Recent genetic evidence indicates that null mutants of the 5' flap endonuclease 1 (FEN1) result in an expansion of repetitive sequences. The substrate for FEN1 is a flap formed by natural 5'-end displacement of the short intermediates of lagging strand replication. FEN1 binds the 5'-end of the flap, tracks to the point of annealing at the base of the flap, and then cleaves. Here we examine mechanisms by which foldback structures within the flap could contribute to repeat expansions. Cleavage by FEN1 was reduced with increased length of the foldback. However, even the longest foldbacks were cleaved at a low rate. Substrates containing the repetitive sequence CTG also were cleaved at a reduced rate. Bubble substrates, likely intermediates in repeat expansions, were inhibitory. Neither replication protein A nor proliferating cell nuclear antigen were able to assist in the removal of secondary structure within a flap. We propose that FEN1 cleaves natural foldbacks at a reduced rate. However, although the cleavage delay is not likely to influence the overall process of chromosomal replication, specific foldbacks could inhibit cleavage sufficiently to result in duplication of the foldback sequence.

Recent biochemical and genetic data have served to illustrate the dual roles of many proteins in DNA metabolism. Enzymes first identified as central components of DNA replication forks, such as the flap endonuclease 1 (FEN1), are now understood to play critical roles in DNA repair pathways (1–5). Our increasing understanding of the mechanisms of these enzymes is revealing the nature of their multiple functions in the cell.

FEN1, a member of the RAD2 superfamily, is a structure-specific nuclease known to be involved in both lagging strand synthesis during DNA replication (6–10) and long patch base excision repair (11). Genetic studies highlight the central role of FEN1 in these cellular processes (12–14). In Saccharomyces cerevisiae, a null mutant of the FEN1 homologue (RAD27/RTH1) is conditionally lethal at high temperatures producing a cellular morphology indicative of an S phase arrest. At the permissive temperature, FEN1 mutants exhibit slow growth and hyper-recombination phenotypes consistent with defects in DNA replication and recombination. Null mutants also have an increased sensitivity to the alkylating agent methyl methanesulfonate but are only moderately affected by UV or ionizing radiation. These characteristics are consistent with participation of FEN1 in base excision repair.

Biochemical analyses have clarified the nature of the FEN1 catalyzed reactions during DNA replication and repair. Reconstitution of lagging strand DNA synthesis in vitro showed that FEN1 is needed to remove the initiator RNA primers of Okazaki fragments (3). FEN1 assists in primer removal through two proposed pathways. First, the nuclease RNase HI cleaves within the RNA primer leaving a single ribonucleotide remaining at the 5'-end of the Okazaki fragment. The FEN1 nuclease removes this ribonucleotide prior to ligation with an upstream Okazaki fragment (9). Alternately, synthesis from an upstream Okazaki fragment may cause the displacement of the RNA primer generating an unannealed 5'-tail or flap structure (15, 16). FEN1 cleaves endonucleolytically at the base of the flap, thereby removing the entire segment of RNA (17–19).

The endonucleolytic activity of FEN1 is thought to be important for the removal of damaged nucleotides in long patch base excision repair (11, 20). During repair of an abasic site, an apurinic/pyrimidinic endonuclease cleaves on the 5'-side of the abasic sugar generating a nick within the DNA (21). We have previously demonstrated that FEN1 cannot remove the abasic sugar (22). Instead, when the damaged sugar and an additional downstream nucleotide are displaced to generate a flap, FEN1 endonucleolytically removes the site of damage as part of an oligomer. The resulting short gap is filled and ligated to complete the repair process.

FEN1 employs a unique cleavage mechanism for substrates containing unannealed 5'-tails or flap structures (Fig. 1). FEN1 removes the flap by recognizing the 5'-end, tracking the length of the tail, and cleaving at the point of annealing (1, 18). Flap substrates composed of either RNA or DNA are readily cleaved by FEN1 (19). However, the unannealed 5'-tail must be single-stranded as the presence of large adducts (18) or annealed primers prevent FEN1 cleavage (18, 23).

