The 3′-Flap Pocket of Human Flap Endonuclease 1 Is Critical for Substrate Binding and Catalysis

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Flap endonuclease 1 (FEN1) proteins, which are present in all kingdoms of life, catalyze the sequence-independent hydrolysis of the bifurcated nucleic acid intermediates formed during DNA replication and repair. How FEN1s have evolved to preferentially cleave flap structures is of great interest especially in light of studies wherein mice carrying a catalytically deficient FEN1 were predisposed to cancer. Structural studies of FEN1s from phage to human have shown that, although they share similar folds, the FEN1s of higher organisms contain a 3′-extrahelical nucleotide (3′-flap) binding pocket. When presented with 5′-flap substrates having a 3′-flap, archaeal and eukaryotic FEN1s display enhanced reaction rates and cleavage site specificity. To investigate the role of this interaction, a kinetic study of human FEN1 (hFEN1) employing well defined DNA substrates was conducted. The presence of a 3′-flap on substrates reduced $K_m$ and increased multiple- and single turnover rates of endonucleolytic hydrolysis at near physiological salt concentrations. Exonuclease- and fork-gap-endonuclease reactions were also stimulated by the presence of a 3′-flap, and the absence of a 3′-flap from a 5′-flap substrate was more detrimental to hFEN1 activity than removal of the 5′-flap or introduction of a hairpin into the 5′-flap structure. hFEN1 reactions were predominantly rate-limited by product release regardless of the presence or absence of a 3′-flap. Furthermore, the identity of the stable enzyme product species was deduced from inhibition studies to be the 5′-phosphorylated product. Together the results indicate that the presence of a 3′-flap is the critical feature for efficient hFEN1 substrate recognition and catalysis.

In eukaryotic DNA replication and repair, various bifurcated nucleic acid structure intermediates are formed and must be processed by the appropriate nuclease. Two examples of biological processes that create bifurcated DNA intermediates are Okazaki fragment maturation (1, 2) and long patch excision repair (3). In both models, a polymerase executes strand-displacement synthesis to create a double-stranded DNA (dsDNA)6 two-way junction from which a 5′-flap structure protrudes. The penultimate step of both pathways is the cleavage of this flap structure to create a nicked DNA that is then ligated. Because the bifurcated DNA structures that are formed in the aforementioned processes can theoretically occur anywhere in the genome, the nuclease associated with the cleavage of 5′-flap structures in eukaryotic cells, which is called flap endonuclease 1 (FEN1), must be capable of cleavage regardless of sequence. Therefore, FEN1 nucleases, which are found in all kingdoms of life (4), have evolved to recognize substrates based upon nucleic acid structure and strand polarity (5, 6).

The Okazaki fragment maturation pathway of yeast has become a paradigm of eukaryotic lagging strand DNA synthesis. In the yeast model, bifurcated intermediates with large single-stranded DNA (ssDNA) 5′-flap structures are imprecisely cleaved by DNA2 in a replication protein A-dependent manner (7). Subsequent to the DNA2 cleavage, Rad27 (yeast homologue of FEN1) cleaves precisely to generate an intermediate suitable for ligation (2). The recent discovery that human DNA2 is predominantly located in mitochondria in various human cell lines (8, 9) suggests that hFEN1 is the paramount 5′-flap endonuclease in the nuclei of human cells. This observation potentially provides a plausible rationale for why deletion of RAD27 (yeast FEN1 homologue) is tolerated in Saccharomyces cerevisiae (10), whereas deletion of FEN1 in mammals is embryonically lethal (11). Recent models wherein mice carrying a mutation (E160D) in the FEN1 gene, which was shown in vitro to alter enzymatic properties (12), have demonstrated...
that FEN1 functional deficiency in mice (S129 and Black 6) increases the incidence of cancer, albeit different types presumably due to genetic background (13, 14). Thus, the function of mammalian FEN1 in vivo is vital to the prevention of genomic instability. In addition to its importance in the nucleus, hFEN1 has recently been detected in mitochondrial extracts (15, 16) and implicated in mitochondrial long path base excision repair (15). Considering the pivotal roles of hFEN1 in DNA replication and repair, it is of interest to understand how hFEN1 and homologues achieve substrate and scissile phosphate selectivity in the absence of sequence information.

Since its initial discovery as a nuclease that completes reconstituted Okazaki fragment maturation (17) and subsequent rediscovery as a 5'-flap-specific nuclease (DNaseIY) from bacteria (18), mouse (19), and HeLa cells (20), FEN1 proteins ranging from phage to humans have been studied biochemically, computationally, and structurally (5, 6, 21). Biochemical characteristics of FEN1 proteins from various organisms have shown that this family of nucleases can perform phosphodiesterase activity on a wide variety of substrates; however, the efficiency of catalysis on various substrates differs among the species. For instance, phage FEN1s prefer pseudo-Y substrates (22, 23), whereas the archaeal and eukaryotic FEN1s prefer 5'-flap substrates (21, 24, 25), which have two dsDNA domains, one upstream and downstream of the site of cleavage, and a 5'-ssDNA protrusion (Fig. 1A). Primary sequence analysis indicates that FEN1 proteins share characteristic N-terminal (N) and Intermediate (I) "domains," which harbor the highly conserved carboxylate residues that bind the requisite divalent metal ions (26–28). Structural studies of FEN1 nucleases from phage to human have been studied biochemically, computationally, and structurally (5, 6, 21). Biochemical characteristics of FEN1 proteins from various organisms have shown that this family of nucleases can perform phosphodiesterase activity on a wide variety of substrates; however, the efficiency of catalysis on various substrates differs among the species. For instance, phage FEN1s prefer pseudo-Y substrates (22, 23), whereas the archaeal and eukaryotic FEN1s prefer 5'-flap substrates (21, 24, 25), which have two dsDNA domains, one upstream and downstream of the site of cleavage, and a 5'-ssDNA protrusion (Fig. 1A). Primary sequence analysis indicates that FEN1 proteins share characteristic N-terminal (N) and Intermediate (I) "domains," which harbor the highly conserved carboxylate residues that bind the requisite divalent metal ions (26–28). Structural studies of FEN1 nucleases from phage to humans (22, 29–36), have shown that the N and I domains comprise a single nuclease core domain consisting of a mixed, six- or seven-stranded β-sheet packed against an α-helical structure on both sides. The α-helices on either side of the β-sheet are "bridged" by a helical arch that spans the active site groove (supplemental Fig. S1). On one side of the β-sheet, the α-helical bundle (αb1) creates the floor of the active site and a DNA binding motif (helix-3-turn-helix) (32). Similarly, the opposite α-helical bundle (αb2) has also been observed to interact with DNA (35). Based on site-directed mutagenesis studies with T5 phage FEN1 (T5FEN1) (37) and hFEN1 (38, 39), and crystallographic studies of T4 phage FEN1 (T4FEN1) (22) and Archaeoglobus fulgidus FEN1 (afFEN1) (35) in complex with DNA, a general model for how FEN1 proteins recognize flap DNA has emerged. The helix-3-turn-helix motif is involved in downstream dsDNA binding, whereas the upstream dsDNA domain is bound by αb2. The helical arch is likely involved in 5'-flap binding (22).

