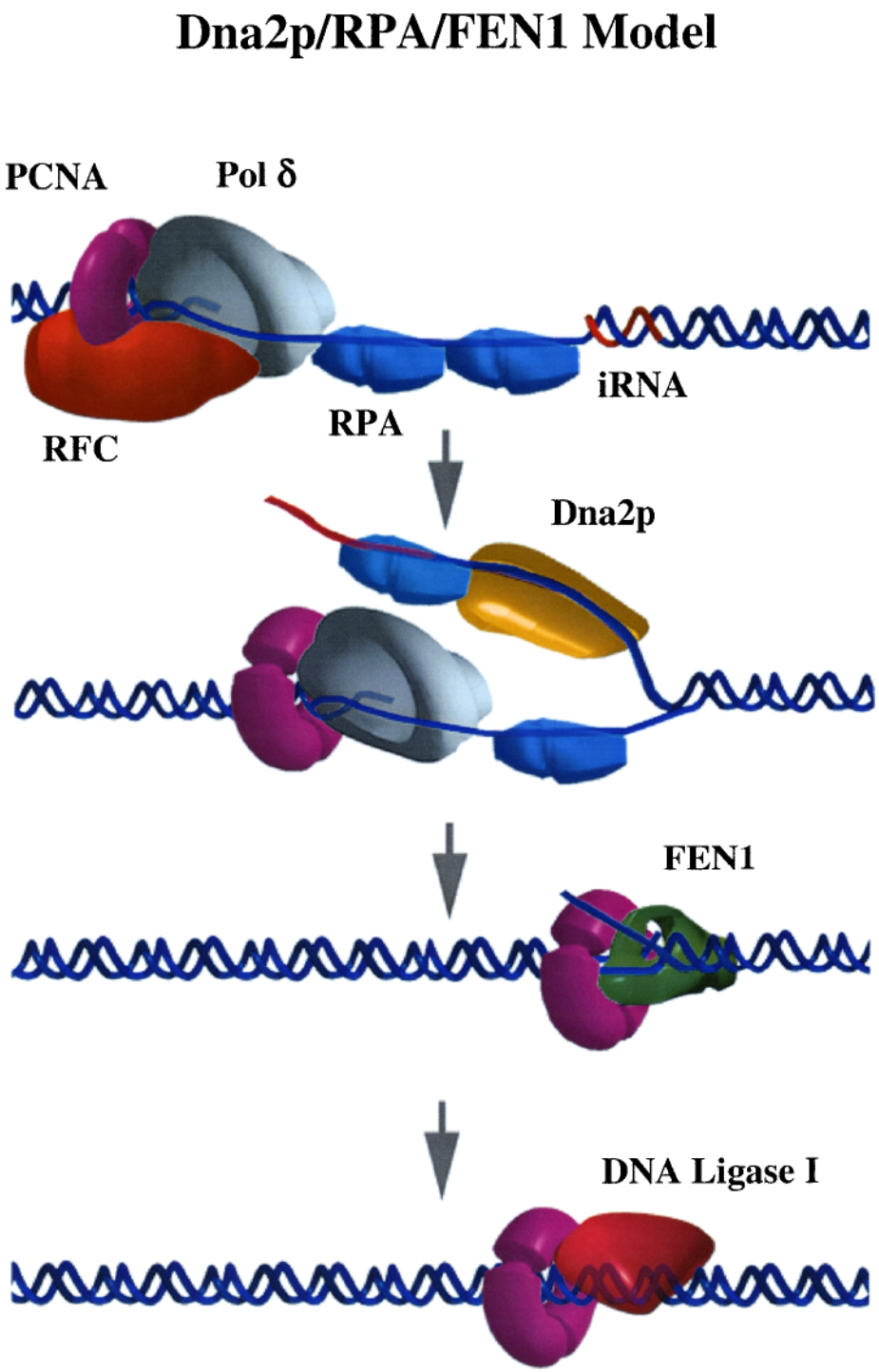

FIGURE 2. The Dna2p/RPA/FEN1 model. Elongation of the upstream fragment results in strand-displacement of the downstream iRNA/DNA into a long flap intermediate. This flap is so long that it will be coated by RPA, which prevents FEN1 cleavage. RPA recruits Dna2 endonuclease, and stimulates Dna2p cleavage within the single-stranded DNA region, generating a short-flap substrate. This Dna2p processed flap is too short for RPA binding but becomes a favored substrate for FEN1 and PCNA in a subsequent reaction. Finally, DNA ligase I and PCNA seal the nick to form an intact DNA strand. All the "entering" and "leaving" proteins for each step have been omitted for clarity. (Figure appears in color online at www.crbmb.com)