The FEN1 family of nucleases is conserved throughout evolution with homologues identified from archaeabacteria, yeast, Xenopus, and mammals (1, 24–28). Biophysical analysis of homologues has begun to provide a structural context for understanding the molecular mechanism of FEN1. Crystal structures of FEN1 homologues from T5 exonuclease (29), T4 RNase H (30), Methanococcus jannaschii FEN1 (31), and Pyrococcus furiosus FEN1 (32) reveal a helical arch or loop above a globular domain containing the active site. This arch may be utilized by the nuclease to track onto the 5'-end of a flap structure (29). Mutational analyses of the loop region in the M. jannaschii FEN1 indicate that this physical structure is critical for both the binding and cleaving of flap substrates (31).

Previous results show that the ability of the FEN1 endonuclease to cleave flap structures is affected by a range of modi-
The addition of large adducts such as bixin-streptavidin complexes, small adducts (cis-platinum), and oligonucleotide primers annealed to the tail, and oligonucleotide sequences repeated to greater lengths (minisatellites) are involved in the mechanism of expansion. Recent studies utilizing flap substrates containing a branch structure permit cleavage by FEN1. These results suggest a tracking process in which the flap is not completely encircled.

In addition to DNA replication and repair, genetic and biochemical data also connect FEN1 with repeat sequence expansion. Many human genetic diseases are characterized by a substantial alteration of the genome. One class of changes observed involve a unique group of sequences referred to as repetitive sequences. These stretches of the genome contain either single, dinucleotide, or trinucleotide sequences repeated in an array of several to about 60 nucleotides (microsatellites) or longer 4–100-nucleotide sequences repeated to greater lengths (minisatellites) (37). Repeat sequences have attracted attention particularly because of their link with a host of genetic conditions called human triplet repeat disorders (38). These disorders include several neurodegenerative diseases such as Huntington’s disease, Friedrich’s ataxia, and some colon cancers. A notable characteristic of these sequences is their ability to adopt higher ordered structures such as hairpin loops both in vitro (39, 40) and in vivo (41).

Although the expansion of repetitive sequences is well described with regard to the alterations seen in patients, information on the mechanism of expansion only recently has become available. First, the nature of the sequence itself influences expansion. Examination of families with Huntington’s disease indicates that the rate of expansion is strongly linked to DNA sequence (38). As a result of the repeating nature of the sequences, regions that expand are able to adopt secondary structure. In addition, an increase in expansion rate is seen if the sequences are located on the lagging strand during DNA replication in yeast (42, 43). It has been observed in yeast that mutations in the proteins FEN1 and replication factor C lead to an increase in the length of repetitive sequences (37, 44–49). These observations suggest that repeat sequences expand during the RNA removal and joining steps of Okazaki fragment processing. Most likely foldbacks formed from the repeat sequences interfere with the required cleavage reaction of FEN1. Because primers annealed to a flap inhibit cleavage, a foldback in the flap is anticipated to inhibit by a similar mechanism. The resulting delay in the removal of flaps with foldbacks leads to an expansion of the repeat. These phenotypes of yeast are relevant to human FEN1 not only because the two nucleases exhibit a high degree of sequence homology but also because human FEN1 will rescue the defects of the null mutant in yeast (50).

The link between the structure of repetitive sequences and the mechanism of FEN1 has led to the proposal of a model to explain repeat expansion (37). During DNA synthesis, displacement by a polymerase or helicase allows repetitive sequences to self-anneal forming secondary structure such as a hairpin loop or foldback within the DNA. Equilibration of this intermediate with the template would form a “bubble” intermediate. Synthesis from the upstream primer and ligation would prevent any further degradation of the extra repeats. Resolution of this structure during replication would lead to expansion (Fig. 1). The model explains expansion of both trinucleotide repeats and the longer minisatellite repeats.

Using a series of substrates designed to adopt secondary structure, we have examined the ability of FEN1 to resolve the foldback and bubble structures formed in the model. In addition, we examined the ability of PCNA and RPA, which stimulate FEN1 activity, to assist in the removal of these intermediates. Results suggest that the model correctly describes a mechanism for repeat expansion.