Unlike phage FEN1s, studies of FEN1s from eubacterial (40), archaeal (21), and eukaryotic origins (41) have shown that the addition of a 3'-extrahelical nucleotide (3'-flap) to the upstream duplex of a 5'-flap substrate results in a rate enhancement and an increase in cleavage site specificity. Moreover, substrates possessing a 3'-flap, which mimic physiological "equilibrating flaps," were cleaved exactly one nucleotide into the downstream duplex, thereby resulting in 5'-phosphorylated dsDNA product that was a suitable substrate for DNA ligase I (21, 41). As postulated by Kaiser et al. (21), the structure of an archaenal FEN1 in complex with dsDNA with a 3'-overhang showed that the protein contains a cleft adjacent to the upstream dsDNA binding site that binds the 3'-flap by means of van der Waals and hydrogen bonding interactions with the sugar moiety (33). Once the residues associated with 3'-flap binding were identified, sequence alignment analyses showed that the amino acid residues in the 3'-flap binding pocket are highly conserved from archaea to human. Furthermore, mutation of the conserved amino acid residues in the 3'-flap binding pocket of hFEN1 resulted in reduced affinity for and cleavage specificity on double flap substrates (42). Although the effects of the addition of a 3'-flap to substrates on hFEN1 catalysis are known qualitatively, a detailed understanding of the relationship between changes in catalytic parameters and rate enhancement by the presence of a 3'-flap is unknown. Here, we describe a detailed kinetic analysis of hFEN1 using four well-characterized DNA substrates and show that the presence of a 3'-flap on a substrate not only contributes to substrate binding (42), but also increases multiple and single turnover rates of reaction in the presence of near physiological monovalent salt concentrations. We also demonstrate that, like T5FEN1, hFEN1 is rate-limited by product release, and thus multiple turnover rates at saturating concentrations of substrate are predominantly a reflection of product release and not catalysis as was previously concluded (39). Furthermore, this study provides insight into the mechanism of hFEN1 substrate recognition.

**EXPERIMENTAL PROCEDURES**

**Protein Expression and Purification—Wild-type human FEN1 (hFEN1, accession code NP_004102) bearing a C-terminal His6 tag was expressed from a pET28b vector in BL21(DE3) and purified as previously described (43). The protein was further purified by anion-exchange chromatography and then dia lyzed against 2 x 1 liter of storage buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, 0.02% NaN3, 1 mM dithiothreitol). The concentration of protein was determined using A280 and the calculated extinction coefficient (22,920 M⁻¹·cm⁻¹). For kinetic assays stocks of hFEN1 (final concentration of 100 nM or 25 μM) were prepared in 50 mM HEPES-K⁺, pH 7.8, 100 mM KCl, 0.1 mg/ml bovine serum albumin, 5 mM tris-(3-hydroxypropyl)phosphine, 50% glycerol and stored at −20 °C.**

**Oligonucleotide Synthesis and Purification**—The DNA oligonucleotides listed in Table 1 were purchased from Integrated DNA Technologies, Inc. through the City of Hope DNA/RNA/peptide core facility. Except for P6 and the fluorescein-labeled (FAM) oligonucleotides, the oligonucleotides were purified as previously described (44), desalted using HiTrap columns, and lyophilized to dryness. After dissolution in TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA), the concentrations were determined from calculated extinction coefficients.

The P6 oligonucleotide was purified by reversed-phase HPLC (Waters Xbridge 10 x 250 mm C-18 column) using buffer A (100 mM triethylammonium acetate, pH 7.2) and buffer B (100 mM triethylammonium acetate, pH 7.2, 90% acetonitrile). The gradient conditions were t = 0 min, 0% B; t = 5 min, 7.5% B; t = 20 min, 10.5% B; t = 25 min, 10.5% B; t = 25.1 min, 100% B; t = 30 min, 100% B; and t = 31 min, 0% B. The purified P6 was dried and repeatedly lyophilized to remove vol-
The amount of product was determined by integration using ImageQuant version 5.2 after denaturing PAGE. All thermal melting experiments were conducted on a Varian Cary 300 scanning spectrophotometer. DNA stock (40 μM) samples were prepared at 1:1.1 ratio of d to T strand (Table 1) in 1× sequence annealing buffer (25 mM HEPES-K+; pH 7.5, 50 mM NaCl, 0.1 mM EDTA), heated to 95 °C, and cooled to room temperature on the bench. Melt samples were prepared by dilution with 1× sequence annealing buffer of stock to give an A260 of 0.6. For bimolecular transitions, ΔH° was determined by linear regression using Prism 5.01 of a plot of the inverse of the melting temperature versus the natural log of the sum of the strand in excess (T-strand; cT-strand) and half the total concentration of the limiting strand (d-strand; c d-strand) (46). R is the gas constant (1.987 cal·K⁻¹·mol⁻¹) (Equation 1).

\[
\frac{1}{T_m} = \frac{R}{\Delta H^\circ} \ln \left[ \frac{c_{T\text{-strand}}}{2} + \frac{c_{d\text{-strand}}}{2} \right] + \frac{\Delta S^\circ}{\Delta H^\circ} \quad (\text{Eq. 1})
\]

Activity Assays Using Radiolabeled Substrates—The indicated d-strand oligonucleotides (Table 1) were 32p radiolabeled at either the 3’ terminus with [α-32P]cordoncytidine 5’-phosphate or the 5’ terminus with [γ-32P]ATP using conventional methods (47), and substrates were similarly prepared as described above. Excess radiolabel was removed using Micro-Biospin 6 columns (Bio-Rad, SSC buffer). Reaction mixtures contained 1 nM radiolabeled substrate in 1× reaction buffer (RB, 50 mM HEPES-K+, pH 7.5, 0.1 mg/ml bovine serum albumin, 5% glycerol, 2.5 mM tris-(3-hydroxypropyl)phosphate), with 8 mM Mg(OAc)₂, 100 mM KCl, and the indicated concentrations of hFEN1. Aliquots of the reaction mixture were removed at the indicated time intervals and quenched by addition of an equal volume of 2× formamide loading buffer containing 20 mM EDTA. Product formation was assessed by phosphorimaging (ImageQuant version 5.2) after denaturing PAGE (20%).