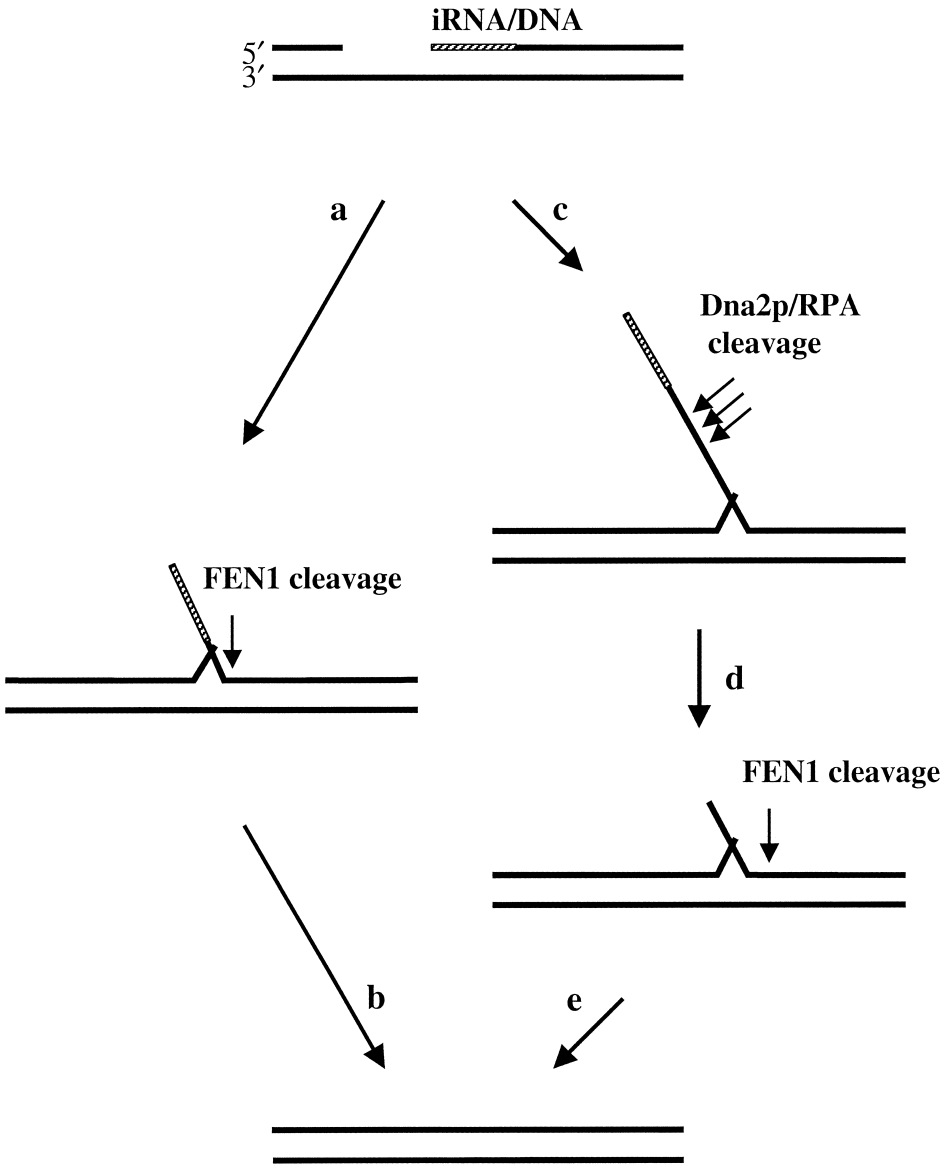

FIGURE 3. The FEN1-only model. (a) Most of the Okazaki fragments are processed via short-flap intermediates and by FEN1/PCNA, (b) allowing the subsequent ligation event by DNA ligase I-PCNA to complete the maturation process. However, when FEN1 activity is compromised or 3′-5′ exonuclease activity of DNA polymerase δ is low, long flaps may arise. (c) A long flap will be coated by RPA, which prevents FEN1 cleavage but recruits Dna2p to generate the favored FEN1 substrate. The resultant short flap will be processed by (d) FEN1-PCNA and (e) DNA ligase I-PCNA subsequently to complete the synthesis.

binding or Dna2p cleavage. This reaction is similar to all the reconstituted reactions reported earlier in SV40 and mouse cells in which FEN1 and/or RNase H were the only nucleases in the system (Goulian *et al.*, 1987, 1990; Ishimi *et al.*, 1988; Waga *et al.*, 1994).

