Effect of Flap Modifications on Human FEN1 Cleavage[†]

Carole J. Bornarth,[‡,§,||] Tamara A. Ranalli,[‡,§] Leigh A. Henricksen,[‡] Alan F. Wahl,[⊥] and Robert A. Bambara*[,‡,@]

Department of Biochemistry and Biophysics and Cancer Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642, and Seattle Genetics Inc., 22215 26th Avenue Southeast, Bothell, Washington 98021

Received June 9, 1999; Revised Manuscript Received August 17, 1999

ABSTRACT: The flap endonuclease, FEN1, plays a critical role in DNA replication and repair. Human FEN1 exhibits both a 5′ to 3′ exonucleolytic and a structure-specific endonucleolytic activity. On primer−template substrates containing an unannealed 5′-tail, or flap structure, FEN1 employs a unique mechanism to cleave at the point of annealing, releasing the 5′-tail intact. FEN1 appears to track along the full length of the flap from the 5′-end to the point of cleavage. Substrates containing structural modifications to the flap have been used to explore the mechanism of tracking. To determine whether the nuclease must recognize a succession of nucleotides on the flap, chemical linkers were used to replace an interior nucleotide. The nuclease could readily traverse this site. The footprint of the nuclease at the time of cleavage does not extend beyond 25 nucleotides on the flap. Eleven-nucleotide branches attached to the flap beyond the footprinted region do not prevent cleavage. Single- or double-thymine dimers also allow cleavage. *cis*-Platinum adducts outside the protected region are moderately inhibitory. Platinum-modified branch structures are completely inert to cleavage. These results show that some flap modifications can prevent or inhibit tracking, but the tracking mechanism tolerates a variety of flap modifications. FEN1 has a flexible loop structure through which the flap has been proposed to thread. However, efficient cleavage of branched structures is inconsistent with threading the flap through a hole in the protein.

Flap endonuclease 1 (FEN1)[1] is a member of a family of structure-specific nucleases that play a crucial role in DNA replication and repair in prokaryotes and eukaryotes (*1*−*3*). The eukaryotic nuclease is involved in Okazaki fragment processing (*4*, *5*) and long-patch base excision repair (BER) (*6*). Biochemical analyses suggest that FEN1 participates with RNase H in the removal of the initiator RNAs of Okazaki fragments (*4*). This is consistent with the observation that a null mutant of RAD27/RTH1, the FEN1 homologue in *Saccharomyces cerevisiae*, exhibits slow growth, sensitivity to methyl methanesulfonate, and hyper-recombination, indicative of defects in DNA replication and repair (*7*). The preferred substrate for FEN1 is a template with a primer having an unannealed 5′-tail or flap structure (*8*). The 5′-end region of Okazaki fragments has been proposed to be displaced into a flap during RNA removal (*3*). This is supported by the demonstration that the RAD27/RTH1 nuclease in *S. cerevisiae* copurifies and interacts functionally with the dna2 helicase (*9*, *10*). This helicase has the appropriate directionality of movement for creating 5′-flap structures.

Damaged bases in DNA are repaired by two general mechanisms, short-patch or long-patch base excision repair (*11*, *12*). Both repair mechanisms are initiated by a DNA glycosylase that generates an abasic site through hydrolysis of the damaged base. The abasic site is then cleaved 5′ to the baseless sugar by an AP (apurinic/apyrimidinic) endonuclease. During short-patch BER, DNA polymerase β mediates removal and replacement of only the damaged nucleotide through a β-elimination reaction. In contrast, long-patch BER involves the removal and synthesis of a segment of DNA approximately two to five nucleotides in length (*13*−*15*). Long-patch BER is thought to repair oxidized, reduced, or fragmented sugars that are inert to the β-elimination reaction employed by the short-patch repair mechanism (*16*). Reconstitution of long-patch BER with purified enzymes requires either DNA polymerase δ or ϵ, proliferating cellular nuclear antigen, replication factor C, DNA ligase I, and FEN1 (*6*). Genetic evidence also supports a role for FEN1 in long-patch BER. Null mutants of FEN1 in *S. cerevisiae* exhibit sensitivity to methylmethane sulfonate, consistent with a BER defect (*7*). Previously, it was shown that FEN1 could not remove a 5′-end baseless sugar. However, if the damaged site is displaced to form a flap, FEN1 will cleave after the abasic site within the downstream DNA (*17*, *18*). Clearly, the ability to cleave a flap substrate is central to the biological function of FEN1.

