Human flap endonuclease 1 (h-FEN1) mutations have dramatic effects on repeat instability. Current models for repeat expansion predict that h-FEN1 protein prevents mutations by removing 5'-flaps generated at ends of Okazaki fragments by strand displacement synthesis. The models propose that hairpin formations within flaps containing repeats enable them to escape h-FEN1 cleavage. Friedreich's ataxia is caused by expansion mutations in a d(GAA)n repeat tract. Single-stranded d(GAA)n repeat tracts, however, do not form stable hairpins until the repeat tracts are quite long. Therefore, to understand how d(GAA)n repeat expansions survive h-FEN1 activity, we determined the effects of h-FEN1 on d(GAA)n repeat expansion during replication of a d(TTC)n repeat template. Initiation within the repeat tract generated significant expansion that was suppressed by the addition of h-FEN1 at the start of replication. The ability of h-FEN1 to suppress expansion implies that DNA slippage generates a 5'-flap in the nascent strand independent of strand displacement synthesis by an upstream polymerase. Delaying the addition of h-FEN1 to the replication reaction abolished the ability of h-FEN1 to suppress d(GAA)n repeat expansion products of all sizes, including sizes unable to hairpin. Use of model substrates demonstrated that h-FEN1 cleaves d(GAA)n 5'-flaps joined to double-stranded non-repeat sequences but not those joined to double-stranded repeat tracts. The results provide evidence that, given the opportunity, short d(GAA)n repeat expansion products rearrange from 5'-flaps to stable internal loops inside the repeat tract. Long expansion products are predicted to form hairpinned flaps and internal loops. Once formed, these DNA conformations resist h-FEN1. The biological implications of the results are discussed.

Repeat expansion mutations are responsible for more than 16 neuromuscular diseases including Friedreich’s ataxia, myotonic dystrophy, and Fragile X syndrome (1). Disease-associated repeat expansions are generated during transmission from parent to child and vary from the addition of a few repeats to the addition of thousands of repeats within tracts that normally contain 20–30 tandem repeats (1). The first models to explain repeat expansion invoked DNA slippage (2) to form loops within the repeat tract during lagging strand DNA synthesis (3–5). A more recent model (6) employs the observation that mutations that disable the flap endonuclease 1 (FEN1) homolog, RAD27, in yeast greatly destabilize DNA repeats; the FEN1 mutations preferentially generate repeat expansions (8, 9). A RAD27 null mutation is responsible for at least a 1000-fold increase in large duplication mutations associated with Okazaki flap processing (10). The model proposes that 5'-flap formation (6), generated by DNA strand displacement synthesis during lagging strand DNA replication, is the primary event mediating the occurrence of repeat expansion. Secondary structures forming within the flap that are resistant to cleavage by FEN1 would protect the flap from repair, resulting in an expansion mutation. Later refinements of the model require conversion to an internal loop conformation to allow ligation of the expanded nascent strand (11).

FEN1 contains both double-stranded DNA 5'-exonuclease and single-stranded DNA 5'-flap endonuclease activities (12, 13) and is predicted to protect against repeat expansion. FEN1 is required for completion of lagging strand DNA replication (14–17). Currently, evidence indicates that RNA primers associated with the 5'-end of Okazaki fragments are displaced during DNA synthesis to produce single-stranded DNA 5'-ends or flaps. Most of the resulting 5'-flap is removed by Dna2 protein; FEN1 trims the remainder of the flap to create a nick for ligation (18). FEN1 hapolinsufficiency in a mouse model lacking one copy of the adenomatous polyposis coli (Apc) gene generated tumors with microsatellite instability (19). Based on the evidence and the latest models of repeat expansion mutagenesis, one might predict that mutations in h-FEN1 cause repeat expansion. Genetic analyses of repeat expansion disease inheritance patterns show, however, that the diseases map to loci containing the repeat expansions associated with each disease (reviewed in Ref. 20), not to the h-FEN1 locus. Furthermore, no mutations in h-FEN1 were detected in a study of patients with the repeat expansion disorder Huntington’s disease (21). Thus, some aspect of the DNA expansion process would appear to be able to defeat apparently normal FEN1 activity.

The biochemistry of h-FEN1 has been studied using synthetic constructs to test the different DNA structures that are subject to h-FEN1 activity (11, 22–25). FEN1 protein gains access to the DNA flap at the free 5'-end (26). After gaining access, FEN1 translocates to the junction of the flap with double-stranded DNA where cleavage occurs (12, 13, 27, 28). Thus, hairpin formation that involves the 5'-end of the DNA flap blocks FEN1 activity in vitro, apparently by denying access to the DNA flap.

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of FEN1 to the single-stranded 5’-end of the DNA flap (23, 24), and is predicted to result in annealing of the hairpinned flap to the template where ligation produces repeat expansion (6). A study using CTG repeat constructs, however, provides evidence that the ability of the flap to be ligated to the upstream DNA fragment is inhibited by a hairpin within the flap (25). Formation of a loop within the expanded repeat tract appears to be necessary to allow ligation of the expansion to the upstream DNA fragment (11).

