Cleavage of Substrates with Mismatched Nucleotides by Flap Endonuclease-1

IMPLICATIONS FOR MAMMALIAN OKAZAKI FRAGMENT PROCESSING*

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Flap endonuclease-1 (FEN1) is proposed to participate in removal of the initiator RNA of mammalian Okazaki fragments by two pathways. In one pathway, RNase HI removes most of the RNA, leaving a single ribonucleotide adjacent to the DNA. FEN1 removes this ribonucleotide exonucleolytically. In the other pathway, FEN1 removes the entire primer endonucleolytically after displacement of the 5'-end region of the Okazaki fragment. Cleavage would occur beyond the RNA, a short distance into the DNA. The initiator RNA and an adjacent short region of DNA are synthesized by DNA polymerase α/primase. Because the fidelity of DNA polymerase α is lower than that of the DNA polymerases that complete DNA synthesis, mismatches occur relatively frequently near the 5'-ends of Okazaki fragments. We have examined the ability of FEN1 to repair such errors. Results show that mismatched bases up to 15 nucleotides from the 5'-end of an annealed DNA strand change the pattern of FEN1 cleavage. Instead of removing terminal nucleotides sequentially, FEN1 appears to cleave a portion of the mismatched strand endonucleolytically. We propose that a mismatch destabilizes the helical structure over a nearby area. This allows FEN1 to cleave more efficiently, facilitating removal of the mismatch. If mismatches were not introduced during synthesis of the Okazaki fragment, helical disruption would not occur, nor would unnecessary degradation of the 5'-end of the fragment.

Mammalian DNA replication proceeds by two distinct processes occurring at replication forks (1). The leading strand is made as a large continuous segment, whereas the lagging strand is synthesized as a series of discontinuous segments called Okazaki fragments. Each segment is individually initiated by a RNA primer. Later, these primers are removed and replaced with DNA, and the fragments are joined into one continuous strand. DNA polymerase α/primase synthesizes every RNA primer (2, 3) and the adjacent 10–20 nucleotides of DNA (4). It is then replaced by either DNA polymerase δ or ε via a process called polymerase switching (5–7). DNA polymerase δ lacks the proofreading 3’-5’ exonuclease activity present in polymerases δ and ε (2). Because DNA polymerase α has no mechanism to remove erroneously inserted nucleotides, it can continue synthesis only by mismatch extension. DNA polymerase α incorporates an incorrect nucleotide at a rate of approximately 1 in every 10,000 nucleotides, whereas the error rate of polymerases δ and ε is at most 1 in 50,000–200,000 nucleotides (8).

Initiator RNA removal is accomplished by a eukaryotic 5’ to 3’ exonuclease/endonuclease before Okazaki fragment joining (9). Most frequently called flap endonuclease-1 (FEN1), this enzyme is also known as RAD2 homologue nucleolyse (10–15). In one proposed pathway, RNase HI-directed cleavage removes almost the entire RNA primer but leaves a single RNA residue. FEN1 exonucleolytically removes this last ribonucleotide (16). However, genetic evidence suggests that alternative pathways exist. Yeast cells are still viable after deletion of the gene that accounts for 75% of RNase H activity (17). In a second proposed pathway, FEN1 alone removes the initiator RNA (18) by its favored exonuclease activity (10, 19). FEN1 endonucleolytic cleavage requires that the substrate has a downstream primer with an unannealed 5’-tail, or flap. FEN1 appears to recognize the 5’-end, slide over the entire length of the tail, and cleave near the junction of the tail with the template. This releases the tail as an intact segment (10, 19). Sometimes, the presence of an upstream primer is also required for FEN1 activity. In fact, for both exonuclease and endonuclease substrates, the upstream primer is often stimulatory (10, 20, 21) but can sometimes be inhibitory (18, 22). For endonucleolytic removal, the RNA must be within a displaced tail. An upstream primer may or may not be present, as appropriate for the particular cleavage site. Displacement of the RNA may be accomplished by a DNA helicase with or without DNA polymerase-directed displacement synthesis from an upstream fragment (18, 23). Depending on how far the tail is displaced, FEN1 can cleave within the RNA, at the RNA-DNA junction, or within the DNA beyond the RNA (18).