According to this FEN1-only model (Figure 3), Dna2p acts only occasionally in the maturation

process and becomes essential only when long flaps are formed. Dna2p-stimulated ligation was only observed when the system was incubated with a primer that already contained a long fixed flap (Ayyagari *et al.*, 2003). Furthermore, this Dna2p-directed processing is optimal with RPA, and this interaction is specific with Rpa1p (the large subunit of yeast RPA). From the kinetic data, FEN1 is a stable component of the maturation complex while Dna2p functions only when the level of FEN1 is reduced enough to produce long flaps that require rescue after RPA coating (Ayyagari *et al.*, 2003).

Since the FEN1-only model supports the formation and cleavage of shorter flap intermediates, and Dna2p is only needed for abnormal flaps, under what conditions would Dna2p be required *in vivo*? This issue was addressed by utilizing two exonuclease-deficient polymerases, polδ-01 and polδ-5DV, that have wild-type polymerase activity but defective 3′-5′ exonuclease activity. Both mutants exhibit increased strand-displacement activity under all the conditions tested (Jin *et al.*, 2003). This suggests that 3′-5′-exonuclease activity of pol δ has a biological function distinct from proof-reading that can prevent excessive strand displacement synthesis, and this 3′-5′ exonuclease activity is proposed to limit the displacement size in yeast (Jin *et al.*, 2003). Genetic data, involving overexpression of Dna2p in a *pol3-5DV/rad27-p/rad51* triple mutant background, show that Dna2p restored the viability of this triple mutant. Double-strand breaks (DSBs) have been proposed to form in the *pol3-5DV/rad27-p* strain, which caused the *pol3-5DV/rad27-p/rad51* triple mutant to be inviable (Jin *et al.*, 2003). This suggests that Dna2p has a role in processing whatever long flaps are generated during maturation and in preventing DSBs. *In vivo*, long flaps may be generated while pol δ is synthesizing across a repeat region that contains secondary structures. Replication of a repeat sequence may cause slippage of pol δ, which would give rise to long flaps. The displaced flap containing repeats may fold into secondary structures that are completely resistant to FEN1 cleavage, and Dna2p will be required to resolve this structure. Overall, in the FEN1-only model (Figure 3) it was proposed that the 3′-5′-exonuclease activity of pol δ is important in preventing the formation of long flaps during nick translation *in vivo*, and the displaced flaps are usually processed by FEN1 (Ayyagari *et al.*, 2003; Jin *et al.*, 2003).

CONCLUSIONS/DISCUSSION

Understanding mechanisms of DNA replication, especially lagging-strand synthesis, is important for determining how the integrity of the human genome is maintained and how it deteriorates during aging and cancer progression (Henricksen and Bambara, 1998). There have been three pathways proposed for Okazaki fragment maturation in eukaryotes to date: RNase H/FEN1 (Dna2p-independent), Dna2p/FEN1 (Dna2p-dependent), and the FEN1-only (Dna2p-semidependent) models (Ayyagari *et al.*, 2003; Bae *et al.*, 2001; Jin *et al.*, 2003; Turchi *et al.*, 1994). Many experimental results support one or the other, but which ones are actually employed by the cell remains to be determined.

The Dna2p/FEN1 model of eukaryotic lagging-strand DNA replication places Dna2p in the role of removing the RNA primer from the displaced 5′-end region of every Okazaki fragment and leaving a shorter DNA flap for FEN1 cleavage. Strongest support for this model comes from results showing that:

1. Strand displacement by human pol δ is limited by human RPA coating of the flap (Maga *et al.*, 2001).
2. An RPA-coated flap stimulates Dna2p but inhibits FEN1 in yeast (Bae *et al.*, 2001).
3. Cleavage of single-stranded DNA can be blocked by primers annealed at the ends, suggesting entry of Dna2p onto the flap from the free single-stranded DNA ends (Bae *et al.*, 1998, 2001; Bae and Seo, 2000).
4. Dna2p is more active on a DNA segment with a 5′-RNA region (Bae *et al.*, 2001; Bae and Seo, 2000).
5. Dna2p and FEN1 interact with each other genetically and physically (Budd and Campbell, 1997).