Determination of hFEN1 Mg(OAc)₂ and KCl Optima with the Double Flap Substrate—Reaction mixtures containing various concentrations of F(5)T3F (2.5, 25, 250, or 2500 nM) were prepared in 1× RB with 8 mM Mg(OAc)₂ and varying concentrations of KCl (0–225 mM). To determine the Mg²⁺ optimum at 100 mM KCl, reaction mixtures were prepared containing 25 nM F(5)T3F substrate in 1× RB (with 100 mM KCl) and varying Mg(OAc)₂ concentrations (2–10 mM). Reaction mixtures were preincubated at 37 °C before initiation by the addition of hFEN1. The reactions were quenched after 6 min by the addition of 100 mM EDTA, 8 mM urea. Reaction progress was assessed by dHPLC using a WAVE System 3500 (Trangenomics, Inc., Omaha, NB) (supplemental Table S1) with fluorescence detector and an Oligo Sep® cartridge as previously described (48). The amount of product was determined by integration using Navigator™ version 1.7 (Trangenomics, Inc.) of substrate and product peaks in fluorescence intensity traces. Cleavage at all substrate concentrations was maintained below 15% to mitigate substrate depletion. Activity was defined as the amount of product produced per unit assay time, where one unit of assay time is 6 min. The normalized activity was defined as the quotient of activity and the enzyme concentration used.

Determination of Steady-state Kinetic Parameters of hFEN1 Catalysis—Reaction mixtures were prepared with varying concentrations of substrates and preincubated at 37 °C before the addition of enzyme to initiate the reaction. The final concentrations of all buffer components were 1× RB with 100 mM KCl, 5 mM NaCl, and 8 mM Mg(OAc)₂. Final substrate/hFEN1 concentrations for reactions containing F(5)T3F, ET3F, G(15)T3F, and F(5)T ranged from 1.3–2000 nM/1.3–200 pm, 2.5–2000 nm/5–400 pm, 5–3600 nm/5–400 pm, and 5–3600 nm/0.1–1600 pm, respectively. Aliquots of each reaction mixture were removed and quenched with 100 mM EDTA, 8 mM urea at eight time intervals between 0 and 8 min. Reaction progress was monitored by dHPLC. All reactions were independently repeated at least six times. Initial rates (ν₀, nmol·min⁻¹) were determined by linear regression of plots of the amount of product versus time. Normalized initial rates (ν₀/[E]₀, min⁻¹) were obtained from the quotient of initial rate and enzyme concentration used. Kinetic parameters kcat and Km were determined by generalized nonlinear least squares using a Michaelis-Menten model (MM, Equation 2) or MM model with a Hill slope (MMH, Equation 3), from which plots of normalized initial rate as a function of substrate concentration were generated (49). (See statistical analysis.)

\[
\frac{v_0}{[E]_0} = \frac{k_{cat}[A]}{K_m + [A]} \quad (\text{Eq. 2})
\]

\[
\frac{v_0}{[E]_0} = \frac{k_{cat}[A]^H}{K_m^H + [A]^H} \quad (\text{Eq. 3})
\]

The contribution of a specific substrate structural feature to the overall binding energy (∆ΔG°) was calculated using Equation 4 (50), R is the gas constant (8.314 J·mol⁻¹·K⁻¹) and T is 310.15 K.

\[
\Delta \Delta G° = -RT \ln \frac{(k_{cat}/K_m)_{F(5)T3F}}{(k_{cat}/K_m)_{subrate}} \quad (\text{Eq. 4})
\]

Single Turnover Rapid Quench Experiments—Rapid quench experiments were conducted at 37 °C using an RQF-63 device from HiTech Ltd. (Salisbury, UK) as previously described (23, 48). Briefly, an 80-μl aliquot of enzyme in reaction buffer (50 mM HEPES-K+, pH 7.5, 0.1 mg/ml bovine serum albumin, 2.5 mM tris(3-hydroxypropyl)phosphine, 8 mM Mg(OAc)₂, 100 mM KCl) was mixed with an equal volume of substrate in reaction buffer. Enzyme was used at a final concentration of at least 8 × Kₘ of the substrate, and final substrate concentrations were 30- to 50-fold lower than Kₘ. After a controlled time delay of 27.5 ms to 51.6 s, 80 μl of quench (1.5 M NaOH, 20 mM EDTA) was added. The quenched reaction mixtures were analyzed as described above for steady-state analyses. The maximal single turnover rate of the reaction was the first-order rate constant (kst,max) derived using nonlinear least squares for a one- or two-phase exponential (Equations 5 and 6, respectively) describing the amount of product formed (Pₙ, nanomolar) per time point (t, milliseconds) (50). (See statistical analysis.)
\[ P_t = P_s(1 - e^{-k_{\text{cat}}t}) \quad \text{(Eq. 5)} \]

\[ P_t = P_{\text{max}}(1 - e^{-k_{\text{cat}}t}) + P_{\text{max}}(1 - e^{-k_{\text{cat}}t}) \quad \text{(Eq. 6)} \]

**Product Inhibition Studies**—The kinetic parameters of hFEN1 with F(5)-T3F were determined at various concentrations of either P6 (0, 50, 100, 500, 1000, and 5000 nM) or Q(T3F) (0, 15, 30, 60, 90, and 120 nM). For each inhibitor concentration, normalized initial rates of reaction \( (\nu_0/\langle E \rangle_0) \) were measured in triplicate at six different concentrations of F(5)-T3F (10, 15, 25, 50, 100, and 1000 nM) using hFEN1 concentrations ranging from 2.5 to 100 pm. Reactions were assayed, and normalized initial rates were determined as described for steady-state analyses. Kinetic parameters \( (k_{\text{cat}} \text{ and } K_{\text{Mapp}}) \) were determined globally by GLS using a Lineweaver-Burke model (Equation 7) from which a plot of the inverse of the normalized initial rate \( (1/\langle E \rangle_0 \nu_0 \text{ minutes}) \) versus the inverse of the substrate concentration \( (1/\langle A \rangle, \text{nanomolar}^{-1}) \) for each concentration of inhibitor was generated.

\[ \frac{[E]_0}{\nu_0} = \frac{K_{\text{Mapp}}}{k_{\text{cat}}} \left( \frac{1}{[A]} \right) + \frac{1}{k_{\text{cat}}} \quad \text{(Eq. 7)} \]

For data exhibiting competitive inhibition, linear regression of a secondary plot of the apparent slope coefficients \( (m_{\text{app}}, \text{min-nm}) \) versus inhibitor concentration \( ([I], \text{nanomol}) \) was used to estimate the secondary slope and ordinate intercept (Equation 8), from which \( K_m \) and \( K_I \) were calculated (49). (See statistical analysis.)

\[ m_{\text{app}} = \left( \frac{K_{\text{Mapp}}}{k_{\text{cat}}K_I} \right) [I] + \frac{K_{\text{Mapp}}}{k_{\text{cat}}} \quad \text{(Eq. 8)} \]

**Statistical Analysis**—Statistical analyses were done using the R packages stats (51, 52) and nlme (53). For data that exhibited heteroskedasticity (steady-state kinetic data and product inhibition data), a power of the mean model was assumed and estimated for the variance model (data), a power of the mean model was assumed and estimated for the variance model (data). In cases in which model selection was required (e.g. Equation 2 versus Equation 3), Akaiake information criteria (AIC) was used to guide model selection. The model that provided the smallest AIC was chosen as the appropriate model unless the difference in AIC was less than 4.