FEN1 may also participate in DNA repair. It is a member of the RAD2 family of repair nucleases (24). This family includes XPG in mammals, RAD2 in Saccharomyces cerevisiae, and RAD13 in Schizosaccharomyces pombe. These homologous nucleases are important components of the nucleotide excision repair pathway and are responsible for the incision 3’ to the damage site (24). FEN1 is homologous to the family members called RAD27 or RAD2 homologue in S. cerevisiae and RAD2 in S. pombe. These family members are smaller than the XPG nucleases and are believed to function in other repair processes (24). A third class of members includes exonuclease I of S. pombe, for which genetic studies suggest a role in mismatch repair.

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repair (24). The FEN1 class enzymes are believed to participate in base excision repair because the null mutant in yeast is sensitive to methylmethane sulfonate (14, 15). In vitro, FEN1 can remove abasic sites (25) and can function in reconstitutions of base excision repair using purified enzymes (26–28). FEN1 can also remove a more diverse group of flap adducts such as cisplatin derivatives (29). Additionally, it may also participate in the removal of monoribonucleotides embedded in chromosomal DNA (30). Finally, FEN1 has been implicated as a part of the mismatch repair pathway (31).

In the current report, we provide evidence that FEN1 participates in the repair of mismatches produced during the priming and synthesis of Okazaki fragments. We show that a single mismatch in an otherwise fully annealed DNA primer changes the pattern of FEN1 cleavage. Typically, on a fully annealed substrate, FEN1 will exonucleolytically cleave the 5'-most nucleotide. We find that a single nucleotide mismatch up to 15 nucleotides in from the 5'-end promotes endonucleolytic cleavages. This constitutes a 5' proofreading process in which the mismatch promotes the nuclease action that leads to its removal.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unlabeled nucleotides were purchased from Amersham Pharmacia Biotech, and [γ-32P]ATP (3,000 or 6,000 mCi/mmol) and [α-32P]dATP (3,000 mCi/mmol) were from NEN Life Science Products. Oligonucleotides were synthesized by Genosys Biotechnologies (The Woodlands, TX). T4 polynucleotide kinase and Sequenase™ version 2.0 were obtained from U. S. Biochemical Corp. RNase inhibitor and snake venom phosphodiesterase were from Roche Molecular Biochemicals. All other reagents were from Sigma Chemical Co.

**Enzyme Purification**—Recombinant human FEN1 was purified as described previously (18, 29). The final preparation was >95% pure as determined by silver-stained SDS-polyacrylamide gel electrophoresis. The final specific activity was 65,000 units/mg, with 1 unit defined as the amount of nucleic acid required to exonucleolytically cleave 4,000 fmol in 30 min at 37°C of a standard exonuclease test substrate consisting of a downstream primer (5'-TTGATGCTGTTTAGTCGAGGAGCAGCAAGAG-3') annealed to a template (3'-GACTTTCGACAGAACGGACGGATCCAGCTGCTAGAGGAC-5').

**Oligonucleotide Substrates**—The sequences of the primers and the structures of the substrates used in this study are depicted in Table I and in the figure legends. Table I depicts template T1 and the downstream primers used in this study. The primers are divided into four groups, depending on the location of the 5' most nucleotide; the 5'-end of all primers in a given group is located at the same place on the template. The first digit of the primer name indicates the group; 1, 2, 3, or 4. To generate a mismatch, each primer has a single base change that forms a single mismatch with the template, T1. The remaining digits in the name indicate the distance of this mismatch in nucleotides from the 5'-end of the primer. For all experiments, the substrate name reflects the use of a specific downstream primer. For example, substrate 2.11 indicates that a primer from group 2 was annealed to the template to create a mismatch at position 11. Control substrates 1.0, 2.0, and 4.0 have no mismatch. Mismatches were made so that nucleotides were paired with themselves (e.g. G was paired with G). The 5'-end of the template extends beyond the downstream primer to permit 3'-end labeling. In substrates 1.T and 1.A, the mismatch is not internal but is, in fact, the 5' most nucleotide, which is mispaired as T-T or A-A, respectively. Substrate 3.6 uses a primer that lacks the three 5'-most nucleotides of the primer in substrate 2.9. Thus, both the location of the 5'-end and the position of mismatch relative to the 5'-end have changed. Substrates made from primers in groups 4 are similar to those made from primers in group 2, except that 25 nucleotides were added to the 5'-end to form an unannealed 5'-tail, the sequence of which is shown in Table I.