Here, we apply an in vitro model of repeat expansion (29, 30) to determine the effects of h-FEN1 on d(GAA)n, (abbreviated GAA) repeat expansion products during DNA replication of a TTC repeat tract in vitro by human polymerase β. GAA repeat expansion in the first intron of the frataxin gene is the most common mutation (98%) causing Friedreich’s ataxia (31). A wide size range of expansion products is generated during DNA replication in vitro (29). Important for this study, small (15–30 base) single-stranded DNA GAA repeat tracts do not form stable hairpins in solution under nearly physiological conditions (32–34) and in yeast (35), whereas longer GAA repeats (>100 bases) do form stable hairpins (29). Therefore, when constituting a flap, small GAA repeat expansions are expected to be targets for h-FEN1 cleavage, allowing us to test whether factors other than hairpin formation in the 5’-flaps involving repeat tracts are required for protection from FEN1 cleavage. Our results demonstrate that h-FEN1 is sufficient to suppress GAA repeat expansion during DNA replication in vitro. To do so, however, required h-FEN1 to be active throughout replication of the GAA repeat tract. H-FEN1-resistant structures were formed if the addition of h-FEN1 to the DNA replication reaction was delayed for even a brief period. The results provide evidence that (GAA)n, primer slippage is sufficient to generate the required 5’-flap repeat expansion intermediate. Formation of the flap does not require strand displacement synthesis by an upstream polymerase. Moreover, experiments with model substrates provide additional evidence that, in the absence of FEN1 activity, relatively short GAA 5’-flaps rearrange to loops inside the double-stranded repeat tract. Larger GAA flaps are predicted to form either internal loops/hairpins or hairpin structures within the 5’-flap. All of these structures are resistant to FEN1. The new results enable refinement of previous models for the expansion pathway. The implications of the new model for genomic instability diseases are discussed.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides**—Oligodeoxyribonucleotides (oligonucleotides) were synthesized by the Lineberger Nucleic Acids Core Facility at the University of North Carolina (Chapel Hill, NC). DNA replication reactions were performed using synthetic oligonucleotide template-primers (Table 1). Repeat template oligonucleotides 1 and 2, d(U(TTC)22)U, and d(U(TTC)20)U, contained a 27- or 30-nucleotide TTC repeat sequence flanked by two 12-nucleotide unique sequences (U′ and U″). The non-repeat template (oligonucleotide 3; Table 1) d(U′(TTC)20)U, contained 30 nucleotides flanked by the U′ and U″ sequences. Replication templates were synthesized with three-carbon tails added to the 3′ ends of the oligonucleotides 4 and 5 (Table 1). Nonreplication (static) substrates for h-FEN1 contained “flap/loop” (so called because, in principle, they could form both flaps and loops when annealed to the templates) oligonucleotides 6–12 (Table 1) annealed to templates. All oligonucleotides were gel-purified to >95% purity using either 10 or 12% polyacrylamide denaturing gels (7.5 M urea), except for oligonucleotides 4 and 5, which were found to be >95% pure by gel electrophoresis. All oligonucleotides were desalted using Roche Quick Spin G-25 columns.

**Enzymes**—Recombinant human pol β was overexpressed in Escherichia coli from a construct (pWL11 in TAP56 E. coli, generously provided by Sam Wilson) and purified to >95% homogeneity based on methods described elsewhere (36). H-FEN1 protein and h-FEN1 constructs (pGET-FCH in BL21(DE3)pLysS E. coli) were generously provided by Sam Wilson and Michael Lieber, respectively. H-FEN1 was >95% pure by SDS-PAGE.

**Enzyme Reactions**—h-pol β replication products were either 5’-end-labeled or internally labeled during replication. To make end-labeled products, primer was 5’-end-labeled prior to annealing using T4 polynucleotide kinase (New England Biolabs) and [γ-32P]ATP (PerkinElmer Life Sciences) and passed through G-25 spin columns (Roche Applied Science) to remove unincorporated [32P]ATP and kinase buffer. To make internally labeled reaction products, primer was not labeled, but [α-32P]dATP (PerkinElmer Life Sciences) was included in the replication reaction. Prior to replication, DNA primer (60 nm) and template (60 nm) molecules were annealed by incubating at 95–100 °C for 5–10 min in 10 mM Tris-HCl (pH 8.0) and 20 mM MgCl2 and allowed to cool slowly to 4 °C. Reaction products contained, in a final volume of 20 μl, 30 nm annealed primer-template, 50 mM Tris-HCl (pH 8.0), 10 mM MgCl2, 20 mM NaCl, 2 mM dithiothreitol, 2.5% glycerol, and 220 nM h-pol β. Reactions with [32P]-end-labeled primers also contained 280 μM of dNTPs, whereas reactions with unlabeled primers contained 28 μM dNTPs, plus 5 μCi of [32P]-labeled dATP. Reactions were assembled on ice, and transferring the reaction to 37 °C immediately after the addition of h-pol β started replication. All reactions were incubated at 37 °C for the lengths of time indicated in the figures. Reactions were stopped by the addition of formamide and EDTA (to a final concentration of 47.5% formamide and 10 mM EDTA) to reactions using end-labeled primers by the addition of EDTA (final concentration 50 mM) to reactions using labeled dATP, followed by cooling to 4 °C. Internally labeled reaction products were passed through G-25 spin columns (Roche Applied Science) to remove unincorporated label, and formamide plus EDTA (final concentration of 47.5% formamide and 10 mM EDTA) were added prior to loading on polyacrylamide gels.

Flap/loop substrates were prepared by annealing repeat and non-repeat templates with oligonucleotides 6–14 (Table 1). The repeat template d(U′(TTC)20)U was annealed to oligonucleotides (oligonucleotides 9–12; Table 1) consisting of 9, 10, 12, or 15 GAA repeats plus 12 nucleotides complementary to the 5′ unique region (U′) in the template. This created template-primers that could, in principle, contain flap expansions in equilibria with loop conformations. For comparison, a nonrepeat template, d(U′(U′)20)U′, was annealed with oligonucleotides (oligonucleotides 6–8; Table 1) complementary to U′ and U″ in the template. This created a template-primer substrate with no flap and fixed flaps of one and three GAA repeats, respectively. h-FEN1 activity was assayed using a control substrate (oligonucleotides 13 and 14 annealed) adapted from Ref. 2. The control substrate contained a 20-nucleotide flap lacking any repeats. Reaction conditions were identical to those of the replication reactions above, except the final reactions contained 10 nm annealed primer-template. h-pol β (but no dNTPs) was included in some reactions using flap/loop substrates to test the effects of h-pol β on h-FEN1 activity. H-FEN1 was added at the start of the reactions, which were at 37 °C for 20 min. Reactions were stopped by the addition of formamide and EDTA (to a final concentration of 47.5% formamide and 10 mM EDTA) and cooled to 4 °C.