**EXPERIMENTAL PROCEDURES**

**Materials—**Oligonucleotides were synthesized either by Integrated DNA Technologies (Coralville, IA) or by Genosys Biotechnologies (The Woodlands, TX). Radionucleotides (γ−32P)ATP (6000 or 3000 Ci/mmol) and (α−32P)dCTP (3000 Ci/mmol) were obtained from NEN Life Science Products. T4 polynucleotide kinase and Klenow fragment of DNA polymerase I (labeling grade) were from Roche Diagnostics. All other reagents were the best available commercial grade.

Recombinant human FEN1 was expressed and purified from *Escherichia coli* utilizing the T7 expression plasmid pET-FCH (34). Recombinant PCNA was expressed in *E. coli* using the expression vector pT7/PCNA (51) or RGS4A (52) and purified. Purified FEN1 and PCNA were dialyzed into storage buffer (30 mM HEPES, pH 7.6 (diluted from a 1 M stock), 30 mM KCl, 20% glycerol, 0.01% Nonidet P-40, 1 mM diithreitol, and 1 mM EDTA) and stored at −80°C. Recombinant human RPA was expressed and purified from *E. coli* using expression vector p11d-trPA (53).

**Oligonucleotide Substrates—**Oligonucleotide primers were designed to form a series of flap or bubble substrates. For flap substrates, the 3’-end of each downstream primer is complementary to the 5’-end of its appropriate template. The 5’-end of the downstream primer forms the unannealed 5’-tail or flap. Within the 5’-end of downstream primers containing secondary structure is an inverted repeat that forms a hairpin loop or foldback. The length of the stem varies from 6–24 nucleotides. Upstream primers anneal to the 3’-end of the template forming a nick at the base of the flap. Bubble substrates contain 25 nucleotides of complementarity at both the 5’- and 3’-ends of the primers, generating an internal unannealed region. Upstream primers anneal to this internal region, forming a nick at the 3’-end of the bubble. Oligomer sequences are listed in Table I. Substrates were constructed as described in the legend.

Prior to annealing, downstream primers were radiolabeled at either the 5’- or 3’-end. Primers (10 pmol) were 5’-end radiolabeled with γ−32P)ATP by T4 polynucleotide kinase as per the manufacturer’s instructions. For 3’-end radiolabeled primers, downstream primers (10 pmol) were annealed to template LAH2.7 (25 pmol), which generates a 5’-overhang and extended with (α−32P)dCTP by Klenow polymerase at 37°C for 3 h. After removal of unincorporated radionucleotides by a Micro Bio-Spin 30 chromatography column (Bio-Rad), all radiolabeled primers were purified by gel isolation from either a 10% or 12% polyacrylamide, 7M urea denaturing gel.

Substrates were generated by annealing a downstream primer, template, and upstream primer at a molar ratio of 1:2:5:5, respectively. A downstream primer and template were placed in 50 μl of TE (10 mM Tris-Cl, pH 8 and 1 mM EDTA) and heated to 100°C for 5 min. The reaction was placed at 70°C and allowed to slowly cool to 25°C. After an upstream primer was added, the mixture was incubated at 37°C for 30 min to 1 h.
D. Downstream primers | \begin{align*} 
D_{\text{control}} & \quad (44-\text{mer}) \quad \text{GACCTGGCCGTCGTGACTGGGAAAACCTGCGG} \\
D_{\text{stem}} & \quad (44-\text{mer}) \quad \text{GCACGTAGACTGGCCGTCGTGACTGGGAAAACCTGCGG} \\
D_{\text{bubble}} & \quad (56-\text{mer}) \quad \text{GCTGTAATAGCCGTCGTGACTGGGAAAACCTGCGG} \\
T_{\text{bubble,2}} & \quad (66-\text{mer}) \quad \text{GCTGTCAGACTGGCCGTCGTGACTGGGAAAACCTGCGG} \\
T_{\text{bubble,2,extend}} & \quad (66-\text{mer}) \quad \text{GCTGTCAGACTGGCCGTCGTGACTGGGAAAACCTGCGG} \\
L_{\text{AH2.7}} & \quad (22-\text{mer}) \quad \text{ACTGCAGTTGGCGTCTGGTAC} \\
U_{25} & \quad (25-\text{mer}) \quad \text{GGCAGGTTTCTCAGCTAGGAC} \\
U_{15} & \quad (15-\text{mer}) \quad \text{TTCCTACGTCAAG} \\
\end{align*} 

\[ a \] Complementary nucleotides involved in formation of secondary structure are underlined. The bold nucleotide represents the first position annealed to the template Tstem.