For 5'-labeling, downstream primers were incubated with T4 polynucleotide kinase and [γ-32P]ATP according to the manufacturer's protocol and then annealed to the template in annealing buffer (50 mM Tris, pH 8, 10 mM magnesium acetate, 50 mM NaCl, and 1 mM diithiothreitol). 5'-end-labeled substrates, downstream primers, and 5'-end-labeled downstream primers were first phosphorylated using T4 polynucleotide kinase and ATP. Then, after annealing to template T1, they were extended by addition to the 3'-terminus using [α-32P]dATP and Sequenase™ version 2.0. Substrates were isolated via 12% native gel electrophoresis (32), eluted from the gel using elution buffer (0.5 mM ammonium acetate, 0.1% SDS, and 0.1 mM EDTA), precipitated in ethanol, and resuspended in annealing buffer or TE, pH 8.0.

**Enzyme Assays**—Assays to monitor cleavage by FEN1 were performed in FEN1 buffer containing 60 mM BisTris (pH 7.0), 5% glycerol, 0.1 mg/ml bovine serum albumin, 5 mM β-mercaptoethanol, 10 mM MgCl₂, and 10 fmol of substrate/reaction in a volume of 25 µl/reaction. Reactions were initiated by the addition of 15 ng (340 fmol) of FEN1/reaction and incubated at 37°C for 30 min. Reactions were stopped with 25 µl of 2× formamide loading dye (98% formamide, 10 mM EDTA (pH 8.0), 0.01% (w/v) each of xylene cyanol and bromphenol blue) and heated at 95°C for 5 min. Controls lacking enzyme were also assayed. Products were separated on either 12% or 18% polyacrylamide, 7 M urea denaturing gel electrophoresis (32) and visualized by autoradiography. DNA size markers were generated by digesting 5'-end-labeled primers with snake venom phosphodiesterase. Any adjustments to the above are noted in the figure legends.

**RESULTS**

**A Single Mismatch Alters FEN1 Cleavage Specificity**—We hypothesized that FEN1 participates in a 5' proofreading reaction linked to DNA replication. In this reaction, FEN1 would remove mismatches generated by DNA polymerase α in the region of DNA just beyond the initiator RNA. On a fully an-
nealed DNA primer, the action of FEN1 is exonucleolytic, whereas on a flap, it is endonucleolytic. Our initial experiments were designed to determine whether the presence of a mismatch would destabilize a primer so that it would appear as a flap to the nuclease. If so, we wanted to measure the distance over which the mismatch could influence cleavage activity.

We first compared the cleavage of a fully annealed control substrate (substrate 1.0) to that of substrates containing a single mismatch either two or three nucleotides from the 5' end (substrates 1.2 and 1.3, respectively) (Fig. 1A). For this and all other experiments in this study, control substrates were identical to the mismatch substrates, except that they were without a mismatch. As expected, on these 5'-end-labeled substrates, FEN1 cleavage in the absence of mismatch resulted in the release of a single nuclease (Fig. 1A, lane 2). Cleavage of fully annealed substrates most often results in exonucleolytic cleavage (10, 11, 21, 22, 30, 33), although with some sequences, additional products from cleavage at internal sites are observed. Apparently, FEN1 has the capacity to invade a small proportion of fully annealed substrates to perform endonucleolytic cleavage. Such minor internal cleavage products were seen with substrate 1.0. When a mismatch was present at the second or third nucleotide, the proportion of products resulting from endonucleolytic cleavage (trimers and pentamers) was greatly enhanced (Fig. 1A, lanes 3 and 4). The mechanism of FEN1 was shifted from predominantly exonucleolytic cleavage to predominantly endonucleolytic cleavage simply by the presence of a single mismatched base pair.

For consistency, mismatched substrates were produced by pairing nucleotides with themselves (i.e., G with G). However, we have noticed that the sequence of the mismatch affects the distribution of internal cleavages, as shown in Fig. 1B. Lane 1 shows the cleavage of control substrate 1.0, as in Fig. 1A. For lane 2, the sequence of the primer was modified to generate a T-T mispair with the template at the 5'-end of the primer (substrate 1.T). For lane 3, the template sequence was modified so that the 5'-end nucleotide of the original primer formed an A-A mismatch with the new template (substrate 1.A). In comparing lanes 2 and 3, the identity of the mismatch clearly alters the ratio of the three major products. There is a higher percentage of pentamer released with the A-A mismatch.