A 10-base pair ladder (Invitrogen) was 5’-end-labeled as described above and used for estimating sizes of replication and expansion reaction products by gel electrophoresis. For determining the sizes of reaction products ~10 nucleotides in length, end-labeled (GAA), primer was partially digested with P1 nuclease (Sigma) in 200 mM NaCl, 50 mM sodium acetate (pH 7.4), 1 mM ZnSO4, and 5% glycerol to generate a 1-nucleotide ladder.

All reaction products were denatured by incubating at 100 °C for 10 min and were separated on 15 or 20% polyacrylamide denaturing gels (7.5 M urea) for 3–4 h at 300–500 V. Gels were run at a constant temperature of 50–55 °C. Bands were visualized using a Storm PhosphorImager (Amersham Biosciences). To determine relative amounts of products of interest, appropriate bands were quantitated using ImageQuant 5.0 image analysis software (Amersham Biosciences). Band densities were corrected for background and expressed as a percentage of the total lane density. The corresponding migration of DNA fragments on a reference lane, as indicated under “Results.” To determine the relative amounts of expansion products per lane, all products larger than the full-length replication product were quantitated and expressed as a percentage of total lane density. To determine the relative amount of products per lane that were ~100 nucleotides long, a rectangle was drawn around the perimeter of the 100-nucleotide marker band and allowed to cool slowly to 4 °C. The marker band was superimposed on each of the lanes of interest, at a position matching the mobility of the 100-nucleotide marker band. Plots of the amount of reaction products were generated with Microsoft Excel software.
Transient Suppression of Repeat Expansion by h-FEN1

RESULTS

Replication Primed from within the Repeat Tract Generates Large Amounts of Repeat Expansion—To characterize the effects of h-FEN1 on triplet repeat expansion during DNA replication, we utilized h-pol β and in vitro replication to generate repeat expansion (bottoms of Figs. 1 and 2) (29, 30). Human pol β is subject to DNA slippage (37) and generates significant amounts of expansion with our template primers (29). We assume that h-FEN1 and DNA polymerase, under our conditions, act independently at opposite ends of the nascent strand. Substrate was composed of two 12-nucleotide flanking sequences and a 30-nucleotide TTC repeat tract (Fig. 1). Replication from a 12-nucleotide unique primer complementary to the upstream 12-base unique sequence Uₕ generated a full-length (unexpanded) product of 54 nucleotides. Replication from a 9-base (GAAₐ)ₙ repeat primer complementary to the (TTC)ₙ repeat tract produced multiple bands, indicating products of eight different sizes (21, 24, 27, 30, 33, 36, 39, and 42 nucleotides in length). The multiple products were assumed to result from the ability of the three-repeat primer to anneal to the 10-repeat template at any one of eight different positions along the repeat tract. The seven products, smaller than the predicted 42-nucleotide full-length product (30-nucleotide repeat tract plus downstream 12-base flanking sequence), were called “partial-length” products. Replication using either primer was completed within the first 5 min (Fig. 1). The amounts of repeat expansion (measured in a given lane from the band density above the fully replicated products, indicated by the arrows in the figure) in both the unique and repeat primer reactions were quantitated and expressed as a percentage of all reaction products (the total band density in the lane). For all time points tested subsequent to 0 min, reactions using the GAA repeat primer generated 7-fold larger amounts of repeat expansion (e.g., 21 ± 3% repeat expansion at 30 min (Fig. 1, lane 7), n = 4) than did reactions using the unique primer (3 ± 2% repeat expansion at 30 min (Fig. 1, lane 13), n = 4). The GAA expansion products have previously been sequenced and were found to be perfect GAA repeats (29). Because of the greater amounts of expansion observed in reactions primed from within the repeat tract, we used GAA repeat priming for all subsequent experiments.