However, other results argue against this model and support the others:

1. Okazaki fragment processing can be reconstituted without Dna2p in various systems (Ayyagari *et al.*, 2003; Goulian *et al.*, 1987, 1990; Ishimi *et al.*, 1988; Jin *et al.*, 2003; Turchi *et al.*, 1994; Waga *et al.*, 1994).
2. The flap made by human pol δ and coated with RPA can be processed by FEN1 (Maga *et al.*, 2001).
3. Mutants in Dna2p in *S. cerevisiae* do not lead to repeat sequence expansions, whereas FEN1 mutants do (Ireland *et al.*, 2000). Expansion in

this case is thought to result from defects in flap processing.
4. FEN1 can cleave within the RNA portion of a flap, whereas Dna2p cannot (Bae *et al.*, 1998; Bae and Seo, 2000; Murante *et al.*, 1996).
5. The *dna2* mutants are not mutagenic (Budd and Campbell, 2000b).

While the RNase H/FEN1 and FEN1-only models seem similar, there are several lines of evidence implying the existence of a second nuclease in a pathway that already requires FEN1.

1. The double-deletion mutant of RNase H and FEN1 in *S. cerevisiae* has more severe growth defects than the single-deletion mutant, suggesting that these two nucleases function cooperatively in a pathway (Qiu *et al.*, 1999).
2. iRNA/DNA synthesized by the pol α complex is about 30 nucleotides long, and synthesis by the pol α complex is more error prone (Hubscher *et al.*, 2002; Kool, 2002). In the RNase H/FEN1 pathway, cleavage of the iRNA by RNase H and subsequently the iRNA/DNA by FEN1 may allow cleavage further into the initiating DNA.
3. The iRNA may form a secondary structure that will block the tracking of FEN1.

In addition, since Dna2p needs a free DNA end to perform endonucleolytic cleavage and its helicase activity requires single-stranded DNA to load (Bae *et al.*, 1998, 2002; Bae and Seo, 2000), the existence of RNase H may be required to process flaps that contain very stable secondary structures without any free ends.

A defining feature that distinguishes the Dna2p-dependent and Dna2p-independent models is the length of a displaced flap. So far, various groups have reported different flap sizes that would support one or the other model. A more in-depth study of the strand-displacement activity by pol δ and PCNA complex will be needed to delineate the correct mechanism for the maturation pathway. However, the FEN1 model proposed by Burgers and colleagues appears to be the best single representation of how most Okazaki fragments are processed and joined during eukaryotic replication since it is consistent with most of the results accumulated so far.

Even though FEN1 is not an essential gene (Sommers *et al.*, 1995), that fact does not challenge its central role in the process. FEN1 has been understood as a required component in Okazaki fragment maturation process by various groups since it is the only enzyme among the three nucleases that possesses the specificity to generate nicked products for the subsequent ligation event (Goulian *et al.*, 1987, 1990; Ishimi *et al.*, 1988; Kao *et al.*, 2002; Waga *et al.*, 1994). Genetic studies have revealed that the function of FEN1 is so important that there are back-up pathways to prevent defects in its crucial metabolic step, such as Exo1p or some other as yet unidentified factors (Johnson *et al.*, 1998; Tishkoff *et al.*, 1997). These enzymes can carry out the function of FEN1, albeit inefficiently, in FEN1 null mutants. The conservation of the FEN1 enzyme in both structure and function within various organisms also argues for its role as a fundamental component of DNA replication (Ceska *et al.*, 1996; Goulian *et al.*, 1987, 1990; Harrington and Lieber, 1994a, 1994b; Hosfield *et al.*, 1998a, 1998b; Hwang *et al.*, 1998; Ishimi *et al.*, 1988; Kenny *et al.*, 1988; Kim *et al.*, 1995; Kimura *et al.*, 2000; Lindahl, 1970; Lindahl *et al.*, 1969; Murante *et al.*, 1996; Qiu *et al.*, 2001; Shen *et al.*, 1997; Sommers *et al.*, 1995; Waga *et al.*, 1994).