These results suggest that the specific nucleotides of the mismatch affect the internal cleavage capacity of FEN1, perhaps by altering the exact amount of primer destabilization or the shape of helical distortion. On the other hand, the pattern of products is similar to those generated when the mismatch was at position 2 or 3 from the 5'-end (Fig. 1, compare A with B). This suggests that the mismatch alters the structure of the substrate around it to favor endonucleolytic cleavage but does not define the position of cleavage. Instead, the nuclease appears to cleave at sites that it favors, irrespective of the exact location of the mismatch.

Internal Cleavages Allow Efficient Endonucleolytic Mismatch Removal—As the mismatch was moved further into the strand, the effect on the distribution of 5' products decreased. Mismatches at 7, 9, 11, 13, and 15 nucleotides from the 5'-end produced a product distribution that differed little from the distribution seen with no mismatch (data not shown). A primer template in which the primer has an unannealed 5'-tail is the preferred substrate of FEN1 (10, 18, 19). We wanted to determine whether a mismatch in the annealed region downstream of an unannealed 5'-tail would alter the pattern of cleavage. We thought the mismatch might effectively lengthen the tail, enabling FEN1 to cleave further internally than it otherwise would. FEN1 can remove initiator RNA endonucleolytically when it has been displaced into a tail (18). The helical disruption caused by a mismatch might allow FEN1 to remove the initiator RNA and a mismatch simultaneously, with an appropriately placed endonucleolytic cut. Fig. 2 clearly shows no difference in cleavage on endonucleolytic substrates with no mismatch (lane 1), a mismatch seven nucleotides into the annealed region (lane 2), and a mismatch nine nucleotides into the annealed region (lane 3). Cleavage of each substrate results in the production of two major products of 26 and 10 nucleotides. The expected product is 26 nucleotides, given that the unannealed 5'-tail is 25 nucleotides. The smaller product is the result of cleavage within the tail. This might occur from either transient annealing between the tail and the upstream portion of the template or the secondary structure within the unannealed tail. The presence of the tail might have facilitated the binding of FEN1 and movement of the nuclease to the site of the mismatch to make an initial cleavage. However, these data provide no evidence that the two features collaborate to favor an altered position of cleavage. Presumably, after the tail has been removed, the mismatch will promote further internal cleavages as suggested by the results shown below.

To perform effective 5' proofreading, FEN1 must cleave beyond the mismatch. It would appear that if the mismatch is sufficiently far from the 5'-end, it cannot be removed by a single cut. However, because the primers examined to this point had been 5'-end-labeled, only the first cleavage event could be observed. A mismatch that is initially deep within a primer becomes increasingly close to the 5'-end as FEN1 cleaves the substrate. As this occurs, the mismatch would be expected to have an increasingly greater influence on the cleavage events.
Although the mismatch is not removed in a single cut, FEN1 may do so through a series of cleavage events. In Fig. 3, substrate 2.9 has a mismatched base pair at position 9. Lane 1 demonstrates that FEN1 can release a monomer, trimer, or hexamer on this substrate. In each event, the mismatch is not removed in a single cut. After FEN1 removes the first three nucleotides, the mismatched base pair at position 9 is now at position 6. We used a second substrate, 3.6, to model this intermediate and examine FEN1 activity. Using substrate 3.6, we can observe the results of subsequent cuts.

Cleavage of substrate 3.6 released monomers, dimers, and tetramers (Fig. 3, lane 2). The mobilities of products in lanes 2 and 3 differ from those in lane 1 because of the sequence difference between substrates 2.9 and 3.6. In particular, released monomer dC migrates farther in lane 2 than does monomer dT in lane 1. To verify the exact lengths of the products from both substrates, we made molecular weight ladders of each primer by digestion with snake venom phosphodiesterase. Because substrate 3.6 already has three nucleotides removed compared with substrate 2.9, the release of a monomer, dimer, etc. in lane 2 is equivalent to cleavage beyond the fourth, fifth, etc. nucleotide in lane 1. Removal of the original mismatch nine nucleotides into the substrate requires the release of a hexamer in lanes 2 and 3. The monomers, dimers, and tetramers released in lane 2 correspond to a second cut 3′ of the fourth, fifth, and seventh nucleotides of the original substrate 2.9. Even this second cut falls short of the goal of mismatch removal. However, the process has now placed the mismatch as little as two nucleotides from the 5′-end.