FEN1 Suppresses Formation of Expansion Products during DNA Replication—Since FEN1 is predicted to play a critical role in suppressing repeat instability (6, 8–10, 19, 38), we examined the effect of h-FEN1 protein on GAA expansion. GAA repeat expansion reactions were titrated with h-FEN1 (Fig. 2A), which was added to the reaction simultaneously with h-pol β, and the amounts of repeat expansion were quantitated (Fig. 2C). The template repeat tract contained nine tandem TTC triplets versus the 10 repeats used above in Fig. 1, allowing us to test the assumption regarding the origin of the partial-length products. A three-repeat primer complementary to a nine-repeat template predicts a full-length product 39 nucleotides long plus six partial-length products (21–36 nucleotides). These seven bands are observed, confirming the origin of the partial-length products. When increasing quantities of h-FEN1 were included in the replication reactions, decreasing amounts of repeat expansion products were observed. As seen in Fig. 2, there was an initial increase in the total amount of expansion at the lowest concentrations of h-FEN1 (30 and 60 nM h-FEN1, lanes 2 and 3) compared with when no h-FEN1 was added (lane 1). Since this increase was small and was not observed in other types of experiments, as shown below, its precise cause is unknown. One possible explanation comes from studies that used a gapped DNA substrate to indicate that FEN1 can stimulate h-pol β activity during long patch base excision repair (39). For all experiments, expansion decreased sharply with h-FEN1 concentrations above 60 nM, reaching an average of an 82% decrease (±6%, n = 5) in total expansion at 240 nM h-FEN1 (lane 6), relative to the maximum expansion observed. Formation of large expansion products was suppressed to a greater extent than was total expansion with increasing h-FEN1 concentrations. For example, at 90 nM h-FEN1 (lane 4), formation of products ~100 nucleotides long was suppressed by 88%, whereas total expansion was suppressed by 28% (both percentages relative to 60 nM h-FEN1, where maximum expansion occurred). Full-length and nearly full-length products were least affected by h-FEN1 activity. h-FEN1 also decreased the amount of partial-length products in a manner that depended on the size of the product (Fig. 2A); smaller partial-length products were affected most, whereas larger products were affected least. Because the repeat primers in these reactions had been 5’-end-labeled, it was also possible to detect the presence of small FEN1 cleavage products. A significant amount of 1-nucleotide product was increasingly visible, since greater quantities of FEN1 were present in the reactions. For example, we observed an average of a 7-fold (±3-fold) increase in the amount of 1-nucleotide products at 120 nM h-FEN1 (lane 5) compared with no h-FEN1. In addition, there was an increase in the amount of products 3 nucleotides (2-fold ± 1-fold at 120 nM h-FEN1), 4 nucleotides (2-fold ± 1-fold at 120 nM h-FEN1), 9 nucleotides, 11 nucleotides, and 12 nucleotides in length. The increases in 9-, 11-, and 12-nucleotide products were not observed in all experiments. 
Because products were end-labeled, the reduction in the amounts of repeat expansion products with increasing h-FEN1 in Fig. 2A were potentially due to the removal of a single nucleotide from the radiolabeled 5’-end of the replication products, rendering the repeat expansion products undetectable. Therefore, replication was performed with unlabeled template-primers in the presence of radiolabeled dATP, generating internally labeled replication products (Fig. 2B). Replication from a GAA repeat primer generated large amounts of repeat expansion products in the absence of h-FEN1 (Fig. 2B, lane 1). Note that the template for these reactions contained 10 TTC repeats, resulting in a 42-nucleotide full-length product. The addition of h-FEN1 suppressed formation of internally labeled repeat expansion products, since it had done with the end-labeled products. In contrast to the reactions with end-labeled products, there was no initial increase in the total amount of expansion at the lowest concentrations of h-FEN1. Increasing concentrations of h-FEN1 had less of an effect on the internally labeled full-length and partial-length products than it did for the end-labeled full- and partial-length products. For example, at 240 nM h-FEN1, there was a 38% decrease in the amount of full-length product observed in the end-labeled reactions (Fig. 2A, lane 6), compared with an 82% decrease in the amount of internally labeled full-length product observed (Fig. 2B, lane 5). In addition, when products were internally labeled, increasing amounts of 8-, 9-, 10-, and 11-nucleotide products were detected with increasing concentrations of h-FEN1 (e.g., 5–9-fold with 120 nM h-FEN1) (Figs. 2B and 3A).

Delaying the Addition of FEN1 Dramatically Decreased Suppression of GAA Expansion—FEN1-resistant structures are predicted to be at risk for producing repeat expansion mutations (6). To test for the formation of such structures during GAA repeat expansion, the addition of FEN1 (to a final concentration of 120 nM) was delayed from 0 to 60 min after the addition of h-pol (Fig. 3, A (lanes 6–11) and B). At 0, 5, 30, and 60 min, respectively, the formation of 100-nucleotide expansion products was suppressed 62, 45, 0, and 0%, relative to no h-FEN1 added. The results demonstrated that the ability of h-FEN1 to suppress expansion was lost if the addition of h-
FEN1 to the replication reaction was delayed. There was a 27% loss of h-FEN1 suppression of expansion after a 5-min delay (100/62 - 45/62 = 27%); complete loss of h-FEN1 suppression was observed if the addition of h-FEN1 was delayed by 30 min. Additional experiments showed a 25% loss of h-FEN1 suppression of repeat expansion after a delay of only 2 min (not shown). Delays less than 2 min were not studied. Partial-length products were still suppressed when the addition of h-FEN1 was delayed. For example, h-FEN1 only lost 40% of its ability to decrease the amount of 21-nucleotide partial-length product when added after a 30-min delay (Fig. 3B) compared with the complete loss of suppression for the 100-nucleotide repeat expansion product. The effects of delayed addition of h-FEN1 on expansion were independent of whether the products were end-labeled or internally labeled. A similar loss of repeat expansion suppression was observed when twice as much h-FEN1 (to final concentrations of 480 nM) added simultaneously with h-pol β. Lanes 6–11, 120 nM h-FEN1, with h-FEN1 added at the time interval indicated after the start of replication with h-pol β. Lane 12, 10-base ladder. B, the delayed addition of h-FEN1 to end-labeled repeat expansion reactions. Reactions used end-labeled (GAA)₃ primer and 120 nM h-FEN1, which was added at the interval indicated after the start of the reaction, and incubation continued for 20 min. At each interval (0–60 min) with or without h-FEN1, the total amount of replication time is equivalent. The arrows indicate the full-length replication products at 39-base length. Reaction conditions are described under “Experimental Procedures.”

Short GAA Expansion Products Form FEN1 Endonuclease-resistant/FEN1 Exonuclease-sensitive Structures—Our results showed that if the addition of h-FEN1 to the replication reactions was delayed, the ability of h-FEN1 to suppress expansion was lost. This suggested a mechanism whereby flap structures sensitive to h-FEN1 form early in replication and, in the absence of FEN1 activity, grow and rearrange into conformations that resist h-FEN1 cleavage activity. Because short GAA repeats do not hairpin (32–35), we studied the susceptibility to h-FEN1 of short predicted expansion products. Synthetic substrates were generated to resemble the potential products of replication from the GAA repeat primer. Control substrates were also generated to contain no flap and fixed flaps of one and three GAA repeats. Oligonucleotides used in these experiments are listed in Table I. Annealed substrates were incubated with increasing amounts of h-FEN1 (Fig. 4), and the resulting cleavage products were quantified, and expressed as a percentage of the total products in each lane, as described under “Experimental Procedures.” Higher concentrations of h-FEN1 were used in these experiments than in the replication experiments to facilitate detection of the weaker 5′-exonuclease activity of h-FEN1, which is highest at nicks in duplex DNA and lower at gaps (≥1 nucleotide) and recessed 5′-ends (12, 13).