**Enzyme Assay**—Assays contained the indicated amounts of substrate and FEN1 in reaction buffer (30 mM HEPES, pH 7.6 (diluted from a 1 M stock), 40 mM KCl, 8 mM MgCl2, 5% glycerol or 0.01% Nonidet P-40, and 0.1 mg/ml bovine serum albumin) in a final volume of 20 μl. Assays were incubated at 37 °C for 15 min and stopped by the addition of 10 μl of termination dye (95% formamide (v/v) with bromphenol blue and xylene cyanol). After heating to 95 °C for 5 min, samples were separated on a 12% polyacrylamide, 7M urea denaturing gel. Products were detected by PhosphorImager (Molecular Dynamics) and analyzed using ImageQuant v1.2 software from Molecular Dynamics. All assays were performed at least in triplicate.

**RESULTS**

We propose that certain FEN1 substrates form secondary structures that influence the kinetics and specificity of cleavage. We examined FEN1 cleavage on substrates with foldbacks in the flap and bubble structure intermediates. This permits us to assess their importance in the context of the model for repeat expansions.

**Cleavage of Flap Substrates Containing Secondary Structure**—To determine whether FEN1 cleaves flaps containing secondary structure, we designed substrates with an inverted repeat within the 5'-end of the flap that forms a stem-loop structure. The length of the annealed region of the stem varied from 6 to 24 nucleotides, and the loop contained 6 nucleotides. To control for the effect of sequence, each of the stem loops had similar nucleotide composition and GC content. The control substrate released a 25-nucleotide product (lanes 6–10), with similar specificity as the flap with no foldback. Cleavage of the 12-nucleotide stem (lanes 11–15) yields both a full-length product from release of the 5'-tail and more significant levels of mono- and dinucleotide products. Cleavage of the 24-nucleotide stem resulted in only the smaller mono- and dinucleotide products (lanes 16–20).

As the foldback stem became more stable with increasing length, FEN1 was blocked from cleaving directly at the base of the flap. If the presence of the base of the flap is necessary for cleavage, the cleavage products would be 24, 36, and 60 nucleotides in length for the 6, 12, and 24 nucleotide stem substrates, respectively. FEN1 is only able to readily cleave the 6-nucleotide stem (lanes 6–10) with similar specificity as the flap with no foldback. Lengths of the annealed region of the stem varied from 6 to 24 nucleotides, and the loop contained 6 nucleotides. To control for the effect of sequence, each of the stem loops had similar nucleotide composition and GC content. The control substrate released a 25-nucleotide product (lanes 6–10), with similar specificity as the flap with no foldback. Cleavage of the 12-nucleotide stem (lanes 11–15) yields both a full-length product from release of the 5'-tail and more significant levels of mono- and dinucleotide products. Cleavage of the 24-nucleotide stem resulted in only the smaller mono- and dinucleotide products (lanes 16–20).
the flap. Instead, FEN1 cleaved the 5’-end of the self-annealed tail, generating mono- or dinucleotide products. These products are expected because the foldback is a substrate for the 5’-exonuclease activity of FEN1 (1, 17). Appearance of the mono- and dinucleotide products also confirms the ability of each sequence to adopt the predicted secondary structure, because FEN1 is an obligate double-stranded exonuclease. As the length of the annealed region increases, so does the stability of the foldback, allowing the nuclease to more efficiently attack the 5’-end. However, cleavage of the 5’-radiolabeled nucleotide prevents further analysis regarding the fate of the stem loop present within the flap. It is possible that FEN1 continued to cleave as an exonuclease until the double-stranded region was sufficiently small that the stem melted, forming a more favorable flap structure for FEN1.