The fact that cleavage did not reach the mismatch was surprising. In substrate 3.6, the mismatch is only six nucleotides from the 5′-end, a distance reached on substrate 2.9 (lane 1). One reason we failed to observe cleavage beyond the mismatch could be because of its distance from the 5′-end. Alternatively, failure to cleave beyond the mismatch may reflect the influence of specific sequences. In many sequence contexts, FEN1 requires an upstream primer for stimulation of cleavage (10, 20, 21). To allow for such stimulation, we added an upstream primer (UP) to substrate 3.6. This upstream primer produces a nick with the nucleotide of the downstream primer that is located immediately 3′ to the mismatch. The presence of this upstream primer did indeed stimulate cleavage after the original ninth nucleotide mismatch, releasing a hexamer from substrate 3.6 (lane 3). Results here show how a combination of several cuts and synthesis from the upstream fragment could collaborate to achieve removal of the mismatched nucleotide.
The FEN1 nuclease has a unique substrate specificity for flap structures. Although FEN1 cleaves exonucleolytically, it prefers a substrate with an unannealed 5'-tail and presumably removes a portion of initiator RNA by an endonucleolytic mechanism (18). If cleavage occurs within the DNA, either as a single cut or as a series of cuts, mismatches incorporated in the DNA by DNA polymerase α could be removed. The mismatch could destabilize the helical structure of the DNA and promote its own removal by relieving any sequence constraints. In fact, the results shown here suggest that the presence of a mismatch promotes extensive endonucleolytic cleavage. The absence of a mismatch would encourage the minimum cleavage before ligation. Apparently, mismatches create a helical disruption, allowing FEN1 to recognize the presence of the mismatch and efficiently remove it. Because the structure-specific requirements of FEN1 cleavage make the template inert to FEN1 degradation (11), FEN1 would be able to distinguish the nascent strand from the template strand and promote the repair of only the incorrectly inserted nucleotide.

One advantage of this FEN1 reaction, which we term 5’ proofreading, is that it can be performed as part of the replication process. In this way, it is similar to the 3’ proofreading function associated with DNA polymerases δ and ε in that errors can be corrected as part of the process in which they were created. Another similarity to 3’ proofreading is that the mismatch creates the substrate for the proofreading nuclease. Because the polymerase-associated 3’ exonuclease is single strand-specific, only mismatched nucleotides are recognized for
degradation. The presence of a mismatched base pair promotes the formation of 5' flaps, the favored substrate of FEN1. This makes FEN1 a logical choice to perform the proposed 5' proofreading reaction. We predict that this process, as a form of replication-coupled mismatch repair, is used in vivo to proofread and remove polymerase α errors from the chromosome.

To test the hypothesis that FEN1 could perform 5' proofreading, we have designed a series of substrates to model potential mismatch intermediates during Okazaki fragment processing. A single mismatch was used to represent errors made by DNA polymerase α in the DNA near the 5'-end of an Okazaki fragment. *In vivo*, an upstream primer might also be present because FEN1 often requires a primer adjacent to the site of cleavage (10, 20, 21). On the other hand, in some sequence contexts, the upstream primer is inhibitory (18, 22). A delay in FEN1 activity would allow synthesis from the upstream Okazaki fragment to approach the sites of cleavage, providing an upstream primer when needed. In most experiments, we have not used an upstream primer. The presence of an upstream primer that is designed to bind competitively with the 5'-region of the downstream primer would create a transient flap structure, allowing for normal endonucleolytic cleavage as opposed to mismatch-directed internal cleavage. This would complicate the interpretation of our results. However, not using upstream primers has at least two consequences: (a) it biases against detecting internal cleavages because synthesis from an upstream fragment would normally collaborate with the mismatch to destabilize the helix structure of the downstream fragment; and (b) it creates the observed uneven cleavage patterns. These arise because some sites can be cleaved readily without an upstream primer, whereas others are upstream primer-dependent. When we used an upstream primer designed to bind adjacent to an internal site on the downstream primer (Fig. 3, lane 3), it had the expected effect of stimulating cleavage and allowing mismatch removal.