The nonrepeat template U₅(T₃)U₄, which was fully complementary to the U₅ and U₄ regions of the template, generating a double-stranded substrate lacking a flap and containing a recessed 5′-end (lanes 2–5). Since the U₅U₄ fragment was radiolabeled at the 5′-end, uncut products appeared as a 42-nucleotide band, which decreased in intensity with increasing amounts of h-FEN1 in the reactions. The most prominent cleavage product for this substrate was a 1-nucleotide fragment (73.4% of total reaction products at 960 nM h-FEN1; lane 5), which is consistent with the 5′-exonuclease activity of h-FEN1 acting on the recessed 5′-end of the double-stranded substrate (13). We annealed the nonrepeat U₅(T₃)U₄ template to oligonucleotides containing one and three GAA repeats at the 5′-end (Table I, oligonucleotides 7 and 8), generating flap substrates containing 3- and 9-nucleotide flaps, respectively. The predominant h-FEN1 cleavage products from the 3-nucleotide flap substrate were 3–4 nucleotides long (lanes 6–9) and 9–10 nucleotides long (lanes 11–14), accounting for a maximum of 68.5 and 65.9%, respectively, of the total products generated at the highest concentrations of h-FEN1. This cleavage pattern is consistent with the 5′-flap exonuclease activity of h-FEN1, which can cleave substrates at the n, n + 1, or n − 1 position of the flap, where n is the number of nucleotides in the flap (12).
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Oligonucleotides used in replication and h-FEN1 cleavage reactions

| Oligonucleotide numbers and names | Size (nt) | Sequence (5’–3’) |
|----------------------------------|----------|-----------------|
| Templates                        |          |                 |
| 1. Ua(TTC)Ub                    | 51       | ACTUTGCTCTTC(TTC)_GGGAGGATGCT |
| 2. Ua(TTC)Ub                    | 54       | ACTUTGCTCTTC(TTC)_GGGAGGATGCT |
| 3. Ua(U)Ub                      | 54       | ACTUTGCTCTTCGAGCTAGTACCTGACATACAGACAGGACTG |
| Replication primers             |          |                 |
| 4. (GAA)3                      | 9        | GAGAGAAGAGAGA |
| 5. Ua                           | 12       | GAATAGCTGAGCT |
| Flag/Loop                       |          |                 |
| 6. Ua                          | 42       | TTTGTATGATGATCTACTGATCTATGACCTGAGACACAGATG |
| 7. (GAA)Ub                      | 45       | GAATCGTATGATGATCTACTGATCTATGACCTGAGACACAGATG |
| 8. (GAA)Ub, Ua                  | 51       | (GAA)TCTGTATGATGATCTACTGATCTATGACCTGAGACACAGATG |
| 9. (GAA)Ub                      | 39       | (GAA)_GAGAGACAGATG |
| 10. (GAA)Ub, Ua                 | 42       | (GAA)_GAGAGACAGATG |
| 11. (GAA)Ub                     | 48       | (GAA)_GAGAGACAGATG |
| 12. (GAA)Ub, Ua                 | 57       | (GAA)_GAGAGACAGATG |
| 13. Control template            | 30       | GGACTCTCCGATCAAGACTG |
| 14. Control flap                | 34       | GAGATCGAAGATGTCCTAACATATAGCAAGAGT |

* Nucleotides (nt) in the 5’ and 3’ unique regions are underlined. Complementary nucleotides in the “control template” and “control flap” oligonucleotides are in boldface type.

Fig. 4. Synthetic d(GAA)ₙ flap substrates equilibrate to form h-FEN1 endonuclease-resistant, exonuclease-sensitive structures. To study effects of h-FEN1 on individual expansion products, synthetic substrates were constructed to resemble predicted flap products generated in GAA expansion reactions. End-labeled substrates (10 nm) were incubated with increasing amounts of h-FEN1 (to final concentrations of 0, 240, 480, and 960 nM h-FEN1) for 20 min at 37 °C, as described under “Experimental Procedures.” Reaction products were separated by 20% denaturing PAGE (7.5 M urea) and visualized by phosphorimaging. Lane 1, 10-base ladder. Lanes 2–15, h-FEN1 added to nonrepeat substrates containing no flap, a one-repeat GAA flap, and a three-repeat (GAA)ₙ flap. Lanes 16–33, h-FEN1 added to repeat-containing substrates with zero, one, three, and six more GAA repeats than TTC repeats. Lanes 34–37, h-FEN1 added to a control flap substrate. Lane 38, 10-base ladder. Lanes 10, 15, 24, and 29, h-pol β (220 nm) was included in the reactions. Schematics of the substrate differences are shown above the gel lanes. The unique sequences are differentiated from repeat sequences by vertical bars.

Cleavage activity appeared unchanged if h-pol β (220 nm) was included in reactions containing 480 nm h-FEN1 (lanes 10, 15, 24, and 29).

The nine-repeat template Ua(TTC)₉Ub was annealed to the oligonucleotide (GAA)ₙUb, which contained nine GAA repeats followed by 12 nucleotides (Ub) complementary to Ua of the template (lanes 16–19). This substrate mimicked the 39-nucleotide full-length replication product generated in Fig. 2A. As was observed with the nonrepeat substrate containing only a recessed 5’-end (lanes 2–5), the most abundant h-FEN1 cleavage product of this substrate was a 1-nucleotide product, comprising up to 95% of all products (lane 19), indicative of h-FEN1 5’-exonuclease activity at a recessed 5’-end. The nine-repeat template was annealed to oligonucleotides (Table I, oligonucleotides 10–12) containing 10, 12, and 15 GAA repeats plus 12-nucleotide complementary to Ua in the template, to generate substrates resembling expansion products with one, three, or six extra repeats in the nascent strand, respectively. When h-FEN1 was added to the substrate with one extra GAA repeat (lanes 20–23), a 4-nucleotide fragment was the most abundant cleavage product, accounting for a maximum of 36.8% of all products. A significant amount of 1-nucleotide fragment was formed as well, comprising up to 12.1% of the total products. This cleavage pattern may indicate the presence of a combination of substrates with 5’-flaps and recessed 5’-ends, sensitive to the endonuclease and exonuclease activity of h-FEN1, respectively. In contrast, substrates containing three (lanes 25–28) and six (lanes 30–33) extra GAA repeats were more sensitive to the exonuclease activity than the endonuclease activity, as demonstrated by the large amount of 1-nucleotide cleavage products generated (up to 60.7 and 91.1% of the total products, respectively). Small quantities of longer cleavage products were formed at 4, 7, and 10 nucleotides for the substrate with three extra repeats (lanes 25–28) and at 4, 7, 10, 13, 16, 19, and 21 nucleotides for the substrate with six extra repeats (lanes 30–33), indicating that a small fraction of these substrates contained flaps of varying numbers of repeats. The longer cleavage products accounted for no more than 2.5% each of the total reaction products for both substrates. There was no apparent difference in resulting cleavage products if h-pol β was included in the 240 nm h-FEN1 reactions containing substrates with one and three extra GAA repeats (lanes 24 and 29).