**RESULTS**

Four Different hFEN1 Substrates Prepared by Various Pairings of Oligonucleotides—To produce substrates whereby the influence of specific structural features on the ability of hFEN1 to catalyze reactions could be reliably quantified, four substrates were designed based upon common upstream and downstream dsDNA domains. To avoid the complication of substrate assembly with varying numbers of oligomers, all substrates were assembled from two oligonucleotides. Two-plate sequences, which form a 3'-hairpin capped by a stable GNA tri-loops (54), created the upstream duplex (Fig. 1A) and differ only by an extra 3'-nt (Table 1). On both variants of template, an identical 20-nt 5'-ssDNA tail could be paired to a labeled d-strand to form an identical downstream heteroduplex in each substrate (Fig. 1, A–E). The sequences at the 5'-termini of the d-strands were varied to produce 5'-flap endonuclease (ENDO) substrates with or without a 3'-flap (Fig. 1, B and E), an exonuclease (EXO) DNA substrate (Fig. 1C) or a 15-nt ssDNA region that ends in a 5- to 10-bp hairpin capped by a GNA tri-loop (fork-gap-endonuclease (GEN) substrate (Fig. 1D)).

The choice of DNA sequence was aided by structure and stability prediction software (55) so that the possibility of alter-
Kinetic Characterization of Human FEN1

**TABLE 1**
The oligonucleotide sequences used in this study

| Oligonucleotide | Type | Sequence                                   |
|-----------------|------|--------------------------------------------|
| P6              | d    | 5’Phos- TTTTTA3’                          |
| Q               | d    | 5’Phos- GTTAGGACTGCTGCTCATC3’             |
| E1             | d    | 5’AGTTAGGACTGCTGCTCATC3’                 |
| E2             | d    | 5’Phos- AGTTAGGACTGCTGCTCATC3’           |
| F(5)           | d    | 5’Phos- TTTTATGTTAGGACTGCTGCTCATC3’      |
| G(15)          | d    | 5’CTGGCACCTTGGAAAAAGCTCGCTGCTGCTCATC3’  |
| T              | T    | 5’GATGACGACGGTCTTAACTGTGGAGGAGAAGCTTCGCTCATC3’ |
| T3F            | T    | 5’GATGACGACGGTCTTAACTGTGGAGGAGAAGCTTCGCTCATC3’ |
| f-N            | d    | (FAM)-5’AGTTAGGACTGCTGCTCATC3’          |
| f-F(5)         | d    | (FAM)-5’TTTTATGTTAGGACTGCTGCTCATC3’     |
| G(15)-f        | d    | 5’CTGGCACCTTGGAAAAAGCTCGCTGCTGCTCATC3’-(FAM) |

*a* Refers to the type of oligonucleotide it is for the establishment of the correct ratio for folding. See "Experimental Procedures."

*b* These two oligonucleotides differ by the absence or presence of a 5’-phosphate monoester.

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**FIGURE 2.** hFEN1 cleavage site selection is predominantly at the two-way junction and shows no evidence for processive cleavage. Reactions containing substrate and the indicated concentration of hFEN1 were incubated at 37 °C, and aliquots were removed and quenched at the times indicated. Schematics of the substrates are shown above the gel image with the position of the radiolabel and the cleavage site indicated by the gray star and arrow, respectively. A, the image is representative of results from substrates labeled on the 3’-termini of the d-strand oligonucleotides and analyzed using a 20% denaturing PAGE (19:1). Size markers (M) allow for cleavage site determination. B, the image shows the results from an hFEN1 assay with the foldback-flap hairpin. The experiment is shown in Fig. 2A. The reaction on a double flap substrate (F(5)-T3F) resulted in a single END product corresponding to reaction one nucleotide into the downstream duplex region, whereas the single flap substrate (F(5)-T) was hydrolyzed mainly at the 5’-flap upstream and downstream duplexes and in the case of the GEN substrate, the foldback-flap hairpin. A concern sometimes raised with hairpin DNA substrates is the possibility of intermolecular rather than intramolecular duplex formation. However, the melting temperatures of transitions assigned as hairpins (T3Fm = 75.5 °C and G(15) m = 80.5 °C) were independent of strand concentration indicating the formation of intramolecular structure (Fig. S2, B, D–F: insets). In contrast, the melting temperatures of transitions assigned as heteroduplexes were concentration-dependent consistent with intermolecular structure (56). Initial Characterization and Optimization of hFEN1-catalyzed Reactions—The site of hFEN1-catalyzed hydrolysis on each substrate was initially characterized using radiolabeled substrates. A typical experiment is shown in Fig. 2A. The reaction on a double flap substrate (F(5)-T3F) resulted in a single END product corresponding to reaction one nucleotide into the downstream duplex region, whereas the single flap substrate (F(5)-T) was hydrolyzed mainly at the 5’-flap upstream and downstream duplexes and in the case of the GEN substrate, the foldback-flap hairpin. A concern sometimes raised with hairpin DNA substrates is the possibility of intermolecular rather than intramolecular duplex formation. However, the melting temperatures of transitions assigned as hairpins (T3Fm = 75.5 °C and G(15) m = 80.5 °C) were independent of strand concentration indicating the formation of intramolecular structure (Fig. S2, B, D–F: insets). In contrast, the melting temperatures of transitions assigned as heteroduplexes were concentration-dependent consistent with intermolecular structure (56). Native gel electrophoresis confirmed that the pairing of oligomers and folding protocol resulted in strand annealing (supplemental Fig. S2A). Furthermore, comparison of first derivative melt profiles (supplemental Fig. S2, B–F) and experimental and predicted thermodynamic parameters from UV melting studies (supplemental Table S2) verified the formation of the ssDNA/dsDNA junction with a minor reaction site 1 nt into the downstream duplex. Because the expected products for flap hFEN1 substrates are well known (39), another indication that the substrates folded as predicted was that they afforded the expected cleavage products and that when allowed to proceed for longer time periods (>8 min), hydrolysis could approach near-completion. In a manner analogous to the double flap substrate, the EXO (E-T3F) and fork-GEN (G(15)-T3F) substrates with a 3’-flap showed cleavage 1 nt into the duplex. In all cases where a 3’-flap was present there was no evidence that the initial 3’-radiolabeled product of hFEN1 action could react further even under multiple-turnover conditions or that the reac-
tion was processive. With a single flap substrate labeled at the 3′ terminus, the minor cleavage product could be due to additional EXO cleavage subsequent to formation of the major cleavage product, but the presence of 5- and 6-nt products when the 5′ terminus is labeled suggests this to be unlikely (data not shown).