For further examination of the effect of secondary structure, substrates were radiolabeled at the 3’-end of the downstream primer and incubated with increasing amounts of FEN1. To determine more precisely the length of secondary structure necessary to influence FEN1 activity, the annealed regions of the stems were made 6, 12, 18, and 24 nucleotides long. Because the length of the annealed downstream region of each substrate is identical, cleavage by FEN1 resulted in the generation of a 19-mer for all substrates (Fig. 3A). FEN1 cleavage was easily detectable on the control substrate and substrates containing 6 and 12 nucleotides (Fig. 3A, lanes 1–15). The ability of FEN1 to cleave the 18- and 24-nucleotide stem substrates is significantly reduced (Fig. 3A, lanes 16–25). At higher levels of FEN1, additional cleavage products smaller than the 19-mer are observed. These are the result of exonuclease cleavage within the annealed double-stranded region of the downstream primer. We also observed some exonuclease-removal of the 5’-end as expected from the results of Fig. 2. Production of the 19-mer does not appear to derive from progressive exonuclease cleavage through the stem, because there are no detectable intermediates between the full-length substrate and the product. These data were quantitated and plotted as the percentage of substrate converted to the 19-nucleotide and smaller products versus the amount of FEN1 (Fig. 3B). It is readily apparent that there is a transition between 12 and 18 nucleotides within the stem that has a major inhibitory affect on FEN1 cleavage. Kinetic analysis of the degradation of 3’-radiolabeled substrate over time produced results consistent with those obtained here by increasing FEN1 concentration (data not shown).

Effect of the Position of the Secondary Structure Relative to the Cleavage Site—We were concerned that the inhibitory effect of foldbacks reflects a steric hindrance by the stem loop of FEN1 interaction with the cleavage site. This would prevent FEN1 from cleaving the substrate and would most likely correlate with the stability of the stem-loop structure. We examined this possibility by varying the distance from the 5’-end of the stem to the first nucleotide annealed to the template (designated b in Fig. 4). Substrates containing the 18-nucleotide stem were lengthened to place 0, 6, or 18 nucleotides between the 5’-end of the stem and cleavage site. Again, the expected product for all substrates is a 19-mer representing the annealed portion of the downstream primer. The control was easily cleaved by FEN1 (Fig. 4, lanes 1–5) as compared with the less efficient cleavage observed for the 18-nucleotide stem at the 6-nucleotide distance (Fig. 4, lanes 11–15). This distance is equivalent to that of the 18-nucleotide stem used in Fig. 3. Placement of the stem at a distance of 0 or 18 nucleotides had little additional affect on FEN1 activity (Fig. 4, lanes 6–10 and 16–20). The cleavage of all three of these substrates is reduced to a similar extent. We conclude that the inhibitory nature of secondary structure results from the requirement of a free 5’-end by FEN1. These results continue to demonstrate the importance of the 5’-end of the substrate for the cleavage mechanism of FEN1.

Cleavage of Trinucleotide Repeats by FEN1—Recently, there has been significant interest in the mechanism of trinucleotide repeat expansion. One model suggests that the ability of these repeats to adopt secondary structure prevents their removal during DNA replication and repair (37). Given that secondary structure is inhibitory to FEN1, we examined the effect of trinucleotide repeats within flap structures. The substrates that we designed contained an increasing number of CTG repeats at the 5’-end of the flap.

Substrates with either 5, 10, or 20 CTG repeats in the flap ((CTG)_5, (CTG)_10, or (CTG)_20) were incubated with FEN1 over time (Fig. 5A). The control substrate, (CTG)_5, was cleaved efficiently by FEN1 (lanes 1–6 and 13–24). The ability of FEN1 to remove the (CTG)_5 was significantly reduced as compared with the other substrates (lanes 7–12). The data were quantitated and plotted as the percent of substrate converted to product versus time (Fig. 5B). During the reaction at 10 min,
FEN1 Cleavage of Flap Secondary Structures