Control substrate with a 20-nucleotide flap lacking repeats, incubated with h-FEN1, resulted in a 19-nucleotide cleavage product (up to 40.0% of total products), consistent with endonuclease cleavage mainly at the n – 1 position in the flap. One-nucleotide products were not detected, indicating that there was no exonuclease cleavage of the flap substrate (lanes 34–37).

DISCUSSION

We studied the effects of h-FEN1 on GAA repeat expansion during DNA synthesis. All polymerases for which it has been measured slip during replication of repetitive sequences (37). This is apparent from the high levels of microsatellite instability in cells deficient in mismatch repair and so unable to repair
the mismatched bases resulting from DNA slippage (reviewed in Ref. 40). Human pol β was used for these studies because the repair-associated enzyme is more prone to slippage than the replicative polymerases (37), and its use thus maximizes the opportunity to observe DNA expansions and to readily detect differences in the amounts and sizes of expansion products. The GAA expansion products were previously shown by DNA sequencing to be perfect GAA repeats (29). Replication was either primed from within the repeat tract or primed from an upstream unique sequence. Priming within a repeat tract models the occurrence of either an Okazaki fragment or an origin of replication within a repeat tract (3, 4). Repeat tracts may be unstable under the supercoiled conditions in the cell (41); the unstable repeats are predicted to serve as preferred sites for replication priming (41). Priming replication from within the repeat tract significantly enhanced expansion of GAA by h-pol β, compared with priming from an upstream unique sequence. The results are consistent with studies in bacteria, yeast, and mammalian cells that predict an association of repeat expansion with lagging strand DNA synthesis (5, 42, 43).

**Modeling the Effects of H-FEN1 on GAA Repeat Expansion**

The dramatic GAA expansion observed with replication using a repeat primer, when compared with replication using a unique primer, indicates that a free 5′-end located within the repeat tract greatly enhances the formation of an expansion product. The GAA expansion results imply that DNA primer slippage is the source of the expansion. Furthermore, the presence of expansion products more than twice the length of the repeat tract indicates that these products are the result of multiple slippage events. For example, the largest expansion products observed in Figs. 1–3 were in excess of 200 nucleotides long. In principle, the largest possible expansion product that could be generated from a single slippage event, occurring during replication of a 30-nucleotide repeat tract flanked by 12-nucleotide unique sequences, is less than 72 nucleotides long. This number accounts for replication of the 30-nucleotide repeat tract, followed by a single slippage of the nascent strand 3′-end back to the beginning of the repeat tract, replication of the 30-nucleotide repeat tract again, and replication of the 12-nucleotide unique region (30 + 30 + 12 = 72 nucleotides). We assume that replication of the downstream flanking unique sequence stabilizes the substrate against additional slippage. Therefore, a 200-nucleotide-long GAA expansion product would be the result of at least six slippage events; each event would add no more than 30 nucleotides to the product. For simplicity, this estimation ignores the fact that a certain number of nucleotides need to remain annealed to the template during slippage, so the actual maximum number of nucleotides added per event may be significantly less than 30.

There are several possible conformations for the slipped expansion intermediates: single-stranded 5′-flaps produced by DNA nascent strand slippage off the end of the template repeat tract, 5′-flaps folded into hairpins, and internal loops and hairpins located within the repeat tract (termed internal loops and hairpins). Short regions of single-stranded d(GAA)n (<50 bases) are predicted not to form stable hairpins, whereas long single-stranded d(GAA)n regions are susceptible to hairpin formation (29). Of the possible conformations for the slipped intermediates listed above, FEN1 is only able to cleave the single-stranded 5′-flaps (24). Since FEN1 is predicted to play a significant role in protecting against repeat instability and in prevention of repeat expansion mutations (6, 8–10, 19, 38), we tested the effect of h-FEN1 on GAA expansion during DNA replication. When added at the start of replication, h-FEN1 dramatically suppressed repeat expansion of GAA nascent strands in a concentration-dependent fashion. The ability of h-FEN1 to suppress repeat expansion during replication from the repeat primer supports the formation of a 5′-flap in the nascent strand, generated by DNA slippage. The flap endonuclease properties of h-FEN1 are well described by biochemical studies (22–24, 26–28), and are represented schematically in the first step of Fig. 5A. It is important to note that our results indicate that when replication is initiated within the repeat tract, flap formation can occur independently of strand displacement synthesis by an upstream polymerase.

With increasing concentrations of h-FEN1, formation of larger repeat expansion products was essentially abolished, and formation of smaller repeat expansion products was also suppressed, but to a significantly lesser extent (Fig. 2). Smaller repeat expansion products were only completely suppressed at high molar ratios of h-FEN1 to DNA (at a FEN1/polymerase/DNA substrate molar ratio of 8:7:1). We expect the molar ratio of h-FEN1 to DNA replication site required for full suppression of expansion to be equal to or less than 1:1 in vivo, where factors such as proliferating cell nuclear antigen are predicted to increase the effectiveness of h-FEN1. Proliferating cell nuclear antigen stimulates FEN1 activity 10–50-fold in vitro (44). Full-length unexpanded products were minimally affected by h-FEN1. The observation of mostly small cleavage products (1–11 bases in length) is consistent with a mechanism in which small slipped products form flaps that are cleaved by h-FEN1 endonuclease. If large flaps were allowed to form and were cleaved by h-FEN1, the cleavage products would be visible as large fragments, some of which would be longer than the full-length unexpanded product (e.g. a 150-nucleotide expansion product with a >100-nucleotide flap). At h-FEN1 concentrations that suppressed expansion, we did not see an increase in any products longer than 11 nucleotides (e.g. lane 6 in Fig. 2A).