We initially hypothesized that mismatches near the 5'-end of a primer would encourage endonucleolytic cleavage by FEN1. However, we were surprised to find that the influence of the mismatch extended for 15 or more residues. This is particularly significant considering that the experimental conditions used here lack factors present in the cell that would favor flap formation. As discussed above, the presence of a growing upstream primer would encourage the displacement of the mismatched region, promoting cleavage. Furthermore, FEN1 in S. cerevisiae has been found to associate with the Dna2 helicase, which moves in the same direction on the template as synthesis (36). This helicase is expected to promote dissociation of the 5'-end sequence of the downstream primer. The action of a homologous mammalian helicase and the presence of other helixdestabilizing proteins such as RPA (37, 38) should contribute to even more efficient internal cleavages. Current technology does not allow us to exactly recreate the conditions present within the cell where 5' proofreading normally occurs. Nevertheless, our results emphasize that the intrinsic structural features of a substrate with a mismatch are sufficient for FEN1 to distinguish it from a fully base-paired substrate. Overall, these considerations are consistent with the proposal that the presence of a mismatch throughout the 10–20 nucleotides of DNA expected to be added by DNA polymerase α will promote FEN1 cleavage. Such an ability would allow FEN1 to preferentially cleave those fragments that require mismatch correction.

For FEN1 to be effective at 5' proofreading, it must not just respond to the mismatched substrate. It must also able to cut beyond the mismatch and remove it. FEN1 can remove distant mismatches by making several cuts in a progression. Using 3'-end labeling, Fig. 4 demonstrates that given enough time and utilizing multiple cleavages, FEN1 can easily cut beyond a mismatch even 15 residues deep. These results present 5' proofreading as a multistep process. As the mismatch is moved further internally, an increasing number of cuts are required to reach the mismatch, and the process becomes less and less efficient. Clearly, at some point, if a mismatch were positioned far enough internally, it would not be reached by FEN1 before the region was ligated into a continuous strand of chromosomal DNA. In such a case, the mismatch would be repaired as usual by post-replication repair processes.

Fig. 5 presents a model illustrating the selective form of replication-coupled repair proposed to be mediated by FEN1 5' proofreading. In the absence of a mismatch, FEN1 activity is minimal after the removal of initiator RNA, whereas the presence of a mismatch encourages the 5' proofreading mechanism. Because of the results shown in Fig. 2, we depict removal of the initiator RNA as a separate step from removal of the mismatch. The results suggest that the creation of a flap does not particularly activate nearby mismatches for cleavage.

The crystal structures of several FEN1 homologues have been solved, and they suggest that FEN1 has an arch or loop structure enabling the nuclease to bind a flap and position the active site for cleavage (39–41). FEN1 may have evolved a strong preference for flap structures to carry out more efficient removal of initiator RNA and to participate in long patch base
excision repair, a process that occurs by a flap mechanism (42, 43). Strong flap specificity explains why mismatches are so effective at stimulating FEN1 activity, as seen in our 3‘-end-labeled time course experiment. A region around the mismatch is converted from an exonucleolytic substrate to the preferred flap substrate. The unannealed region can be removed quickly, allowing the nuclease to progress further on the substrate than if it were completely annealed.

Deletion of the yeast FEN1 caused an increase in the expansion frequency of trinucleotide repeats (44). This suggests that a mutation of FEN1 may be involved in human diseases such as myotonic dystrophy, Huntington’s disease, several ataxias, and fragile X syndrome, all of which involve the expansion of repeat sequences (44). The role that FEN1 may play in DNA replication and repair and in preventing human diseases indicates the value of examining the exact mechanisms of this enzyme and defining the complete range of its substrate specificity.

In this report, we describe the specificity of FEN1 for DNA substrates with mismatches near the 5‘-end of a primer. We show that the presence of a mismatch alters FEN1 specificity, promoting the nuclease to remove the mismatch. We propose that this activity has evolved to remove mismatches introduced by DNA polymerase α during the initiation of Okazaki fragments. In effect, FEN1 would then be the proofreading nuclease for DNA polymerase α.

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