Because previous studies showed that GEN substrates could undergo EXO and ENDO cleavage (39), the fork-GEN reaction was also studied using a 5′-radiolabeled substrate. In the presence of the 3′-flap, the product of fork-GEN hydrolysis was predominantly ENDO (Fig. 2B). However, trace amounts of EXO product were detected. This EXO product could have resulted from EXO cleavage of the intact substrate or the ENDO product. In contrast, a fork-GEN substrate lacking the 3′-flap (G(15)-T) required far more enzyme to observe cleavage within the same time frame and underwent considerably more EXO reaction at the 5′-flap hairpin. Nonetheless, ENDO cleavage was still observed.

Subsequently, hFEN1 reactions were analyzed using fluorescein-labeled substrates and reversed-phase ion-pairing denaturing-HPLC with fluorescence detection. This technique has been previously used for the kinetic characterization of T5FEN1 (48). Using substrates labeled with an alkyl-fluorescein moiety attached at either the 5′ or 3′ terminus, separation was achieved for all four hFEN1-catalyzed reactions (supplemental Fig. S3). Comparison of the product retention times to synthetic standards confirmed that the products were consistent with those observed using traditional methodology (data not shown). Thus, the presence of the fluorescein label on the substrates did not interfere with cleavage site selection.

**The Steady-state KCl Optimus of hFEN1—** Previous studies of mammalian FEN1s using single flap substrates showed that addition of monovalent salts to the assay buffer decreased FEN1 activity (19). However, the monovalent salt optimum for hFEN1-catalyzed reaction with the so-called physiological “double flap” substrates like F(5)-T3F has not been investigated. Furthermore, because protein-nucleic acid interactions are dominated by electrostatics (58), and kinetic studies are conducted with a wide range of substrate concentrations, the effect of the substrate concentration on the apparent KCl optimum was examined. In contrast to earlier studies with single flap substrates (19), the hFEN1-catalyzed hydrolysis of the double flap substrate (F(5)-T3F) was found to be mildly stimulated rather than inhibited by the addition of monovalent salt. Conducting the experiment at several concentrations of the double flap substrate (2.5–2500 nM), the KCl optimum for FEN1 was shown to progressively increase from 75 mM to 150 mM (Fig. 3). Thus, the monovalent salt optimum for hFEN1 under steady-state was a function of the substrate. Because the melting temperature of heteroduplexes and hairpins is also a function of monovalent and divalent salt concentrations, it was possible that, although the Mg2+ concentration was constant, the substrate was partially unfolded at low salt. Thus, the initial increase in activity could have been due to an increase in substrate stability at the assay temperature. However, DINAMelt simulations (57) for the heteroduplex domain and of the hairpin using UNAFold (55) produced melting temperatures greatly in excess of the assay temperature. For subsequent kinetic studies, we chose to use 100 mM KCl for kinetic assays, because it was reasonably efficient for all substrate concentrations tested. Furthermore, because the hFEN1 Mg2+ optimum was originally determined in the absence of added monovalent ions, the Mg2+ optimum was reassessed in the presence of 100 mM KCl. However, its presence did not influence the optimum (data not shown); thus, subsequent studies used 8 mM Mg(OAc)2.

**Steady-state Kinetic Parameters Demonstrate the Importance of the 3′-Extrahelical Nucleotide—** To determine the steady-state kinetic parameters for all four fluorescent substrates, normalized initial rates of reaction were measured at least six times over a range of substrate concentrations above and below the expected K_m values. In cases where more than one product was present (F(5)-T and to a far lesser degree F(5)-T3F), the initial rate was estimated using the sum of all products. The data for the double flap (F(5)-T3F), EXO substrate with 3′-flap (E-T3F), and single flap (F(5)-T) were appropriately modeled using the MM equation (supplemental Fig. S4, A, B, and D), whereas the data for the fork-GEN with 3′-flap (G(15)-T3F) substrate were best modeled using the MM model with a Hill coefficient (MMh), because the initial rate of reaction continued to increase with substrate concentration (Fig. S4C). The Hill coefficient for the G(15)-T3F data fit using the MMh model was 0.7. Catalytic parameters of the fluorescent substrates are shown in Table 2. For comparison reactions were carried out with 3′-radiolabeled substrates under the same buffer conditions at substrate concentrations well below K_m. The normalized initial rate of reaction under these conditions was an approximation of k_cat/K_m and was observed to be similar to the analogous measurement using fluorescent substrates (data not shown).

**FIGURE 3. The steady-state KCl optimum of hFEN1 is a function of substrate concentration.** A plot of the normalized activity at 2.5 mM (open circles), 25 mM (filled triangles), 250 mM (filled squares), and 2500 mM (filled circles) F(5)-T3F versus added KCl concentration. The points are the average of three replicates. Activity is the number of moles of product formed per unit assay time, and normalized activity is the activity divided by the hFEN1 concentration used.

Both the EXO reaction of the nicked DNA substrate that possesses a 3′-flap (E-T3F) and the ENDO reaction of the double flap substrate (F(5)-T3F) proceeded with similar catalytic efficiency (k_cat/K_m). However, in comparison to the double flap substrate, the turnover number and K_m for the EXO substrate were reduced 2.3- and 1.4-fold, respectively. Because the only difference between these two substrates was the absence of a 5′-flap (Fig. 1, B and C), the loss of the 5′-flap when the 3′-flap was present did not drastically reduce the efficiency of the reaction unlike a previous study with an EXO substrate lacking a 3′-flap (39). In contrast, the loss of the 3′-extrahelical nucleo-
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TABLE 2
Catalytic parameters for hFEN1-catalyzed reactions

| Substrate | $k_{\text{cat}}$ | $K_m$ | $k_{\text{cat}}/K_m$ | $k_{\text{STmax}}$ | $k_{\text{STmax}}/k_{\text{cat}}$ | $\Delta G_n$ |
|-----------|-----------------|-------|----------------------|-------------------|-----------------------------|-----------|
|           | min$^{-1}$ | nm    | nm min$^{-1}$ | min$^{-1}$ | min$^{-1}$ | kMol$^{-1}$ |
| F(5)-T3F | 164 (5.1) | 44.0 (4.0) | 3.7 | 1350 (72) | 8.2 | 0.9 |
| E-T3F    | 71.4 (1.6) | 27.5 (2.1) | 2.6 | 631 (67) | 8.8 | 5.9 |
| G(15)-T3F | 81.6 (3.5) | 185 (33) | 0.44 | 638 (110)$^{d}$ | 7.8 | NS$^{*}$ |
| F(5)-T   | 20.0 (0.6) | 185 (18) | 0.11 | 225 (28)$^{d}$ | 11 | 9.1 |

$^a$ $k_{\text{STmax}}$ is the maximum single turnover reaction rate.
$^b$ The relative differences in the Gibbs free energy of transfer were calculated according to Equation 4. See "Experimental Procedures."
$^c$ The reported $k_{\text{cat}}$ and $K_m$ are derived from the Michaelis-Menten model with a Hill slope, which was 0.7 (0.04) for the fit.
$^d$ The value reported corresponds to the initial phase of the exponential. The value for the second phase of the exponential is 14.2 (3.3) min$^{-1}$.