[†] This research was supported by National Institutes of Health Grant GM24441 and Fellowship Grant GM18961 (to L.A.H.).

* To whom correspondence should be addressed: Department of Biochemistry and Biophysics, University of Rochester Medical Center, 601 Elmwood Ave., Box 712, Rochester, NY 14642. Phone: (716) 275-3269. Fax: (716) 271-2683.

[‡] Department of Biochemistry and Biophysics, University of Rochester School of Medicine and Dentistry.

[§] These authors contributed equally to this work.

[||] Present address: Division of Biological Sciences, Sections of Microbiology and Molecular and Cellular Biology, University of California, Davis, CA 95616.

[⊥] Seattle Genetics Inc.

[@] Cancer Center, University of Rochester School of Medicine and Dentistry.

[1] Abbreviations: AP, apurinic/apyrimidinic; BER, base excision repair; CDDP, *cis*-diamminedichloroplatinum; FEN1, flap endonuclease 1; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; NP-40, Nonidet P40; nt, nucleotide.

10.1021/bi991321u CCC: $18.00
Published on Web 09/11/1999

Previous analyses suggested that proper loading onto the flap structure is an important prerequisite for cleavage. Eukaryotic FEN1 was unable to cleave a substrate in which the 5′-region of the tail could anneal to the template, i.e., a bubble substrate (*19*). Primers annealed to the flap were inhibitory with respect to cleavage (*20*, *21*). Also, biotin−streptavadin complexes on the flap prevented cleavage (*20*). These observations are consistent with the idea that the nuclease tracks along the flap from the 5′-end to the site of cleavage. On the other hand, previous work has shown that FEN1 is able to cleave flaps containing small covalent adducts, 5′-end modifications, and RNA segments (*20*, *22*). Apparently, some modifications of flap structure are tolerated by the tracking mechanism, while others are not.

The crystal structure of the FEN1 homologue T5 exonuclease (*23*) revealed a helical arch, creating a hole in the protein. The authors suggested that the nuclease threads onto the substrate by sliding the 5′-end of the flap through the arch (*23*). Several other crystal structures of FEN1 homologues have been determined, including T4 RNase H (*24*), *Methanococcus jannaschii* FEN1 (*25*, *26*), and *Pyrococcus furiosus* FEN1 (*27*). Each crystal structure reveals an arch or loop, supporting the idea that this structure is important for the tracking mechanism of FEN1. Mutational analyses of the loop region in the *M. jannaschii* FEN1 suggest that this loop is critical in both the binding and cleavage of flap substrates (*25*).

Although biochemical and structural data are consistent with a tracking mechanism for FEN1, it has not been determined whether FEN1 is obligated to traverse the length of a flap prior to cleavage. The enzymatic footprint of FEN1 at the site of cleavage has been determined. The results indicate that that on a long flap (73 nucleotides) FEN1 contacts only the 25 nucleotides near the cleavage point (*22*). Yet modifications along the tail beyond the footprint affect FEN1 cleavage. We have generated a variety of substrates designed to address the interactions of FEN1 with the single-stranded flap to better define the mechanism of FEN1 cleavage. In the analysis that follows, we measured the effect of flap modifications, inside and outside of the footprinted region, for their ability to influence the efficiency of flap cleavage. Experiments were designed to determine whether the nuclease tracks from the 5′-end of the flap to the site of cleavage and whether such tracking involves threading the flap through a hole in the protein.