The production of mostly 1-mers (Fig. 2A), also suggested that h-FEN1 exonuclease activity might have contributed significantly to the suppression of repeat expansion. Results indicate, however, that the endonuclease rather than the exonuclease activity of h-FEN1 was responsible for suppressing the formation of expanded products during replication for the following reasons. First, the unexpanded full-length replication products, which contain recessed 5′-ends that are substrates for the exonuclease activity of h-FEN1, were relatively resistant to h-FEN1 compared with both the expanded and partial-length products (Fig. 2). Second, the activity of h-FEN1 for recessed 5′-ends is relatively weak when compared with its activity for 5′-flaps (12, 13). Third, internally labeled and end-labeled expansion products were similarly suppressed by h-FEN1 when it was added at the start of replication (Fig. 2). Thus, the significant production of small h-FEN1 products when FEN1 is added at the start of replication implies that h-FEN1 recognizes the initial slippage event that causes a flap and removes the hanging nucleotides.

The observed pattern of suppression of GAA expansion by h-FEN1 and the sizes of cleavage products generated suggest the following mechanism. If h-FEN1 is present, short 5′-flaps resulting from small DNA slippage events are continuously cleaved, preventing the growth of smaller expansion products into larger ones. At higher concentrations of h-FEN1, virtually all slipped 5′-flaps would be cleaved as soon as they form, and expansion would be almost completely suppressed (as seen in Fig. 2, A and B, lanes with 240 nM h-FEN1 added). At intermediate concentrations of h-FEN1 (as in Fig. 2, A and B, lanes with 120 nM h-FEN1), many of the small slipped expansion products would be cleaved immediately after their initial formation, but a certain percentage of these products would escape cleavage and undergo another slippage event. Some of
these “twice-slipped” products would then be cleaved by h-FEN1, which is still present in the reaction, but a percentage would escape cleavage once more and undergo a third slippage event. Thus, if the concentration and efficiency of h-FEN1 remains constant, the number of products to escape cleavage and undergo each subsequent slippage event would decrease exponentially as the number of events increases. This exponential decrease would result in a shift in the distribution of expansion products, such that formation of larger expansion products would be suppressed to a much greater extent than would smaller products. This behavior was observed with increasing concentrations of h-FEN1 during replication (Fig. 2, A and B).

The distribution of expansion product lengths after treatment with h-FEN1, however, appears to have been the result of additional effects. Replication and expansion was finished within 2 min (Fig. 1). Thus, the expansion products would have been subjected to h-FEN1 cleavage activity for the remaining reaction time of 18 min (Fig. 2A) and 28 min (Fig. 2B). The apparent resistance of the shorter length expansion products to 120 nM h-FEN1 suggests the possibility that the short products form structures that are resistant to h-FEN1.

**Effects of H-FEN1 Delay on GAA Repeat Expansion**—To study the formation of h-FEN1-resistant expansion products, the addition of h-FEN1 was delayed relative to the start of the replication reaction (Fig. 3). GAA repeat expansion products were found to increase in resistance to h-FEN1 the later h-FEN1 was added during the replication reaction. This effect was observed even at high concentrations of h-FEN1 (at a FEN1/substrate molar ratio of 8:1). Longer GAA repeat expansion products are capable of forming stable hairpins (29), so their resistance to the delayed addition of h-FEN1 may be explained by hairpin formation within a long 5′-flap (23, 24). All of the GAA repeat expansion products were resistant to h-FEN1, however, including the short repeat expansion products that do not form stable hairpins under our conditions (32–35). To confirm this resistance to h-FEN1, we generated double-stranded synthetic DNA substrates to resemble either the full-length replication products or selected small expansion products and tested the effect of h-FEN1 on these substrates. Generation of the substrates mimicked the situation above in which repeat expansion products were formed in the absence of h-FEN1 because the addition of h-FEN1 to the replication reaction was delayed. The synthetic substrates allowed us to observe the effects of h-FEN1 on individual products in the absence of replication. Control substrates containing either nonrepeat flaps or fixed GAA flaps were similarly susceptible to cleavage by the endonuclease activity of h-FEN1, confirming that short GAA flaps do not form hairpins under our conditions. In contrast, repeat substrates containing zero-, three-, and six-repeat expansions were relatively resistant to h-FEN1 exonuclease activity but sensitive to h-FEN1 endonuclease activity, suggesting that these substrates contained mostly recessed 5′-ends because the 5′-flaps had been converted to loops within the double-stranded repeat tract (Fig. 4). Synthetic substrate containing only one extra GAA repeat (Fig. 4, lanes 20–24) was sensitive to both the endonuclease and exonuclease activities of h-FEN1, implying the presence of a mixture of loop- and flap-containing products.