Fig. 4. The location of secondary structure does not increase FEN1 cleavage. The distance from the 5′-end of the foldback to the cleavage point may influence the ability of FEN1 to resolve these structures. Substrates containing a 12-nucleotide foldback with 0, 6, or 18 nucleotides between the foldback and cleavage point were incubated with increasing amounts of FEN1 (10, 50, 100, and 500 fmol) at 37 °C as described under “Experimental Procedures.” Reactions in lanes 1, 6, 11, and 16 contain only a substrate. A schematic diagram of the substrates is depicted above the figure. The length (in nucleotides) of distance between the 5′-end and the cleavage site is designated as b. The control substrate contains a standard flap structure. The size (in nucleotides) of substrates and location of cleavage products are as indicated. Annealing primers Dcontrol, Tstem, and U25 generated the control substrate. Each foldback substrate contains D18 stem/0 gap, D12 stem, or D18 stem/18 gap annealed to Tstem and U25.

FEN1 cleaved ~45, 41, and 35% of the control, (CTG)5, and (CTG)10, respectively. By comparison, only 3% of the (CTG)20 substrate was converted to product by FEN1. The inhibition of FEN1 by the (CTG)20 most likely reflects the ability of this sequence to form a stable secondary structure (54). As with the stem-loop structures used in the previous experiments, we do not observe any intermediates resulting from the exonucleolytic degradation of the 5′-end of the substrate. However, we were able to observe exonucleolytic degradation of the (CTG)20 at extremely high levels of FEN1 (data not shown).

Cleavage by FEN1 of Intermediates Proposed to Form during Repeat Expansion—The model of repeat expansion (Fig. 1) includes an intermediate in which the flap anneals to the template forming a bubble. Because a free 5′-end of the flap appears to be required for efficient FEN1 activity (23), the bubble is expected to be a poor substrate. It may not be completely inert, however, because FEN1 can degrade the annealed 5′-region exonucleolytically until the flap becomes single-stranded.

To examine the course of events when FEN1 encounters such an intermediate, we created a model bubble substrate. The top strand has 25-nucleotide regions at both its 5′- and 3′-ends annealed to the bottom strand, with a central 30-nucleotide unannealed bubble region. The bottom strand has a central 25-nucleotide unannealed region. The different lengths of the top and bottom single strands are designed to encourage the strands to physically separate into structures resembling flaps. The control substrates contain a different bottom strand lacking an upstream primer resulted in products of differing sizes. These products arise because nucleotides within the long flap transiently anneal to the template upstream of the base of the flap, generating alternate cleavage sites. The presence of the upstream primer blocks transient annealing of the downstream primer to the template, eliminating the longer cleavage products seen in the absence of the upstream primer.
A 5' substrate. The control contains primer D control annealed to Tstem (lanes 1–7). At these levels of FEN1, very low amounts of cleavage are observed on the control substrate. However, addition of PCNA results in a dramatic increase in cleavage products (compare lane 2 with lane 5). Stimulation of FEN1 by PCNA is seen for the 6- and 12-nucleotide stem substrates (lanes 6–15). However, little enhancement occurs on the 18- and 24-nucleotide stem structures (lanes 16–25). Although PCNA can stimulate FEN1, PCNA is unable to alter the substrate specificity for cleavage and reduce the negative effects of secondary structure. The data for the control and the 12- and 24-nucleotide stems were quantitated and plotted as a fold stimulation of FEN1 cleavage versus PCNA concentration (Fig. 7B). The results indicate that although FEN1 cleavage is substantially increased by PCNA, the level of cleavage of a 12-nucleotide foldback substrate was not enhanced relative to that of the control. In fact, the presence of PCNA was unable to generate any cleavage from the nearly inert 24-nucleotide foldback substrate. It is readily apparent that the capacity for PCNA to stimulate FEN1 is limited by the intrinsic ability of the nuclease to cleave a particular substrate. PCNA improves the reactions performed by FEN1 without altering its specificity. High levels of PCNA are needed for these reactions because loading of PCNA onto a linear substrate is dependent on diffusion.