This substrate was not applicable to this analysis due to changes in flap length and the presence of a hairpin.


tide had a much larger effect on the kinetic constants. The turnover number for the "single flap" substrate (F(5)-T) was reduced 8.7-fold, whereas the $K_m$ value increased 4.2-fold when compared with its cousin, F(5)-T3F (Fig. 1, B and E). Taken together, the removal of the 3'-flap from the substrate altered the kinetic parameters of hFEN1-catalyzed reaction to a greater extent than the loss of the ssDNA 5'-flap. Although hFEN1 cleavage site selection on the fork-GEN (G(15)-T3F) and double flap (F(5)-T3F) substrates was shown to be analogous, the turnover number was approximately half that of F(5)-T3F, and the $K_m$ was ~4-fold higher than the values for the double flap substrate. Thus, the presence of a hairpin structure in the flap-strand adversely affected the efficiency of the reaction. However, in comparison to the single flap substrate, the decrease in the turnover number caused by the presence of a hairpin structure was surprisingly smaller than that observed with the loss of the 3'-extrahelical nucleotide. Apparent second-order rate constants ($k_{\text{cat}}/K_m$) have been used as a measure of substrate specificity (50). From the data discussed above, the order of substrate preference was double flap (F(5)-T3F) > EXO with 3'-flap (E-T3F) > fork-GEN with 3'-flap (G(15)-T3F) > single flap (F(5)-T).

Single Turnover Experiments Indicate That Product Release Is the Predominant Rate-limiting Step—Product release has been shown to be partially rate-limiting for T5FEN1-catalyzed reactions (23). To determine if this was also the case for hFEN1, single turnover (ST) experiments were performed using a quench flow apparatus under conditions whereby [hFEN1] $\gg$ $K_m$ $>$ [substrate] (23, 48). Reactions were initiated by combining equal volumes of enzyme and substrate in magnesium-containing buffer and quenched with sodium hydroxide. All ST data were initially modeled using a one-phase exponential (Equation 5). Although the data for F(5)-T3F, E-T3F, and F(5)-T were fit with a one-phase exponential, the G(15)-T3F data (supplemental Fig. 5S) was better modeled using a two-phase exponential (Equation 6). To ensure that the measured rates were not influenced by enzyme substrate association, an alternative mixing experiment was conducted where enzyme and substrate were pre-mixed in the absence of divalent metal cofactor and then reaction initiated by addition of metal cofactor. This alternative mixing protocol produced the same rate of reaction (data not shown).

The maximal ST rates of reaction ($k_{\text{STmax}}$) for the four substrates varied from 200 to 1400 min$^{-1}$ and were faster than the respective steady-state turnover number ($k_{\text{cat}}$) (Table 2). Thus, all four steady-state hFEN1-catalyzed reactions studied herein were rate-limited by product release. The degree to which product release attenuates T5FEN1 multiple turnover rates has previously been assessed from the quotient of the ST rate and turnover number ($k_{\text{STmax}}/k_{\text{cat}}$) (23). For the four hFEN1 substrates studied, the extent to which product release attenuated multiple turnover (MT) rates varied ~7- to 11-fold (Table 2). In summary, the $k_{\text{cat}}$ for all four hFEN1-catalyzed reactions studied was not a measure of catalysis as was concluded in previous studies (39) but was instead a measure wholly or partially of the rate of enzyme product release.

Reactions Catalyzed by hFEN1 Are Susceptible to Product Inhibition—Because product release was rate-limiting in hFEN1 MT experiments, it was of interest to determine the order in which hFEN1 released its two products. The expected patterns of product inhibition (i.e. competitive, linear-mixed/ non-competitive, or uncompetitive inhibitors) have been predicted for Uni Bi enzymes (59, 60). Thus, we reasoned that such a study with hFEN1 could shed light on the catalytic cycle of hFEN1. As previously shown (Fig. 2A), the products from hFEN1-catalyzed reaction with the double flap substrate, F(5)-T3F, are a 6-nt product (P6) and a 19-nt product (Q) that under native conditions remains base paired with the T3F template hairpin (Q-T3F) (Fig. 4, A and B). For product inhibition studies, we measured the normalized initial rates of reaction for F(5)-T3F in the presence of P6 or Q-T3F inhibitor. Due to the limited number of substrate concentrations tested ($n = 6$ concentrations of F(5)-T3F per concentration of P6 or Q-T3F), a Lineweaver-Burke model was used to determine if inhibition was occurring, what type of inhibition was occurring, and to estimate the kinetic parameters. Although deriving parameter estimates from double reciprocal plots has been discouraged due to error redistribution upon taking the reciprocal of the data, an appropriate weighting scheme was applied to mitigate these effects (61).

Results from the study using the P6 product indicated that P6 was not an inhibitor of the F(5)-T3F forward reaction even at concentrations as high as 5 μM (data not shown). Thus, the P6 product has a very weak to no affinity for the free enzyme or enzyme intermediate in the hFEN1 catalytic cycle under the conditions tested. On the other hand, Q-T3F acted as a competitive inhibitor with respect to F(5)-T3F (Fig. 4C). Further support for this conclusion was derived from a secondary plot of the apparent slopes of each line versus the respective inhibitor concentration (Fig. 4C, inset), which showed the expected trend for increasing concentrations of inhibitor (49). In addition, the secondary plot allowed for determination of the inhibition con-
A.  
\[ E + A \xrightleftharpoons[k_{\text{on}}]{k_{\text{off}}} [EA] \xrightarrow{k_{\text{cat}}} [EPQ] \xrightarrow{k_{\text{release}}} E + P + Q \]

B. (F(5))-T3F  
5′- end label

Q•T3F  
5′- end label

P6  
5′- end label

C.  