If the FEN1-only model is likely the major pathway, why is Dna2p essential? One explanation for this is that Dna2p only deals with the situation when the flap gets too long, and even infrequent long flaps have lethal consequences for the cells if the Dna2p enzymatic activity is impaired. In addition, millions of Okazaki fragments have to be generated and joined during the S phase of the eukaryotic cell division cycle. Although the percentage fragments with long flaps may be low, their actual numbers may be substantial. Another view is that Dna2p acts in only one of several parallel pathways for processing the iRNA/DNA. This would explain why Dna2p and FEN1 interact with each other genetically, and the overproduction of one rescues the phenotypic defects of the other (Budd and Campbell, 1997). Dna2p would then have to be essential because it also carries out a critical function in DNA metabolism beyond its role in the replication fork.

Besides DNA replication, Dna2p has been proposed to participate in other DNA metabolic pathways, such as double strand break repair (DSBR), telomere replication, stalled replication, and destabilization of minisatellites in the genome (Budd and Campbell, 2000b; Choe *et al.*, 2002; Lopes, 2002; Weitao *et al.*, 2003). Dna2p mutants show sensitivity to ionizing radiation, suggesting that the enzyme participates in DSBR (Budd and Campbell, 2000b). Dna2p is sequestered at the telomere and colocalizes with a telomere capping protein, Sir3p (Silence Information Regulator) (Choe *et al.*, 2002). Ku, an important component in the DSBR/NHEJ (Non-Homologous End Joining) pathway, also binds to Sir3p, and upon induction of DSBs Dna2p

and Ku mobilize throughout the cell (Budd and Campbell, 2000a; Choe *et al.*, 2002). In addition, overexpression of Dna2p rescues the defects in the *pol3-5DV/rad27-p/rad51* triple mutant in yeast (Jin *et al.*, 2003). In *dna2-2* (helicase-deficient mutant), replication pausing and Holliday junction formation are increased, and they are accompanied by DSB formation at the naturally occurring replication fork block in ribosomal DNA (Weitao *et al.*, 2003). All of these observations would support a role for Dna2p in DSBR. The requirement for Dna2p in *de novo* telomere synthesis and synthetic lethality of *dna2*-2 with *ets*Δ (not encoding the telomerase catalytic subunit) also implies that Dna2p has a role in telomere replication (Choe *et al.*, 2002). Perhaps the interaction between FEN1 and Dna2p is functional during recombination or DSBR, instead of DNA replication.

We also speculate that an additional protein component could be missing in reconstitutions of Okazaki fragment processing, preventing us from having a deeper understanding of Dna2p biology. One such factor could be either the Werner's protein (WRN) or Bloom's protein (BLM). The counterpart of these two helicases in *S. cerevisiae* is the Sgs1p helicase. It was found that Sgs1p and Dna2p interact with each other genetically, the *dna2-1/sgs1*Δ strain exhibits more severe defects in growth, and it is more sensitive to X-rays and UV irradiation (Budd and Campbell, 2000b). Human BLM suppresses the phenotypic defects of the *dna2-1* mutant in yeast, and it interacts with both yeast Dna2p and FEN1 physically (Imamura and Campbell, 2003). Moreover, WRN interacts with FEN1 and stimulates its activity (Brosh *et al.*, 2001). Maybe WRN or BLM interacts with both Dna2p and FEN1 in a DNA repair pathway, which could define the essential role of Dna2p in the cells. These observations also imply that the helicase activity of Dna2p in combination with that of other proteins is important for Dna2p function during replication. A recent study by Campbell and colleagues indicated that a helicase-deficient strain, *dna2-2*, is synthetically lethal with a null mutant of a DNA helicase involved in ribosomal DNA replication, *rrm3*Δ (Weitao *et al.*, 2003). This would suggest that the helicase activity of Dna2p is important in ribosomal DNA repeat synthesis.

Such possibilities do not exclude a model in which all three nucleases, FEN1, Dna2p, and RNase H, work together in a "replisome" or "synthesome" context with all the replication proteins interacting with each other in a megacomplex. There are several such complexes isolated from different cell lines so far (Coll *et al.*, 1996; Jiang *et al.*, 2002; Lin *et al.*, 1997), although participating proteins in these complexes have yet to be fully clarified. The three pathways we considered may still coexist, and, depending on which situation is encountered by the replication machinery, different enzyme combinations would be employed to process the flap intermediates. Much of the precise mechanism of how iRNA/DNA is removed remains to be determined. Many more experiments will be needed to fully understand this fundamental process.

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