In addition to PCNA, the single-stranded DNA-binding protein RPA also stimulates FEN1, although to a lesser degree (–2–3-fold) (60). The ability of RPA to bind DNA and cause local unwinding may aid in the formation of a single-stranded flap better suited to FEN1 cleavage. We tested the ability of RPA to stimulate cleavage of foldbacks under standard conditions for FEN1 cleavage and under varying salt and MgCl₂ concentrations. Although we observed a moderate increase in the activity of FEN1 in the presence of RPA on control sub-

![Image](http://www.jbc.org/)
inhibitors of cleavage. For example, 20–30% inhibition does not occur in our assays until the complementarity of the foldback is 12 nucleotides. The likelihood of a perfect hairpin of 12 nucleotides forming in a region of, for example, 60 nucleotides is about 1 in 1000. This makes the opportunity for formation of a large foldback infrequent within the genome. Of course, because of their large numbers, the 5'-ends of Okazaki fragments will not be far from any site that can form a very stable hairpin.

Considering the deleterious effects of sequence expansions, we expect that the normal processes of DNA replication and repair have evolved to resist expansion by preventing steps in the expansion mechanism. Taking this view suggests explanations for two puzzling features of the current model of Okazaki fragment processing. First, why does FEN1 have an exonuclease activity? It would appear to be able to perform all of its functions in both Okazaki fragment processing and DNA repair as an endonuclease. The exonuclease function may be retained to degrade the annealed regions of flap foldbacks and bubble substrates. Our results show that the double-stranded portions of these substrates can yield to progressive exonucleolytic degradation from the 5'-end. This produces shorter complementary regions, which are more prone to transient unannealing that allows entry of FEN1. It is possible that this function alone provides the necessary evolutionary pressure to retain exonuclease activity.

Second, reconstitution of efficient Okazaki fragment processing in vitro strongly suggests a role for RNase H. Nevertheless, deletion of RNase H activities in yeast have little effect on the efficiency of Okazaki fragment processing (61). Because the primer displacement pathway apparently back up the use of RNase H, is the role of RNase H in DNA replication simply to provide an alternative pathway for RNA removal? A different possibility is that it is necessary to efficiently degrade foldbacks when Okazaki fragments still containing the initiator RNA are displaced. In this case, the foldback would be partially RNA. Annealed 5'-triphosphorylated RNA segments resist the exonuclease activity of FEN1. RNase H, however, readily degrades them. In this case, RNase H would not appear to be necessary for cellular viability but might help to control genome stability.

Results presented here provide further evidence that FEN1 tracks down the flap to the position of cleavage, because blockage of the 5'-end region during either a foldback or bubble formation is inhibitory. Cleavage by FEN1 previously was shown to be inhibited by primers annealed to the flap, by biotin-streptavidin complexes on the flap and by some chemical adducts (18, 23, 34). Analysis of the structures of members of the FEN1 family of nucleases has revealed the presence of a helical arch or loop in the protein just adjacent to the proposed site of catalytic activity. This led to the proposal that FEN1 threads the flap through the hole created by this structure, sliding to the site of cleavage. Measurements showing that the hole was appropriate in size for single-stranded but not double-
stranded DNA appeared to explain inhibition by annealed primers (29). However, we have recently shown that branch structures on the flap, far from the site of cleavage, are not inhibitory (34). This result would appear to rule out a threading mechanism, in favor of a tracking process that allows for certain large flap modifications. Proving with absolute certainty that a tracking process occurs remains an elusive goal. Virtually every relevant experiment involves blocking the movement of FEN1 on the flap. Results presented here also fall into that category. However, any modification of the flap could also potentially influence interaction of FEN1 with the cleavage site. At this point, a preponderance of evidence, rather than a definitive experiment, supports the tracking mechanism.

Overall, our results show how flap secondary structure and bubble formation can promote steps in a proposed mechanism for repeat sequence expansion. A recent report by Spiro et al. (54) also provides convincing evidence that triplex repeat sequences form structures resistant to FEN1 activity, providing (55) also provides convincing evidence that triplex repeat sequence expansion. A recent report by Spiro et al. (54) also provides convincing evidence that triplex repeat sequences form structures resistant to FEN1 activity, providing convincing support for the proposed model. Furthermore, the inhibition of FEN1 by these structures provides explanations for the exonuclease activity and the apparent role of RNase H in Okazaki fragment processing.

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