![Graphical representation of the kinetic parameters for the reaction](image)

**FIGURE 4.** One of the products from the F(5)-T3F reaction is a competitive inhibitor. A, schematic of an hFEN1-catalyzed reaction shown in Cleland nomenclature illustrating the possible intermediates, where \( E, A, P, \) and \( Q \) represent enzyme, substrate, product 1, and product 2, respectively. The enzyme-substrate and enzyme-product complexes are shown in brackets. B, hFEN1 predominantly cleaves the dST3F substrate into a 6-nt ssDNA 5′-product (P6) and a dsDNA 3′-product (Q-T3F). C, Lineweaver-Burke plot of the reciprocal of normalized initial rate versus the reciprocal of the substrate concentration at various concentrations of the 3′-product Q-T3F (black, 0 nM; cyan, 15 nM; green, 30 nM; orange, 60 nM; purple, 90 nM; and magenta, 120 nM). Data were best modeled as a competitive inhibitor (model ii) with an estimated variance power of 2 × 0.64 with 121 degrees of freedom. The residual standard error was 0.03. Inset: plot of the apparent slope (\( m_{\text{app}} \)) of the line from the Lineweaver-Burke model versus the concentration of Q-T3F (59, 60). The parameter estimates for \( k_{\text{cat}}, K_{m}, \) and \( K_{i} \) from the model are 172 min⁻¹, 44 nM, and 75 nM, respectively. D, hypothetical catalytic cycle of hFEN1 based on data herein. When FEN1 \([E] + \) substrate \([A]\) form a stable enzyme-substrate complex \([EA]\) \( k_{\text{on}} \), the complex can either dissociate \( (k_{\text{off}}) \) or rearrange to create a cleavage-competent complex \([\text{E}^\prime]\) \( 40 \). When the scissile phosphate is cleaved presumably via a “two-metal-ion” mechanism (75), the ssDNA 5′-product \([P]\) dissipates quickly to create a stable enzyme product complex \([\text{EQ}] \). Subsequent dissociation \( (k_{\text{release}}) \) of the enzyme-product complex regenerates free enzyme for another catalytic cycle. Only under conditions of initial rate can the enzyme-product re-association be ignored. In such a reaction scheme, the macroscopic \( K_{m} \) parameter would be a combination of all microrate constants within the bracket and may be thought of as a dissociation constant for all enzyme-bound intermediates (50). The macroscopic \( k_{\text{cat}} \) term would mainly be a reflection of enzyme-product release as indicated by it being placed above this step but may be attenuated by the first order microrates within the bracket (23). The maximal single turnover rate \( (k_{\text{Stmax}}) \) would reflect one or some combination of the first order microrates indicated by the bracket.

**DISCUSSION**

**Kinetic Parameters Show That the 3′-Flap Is a Critical Structural Element for hFEN1-catalyzed Reaction**—The results presented here demonstrate that at near physiological monovalent salt conditions and in the presence of a 3′-extrahelical nucleotide mimicking nucleic acid structures present during lagging strand DNA replication (41), the hFEN1 reaction of substrates with or without a 5′ ssDNA flap proceeds with comparable efficiency. On the other hand, the absence of the 3′-flap results in a decrease in catalytic efficiency. The energetic contribution of the ssDNA 5′-flap and the 3′-extrahelical nucleotide (\( \Delta G_{\text{ep}} \)) can be assessed using the ratios of the second-order rate constants (50) for carefully designed substrates that differ by one structural element. Although previous studies have emphasized the importance of the ssDNA 5′-flap structure to hFEN1 substrate discrimination (62), the loss of the 3′-flap is approximately nine times more detrimental to hFEN1-catalyzed reaction than a 5′-flap (Table 2), thereby implying a preeminent role for the 3′-flap interaction.

The most surprising result of this study is that the presence of a secondary structure within the 5′-flap region in the form of a very stable hairpin loop is tolerated and produces relatively small changes in catalytic parameters provided the fork-GEN substrate is presented with a 3′-extrahelical nucleotide. The results allow two possible mechanisms for the observation of GEN-type activities to be ruled out. First, the GEN-type reaction observed here is predominantly the result of reaction of the intact substrate. Most previous GEN-type substrates, such as simple duplex-gap substrates, underwent considerable EXO reactions and reacted slowly (39). One potential explanation for the GEN phenomenon on these simple substrates is that progressive EXO reaction eventually revealed a 5′-ssDNA flap substrate. This is not the case with the fork-GEN substrate with a 3′-flap (G15)-T3F, where the dominant reaction monitored by 5′ labeling corresponds to reaction at the junction and results from 3′-end-labeling show that the cleavage occurs 1 nt into the downstream duplex in a manner analogous to reaction on a double flap. Furthermore, the rates of reaction observed are not consistent with multiple catalytic cycles. Second, the stimula-
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...tion and increase in cleavage site specificity of the reaction of the fork-GEN substrate with and without a 3'-flap are analogous to those observed when comparing single and double flaps. Because the binding site for the 3'-extrahelical nt is in the upstream binding site, the fork-GEN with 3'-flap reaction does not proceed by binding the flap-hairpin duplex in the FEN1 upstream binding site (37).

Although the data have ruled out two possible explanations for fork-GEN reaction, the observed deviation from hyperbolicity for the steady-state data and the need for a two-phase exponential for the ST data on the fork-GEN substrate poses new questions. In ST experiments where $[E] \gg [S]$, EXO and ENDO reaction may occur in parallel in a manner analogous to that observed with G(15)-T (Fig. 3B). In the dHPLC assay with G(15)-T3F (supplemental Fig. S3), only the ENDO product is observable. Thus, one possible explanation for the two-phase exponential is that the first phase of the exponential is defined by hFEN1-substrate complexes that are ENDO-competent, whereas the slow phase of the curve is the ENDO cleavage rate attenuated by EXO-competent complexes dissociating and then reforming an ENDO-competent complex. The deviation in hyperbolicity can be caused by a number of factors (58, 60, 61). However, with a substrate that can form alternative enzyme substrate complexes, the deviation in hyperbolicity under steady-state conditions ($[E] \ll [S]$) may be caused by alternative binding modes that become more prevalent as the substrate concentration is increased. Obviously, further study of this phenomenon is warranted.

The apparent second order rate constants for hFEN1 hydrolysis of EXO and ENDO reaction with the 3'-flap (E:T3F, $4.5 \times 10^7$ M$^{-1}$ s$^{-1}$ and F(5)-T3F, $6.2 \times 10^7$ M$^{-1}$ s$^{-1}$, respectively) approach those normally associated with rate-limiting encounter of enzyme and substrate of $10^{-7}$-$10^{-8}$ M$^{-1}$ s$^{-1}$ (50). For diffusion-controlled reactions to occur, the rate of dissociation of the enzymesubstrate complex has to be slower than any forward process in the catalytic cycle. One possibility is that the 3'-flap stabilizes the hFEN1-DNA complex such that hFEN1 catalysis comes close to possessing these features. This is consistent with previous reports that hFEN1 displays a higher affinity for double flap substrates as determined by gel shift assays (63). Alternatively, the presence of the 3'-flap could also increase a first order rate after initial enzyme-substrate complex formation (i.e. catalysis or conformational change). According to the data presented here, the presence of the 3'-flap on a 5'-flap substrate both lowers $K_m$ and stimulates both multiple and single turnover rates of reaction, thereby suggesting that this interaction may contribute to both ground state substrate binding and subsequent first-order steps in the catalytic cycle of hFEN1 (Fig. 4D).