The resistance of both long and short equilibrated repeat expansion products (Fig. 3) and short synthetic repeat expansion substrates (Fig. 4) to h-FEN1 endonuclease activity argues for a multistep pathway as shown in Fig. 5A. Step 1 of this pathway predicts that DNA slippage initially results in a 5′-flap as the kinetically favored intermediate, independent of strand displacement synthesis. Production of this required intermediate explains why repeat expansion is suppressed if h-FEN1 is present from the start of replication of the repeat tract. If h-FEN1 activity is delayed, step 2 (Fig. 5A) predicts a rearrangement of the template and nascent strand structures.
to hide the end of the 5′-flap. (The lowercase letters in 2a, 2b, and 2c indicate the different structures predicted to be in equilibrium in step 2.) We propose that when the addition of h-FEN1 is delayed, the inability of h-FEN1 to cleave short expanded products results from the formation of an internal loop (step 2b) or a series of small loops in the nascent strand that effectively absorb the flap, making even the smallest repeat expansion resistant to FEN1. An alternative possibility is a folding back of the short flap onto the duplex to form a triplex structure. However, the relatively short 6- and 9-nucleotide expansions are inconsistent with triplex formation because of the need to span the fold-back coupled with the need to form a stable structure. Moreover, studies with synthetic oligonucleotides show that GAA/TTC complexes do not form B′R′ triplexes (34, 45, 46) as would be required to form a triplex by fold-back of the GAA flap. Whereas short expansion products are protected from h-FEN1 by formation of loops inside the repeat tract (internal loops), long GAA expansion products may form long 5′-flaps that hairpin if h-FEN1 activity is delayed (step 2a). In addition, long expansion products may form internal loops similar to those predicted for short expansion products. Step 3 (Fig. 5A) predicts the continued expansion of the products to sizes that can form hairpins. Hairpin structures in the flap and in the repeat tract may be in equilibrium (3a).

The observation that h-FEN1 suppresses formation of large expansion products (Fig. 2) argues that most long expansion products are prevented from forming in vivo. Thus, on the one hand, short expansions and contractions products are predicted to predominate in vivo. On the other hand, if long flaps have the opportunity to hairpin, this enables them to persist in the presence of FEN1. This persistence predicts that some small number of molecules will reequilibrate to move the hairpin inside the repeat tract (3a) to form an annealed 5′-end that can be ligated to the upstream nascent strand, generating large expansion mutations (Fig. 5A) (25).

Effects of h-FEN1 on Partial-length Products—In addition to the repeat expansion products, the partial-length replication products were also highly susceptible to h-FEN1. Susceptibility to h-FEN1 increased with the decreasing size of the partial-length product and conversely with the increasing size of the unpaired single-stranded DNA region in the template strand. The results implied that the partial-length products were susceptible to h-FEN1 because of the single-stranded DNA region of the TTC repeat tract available in the template. Under our reaction conditions, single-stranded DNA containing 17 or more TTC repeats (≥51 bases) is able to form stable hairpins (29), which implies that transient hairpin formation is possible in shorter TTC repeat lengths. Therefore, our results suggest a mechanism (Fig. 5C) whereby transient TTC loop and hairpin formation in the template single-stranded DNA region could induce a 5′-flap in the nascent strand that is a target for h-FEN1 flap endonuclease activity. By loop (these structures have also been called bulges), we mean one or more tandem nucleotides rotated out of the helix (47). It is worth noting that the experimental results and the mechanism summarized in Fig. 5C predict that FEN1 activity may contribute to deletion mutations within the repeat tract in vivo if the 5′-end of the FEN1-cleaved DNA can be ligated to an upstream nascent strand before the TTC loop dissolves.

Comparison with Prior Models—We compared our results with prior replication models for repeat expansion that are based on DNA slippage (3–5) and based on 5′ DNA flap formation caused by displacement DNA synthesis (6, 11). The comparison generates a novel flap DNA slippage model wherein 5′-flap formation is the result of DNA slippage and replication (Fig. 5A). In this newly refined model, DNA displacement synthesis to form the flap is not required. This removes a limitation of the flap strand-displacement model that the size of the expansion is limited to the amount of DNA displaced. In addition, the flap strand-displacement model predicts that flaps persist by the formation of FEN1-resistant hairpins and triplex structures (6). For GAA repeats, we found evidence that, in the absence of h-FEN1, short flaps rearrange to form loop structures within the repeat tract that are resistant to FEN1. The small loops may be substrates for mismatch repair until they grow large enough to form hairpins (35). The 5′-ends of these structures can be ligated to the progressing upstream Okazaki fragment. On the other hand, if long GAA expansion products form flaps that become resistant to h-FEN1 by hairpin formation (29), these structures cannot be ligated to the upstream Okazaki fragment unless some percentage of the molecules are converted to internal hairpins/loops (11). Thus, the refined model predicts that h-FEN1 has a major role in protecting against both the short expansions found in Huntington’s disease and Kennedy’s disease as well as the large expansions that characterize myotonic dystrophy, Friedreich’s ataxia, and SCA10 (1, 20).

Effects of h-FEN1 on Expansion Products Predict Biological Consequences—We speculate that the repeat expansion pathway outlined in Fig. 5A may help explain why haploinsufficiency of mouse FEN1, when combined with a mutation in the adenomatous polyposis coli (Apc) gene in mice, gives advanced stage tumors all showing microsatellite instability (19). Mice that are heterogeneous for the mutated Apc gene alone have a tumor predisposition phenotype; they develop colonic polyps and adenosomas of the small intestine that do not show microsatellite instability (19). Reduction in the expression of FEN1 resulting from haploinsufficiency may result in delayed activity of FEN1 protein at random Okazaki fragments. For GAA repeat expansion, and perhaps for expansion of other repeat sequences as well, the required production of a short flap is followed by the stable rearrangement of the expansion product to a double-stranded DNA repeat tract with loops that can no longer be cleaved by FEN1. The rearrangement to a structure with loops either requires the absence of FEN1 activity because of limiting amounts of protein or because of a defect in FEN1 function. The resulting increase in repeat instability combined with the Apc tumor predisposition would therefore enable tumor progression. The repeat expansion pathway also offers an explanation as to why mutations in h-FEN1 have not been associated with the repeat expansion diseases (20, 21). Too little FEN1 activity is greatly destabilizing or lethal (19), whereas a transient delay in h-FEN1 protein reaching a particular Okazaki fragment because of a reduction in h-FEN1 gene expression is predicted to provide the opportunity for repeat expansion to occur (Fig. 5A). Thus, amounts of h-FEN1 sufficient to saturate all sites primed for replication may be necessary to prevent DNA repeat instability.

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