For all the substrates tested here, the maximal single turnover rates of reaction ($k_{S_{\text{max}}}$) are greater than the respective MT rates ($k_{\text{cat}}$). This identifies the predominant rate-limiting step at saturating substrate concentrations in multiple catalytic cycles as product release. Moreover, whereas the short 5'-product of a double flap substrate (P6) is not an inhibitor of the reaction, the longer 3'-product (Q-T3F) is a competitive inhibitor, thereby identifying the stable enzyme-product species. Curiously, although the 3'-flap is involved in enzyme-substrate stabilization and would therefore be expected to contribute to enzyme-product stabilization as well, the rate-limiting step for the single flap substrate (F(5)-T) is also product release, and the extent to which the 3'-products of single flap hydrolysis retard MT rates (Table 2) is surprisingly greater than 3'-products with the 3'-flap. Similar effects have also been observed with T5FEN1 (23), which lacks a 3'-flap binding site. Thus, the predominant features that confer enzyme-product stability likely arise from structural features elsewhere in the substrate.

Comparison of the differences in maximal ST rates for various substrates showed that the $k_{S_{\text{max}}}$ for EXO and fork-GEN reactions was 2-fold lower than that of the double flap substrate. Interestingly, this 2-fold decrease for both substrates correlates with the approximate 2-fold decrease in $k_{\text{cat}}$ observed in MT experiments. In a similar fashion, the approximate 6-fold difference in the ST rate for the single flap compared with the double flap roughly correlates with the 8-fold decrease in the MT rate. These observed correlations suggest that the first order rates associated with the conversion of the intermediates subsequent to initial enzyme-substrate complex formation, but preceding the enzyme-product complex release, attenuate the overall steady-state turnover rate (Fig. 4D).

The Addition of Monovalent Salt Does Not Inhibit hFEN1-catalyzed Hydrolysis of Double Flap Substrates—Due to the predominance of electrostatics in their mode of interaction, most protein-nucleic acid complexes are destabilized when the ionic strength of the buffer is increased (58). Mammalian FEN1s have traditionally been studied at low monovalent salt concentrations (19, 39), conditions that should favor stability of the enzyme-substrate complex. However, hFEN1-catalyzed hydrolysis of the double flap substrate is remarkably robust in the presence of monovalent salt; in fact, reaction at higher substrate concentrations is mildly stimulated at near physiological KCl concentrations. The stimulation observed at higher substrate concentrations under $k_{\text{cat}}$ conditions might reflect the destabilization of the enzyme-product complex. Destabilization of enzyme-product complex would accelerate the rate of multiple turnover reactions at high substrate concentrations until enough salt is added to significantly destabilize the enzyme-substrate complex so that substrate is no longer saturating. However, at sub saturating substrate concentrations ($k_{\text{cat}}/K_m$ conditions), hFEN1 hydrolysis of the double flap substrate is not retarded, but rather stimulated by addition of physiological amounts of KCl, despite the predominance of electrostatic interactions in the hFEN1-substrate complex (38). Even a diffusion-controlled reaction should be inhibited by salt eventually, if the interactions are predominantly electrostatic (64, 65), albeit the concentration required to achieve this may not have been reached in this study. Although the origins of monovalent salt stimulation of hFEN1 activity with a double flap substrate deserve further investigation, this property may in part be conferred by the non-ionic 3'-flap interactions.

Mechanism of hFEN1 Substrate Recognition—Several mechanisms have been proposed to suggest how FEN1 proteins recognize the substrate and select the scissile phosphate. The model predominantly accepted for mammalian FEN1 proteins has long been the threading or tracking model, wherein the protein recognizes the free 5'-ssDNA tail and threads the...
ssDNA strand through the helical arch. Upon reaching the junction, the protein is hypothesized to cleave the appropriate scissile phosphate (62, 66, 67). Based on work with the 5′-3′ exonuclease domain of Escherichia coli DNA polymerase I, Joyce and co-workers proposed that FEN1 proteins initially recognize or capture the two-way junction and then thread the helical arch with the ssDNA 5′-flap (40). Both models are difficult to reconcile with GEN-type activities, because crystallographic analyses of FEN1 structure show that duplex DNA is too large to traverse a structured arch (32, 33, 67). In light of the GEN activity, Chapados et al. proposed that instead of threading through the helical arch, the helical arch clamps the flap structure (35). However, a recent structure of bacteriophage T4FEN1 bound to a pseudo-Y DNA shows that the 5′-flap appears to pass through the arch (22). Furthermore, although a portion of the T4FEN1 arch is disordered, the 5′-flap makes extensive contacts with arch residues. Based on our work and recent structural observations (22), we believe that the model proposed by Joyce and co-workers (40) is consistent with the data we have presented here. First, we have shown that hFEN1 is capable of cleaving a substrate lacking a 5′-flap (E13T3F) almost as well as one with a flap. Second, the 3′-product Q13T3F, which is a poor alternative substrate, is a competitive inhibitor with respect to the double flap substrate, whereas the 5′-product, P6, is not.

Although the data can argue for initial substrate capture in vitro by recognition of the two-way junction, how the scissile phosphate is selectively placed in the active site to create the cleavage-competent complex (40) is yet to be determined. Because the hFEN1 helical arch is composed mainly of positively charged and hydrophobic amino acids, it is conceivable that, after the capture step, conformational fluctuations in the helical arch could “tease” the ssDNA flap into the archway. However, cleavage of fork-GEN substrates would argue against this. Although UV thermal melting shows that the G15 hairpin is quite stable in solution, one cannot guarantee the stability of the G(15) hairpin once the two-way junction with 3′-flap is reformed (7.3 × 10^6 M⁻¹ s⁻¹) observed for fork-GEN substrates with a 3′-flap argues the plausibility of a biological significance to this hFEN1 activity, especially as proliferating cell nuclear antigen has previously been shown to stimulate it (69–72). Furthermore, most secondary structures presented within flaps in a biological context, such as those resulting from triplet repeats, are unlikely to be as stable as the very stable hairpin introduced here. Thus, other factors such as mismatch repair protein stabilization of trinucleotide repeat fold-back hairpins likely contribute to trinucleotide repeat expansion and contraction phenotypes in vivo (73).

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