EXPERIMENTAL PROCEDURES

Materials

The synthetic branched oligonucleotides were supplied by Bristol-Myers Squibb. All other oligonucleotides were obtained from Genosys Biotechnologies (The Woodlands, TX). Nickel NTA agarose resin was obtained through Qiagen. The acrylamide that was used was the sequencing gel mix from Boehringer Mannheim. Radionucleotides [α-^{32}P]dGTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were from Dupont NEN. All other reagents were from Sigma.

Methods

Oligonucleotide Substrates. In all substrates (Table 1), the 3′-end regions of downstream primers are homologous with the 5′-ends of their respective templates. Once annealed, these primers create substrates with unannealed 5′-tails as shown in the figures. Each respective upstream primer was annealed to its template to create a nick at the base of the unannealed 5′-tail of the downstream primer. Prior to annealing, each of the downstream primers (except those in Figure 2) were 5′-phosphorylated and radiolabeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (Boehringer Mannheim) as per the manufacturer's instructions. The DNA substrate in Figure 2 was radiolabeled at the 3′-end using [α-^{32}P]dGTP, Sequenase, version 2.0, and purified over a G-250 Sephadex minicolumn. All substrates were gel purified on a 12% polyacrylamide, 7 M urea denaturing gel prior to annealing.

Substrates containing thymine dimers (Figure 5) were constructed from 11-mer oligonucleotides ligated to the 5′-end of a 42-mer. The procedure for dimerization and purification of dimerized oligonucleotides was described previously (*28*). One 11-mer primer containing the thymine dimer was annealed next to a 42-nucleotide downstream primer previously labeled with [γ-^{32}P]ATP at the 5′-end. The two primers were treated with T4 DNA ligase for 1 h at 37 °C to form the downstream primer $D_{1TTDIMER}$. The downstream primer $D_{2TTDIMER}$ was generated by ligation of two 11-mers to the radiolabeled 42-mer. Both $D_{1TTDIMER}$ and $D_{2TTDIMER}$ were gel purified on a 12% polyacrylamide, 7 M urea denaturing gel and annealed to the appropriate template (T_{DIMER}) and upstream primer (U_1) to form the final flap substrate.

Platination of DNA Substrates. DNA oligonucleotides were platinated as described previously (*29*). Briefly, an excess of *cis*-diamminedichloroplatinum (CDDP) was incubated with DNA in platination buffer (3 mM NaCl and 1 mM Na_2HPO_4) for 18 h in the dark at 37 °C. Platinated DNA was separated from nonplatinated DNA using 18% polyacrylamide, 7 M urea sequencing gel electrophoresis and gel purified. Because platinated DNA migrates more slowly, the resulting purified substrate is >99% platinated (*30*). When DNA had multiple sites for platination, the platination product with the slowest mobility was purified. It represents complete adduction of available sites.

Purification of Human FEN1. Recombinant human FEN1 was obtained from bacteria using the T7 RNA expression system (*31*) and purified using the procedure of Barnes and colleagues (*22*). Briefly, plasmid pET-FCH (*32*) was transformed into *Escherichia coli* strain BL21(DE3)/pLysS (*31*), grown at 37 °C, and induced with a final IPTG concentration of 1 mM for 5 h. Cells were collected by sedimentation and lysed by three passages through a French press. The recombinant protein, which contains the entire amino acid sequence for human FEN1 with a histidine tag at the C-terminal end, was purified by nickel NTA agarose chromatography and hydroxyapatite chromatography (*22*). Active fractions were pooled and further purified by CM-Sepharose chromatography as described for calf FEN1 (*33*). The final preparation was >95% pure according to analysis by SDS−polyacrylamide gel electrophoresis and staining with silver. Purified enzyme was dialyzed into a storage buffer [20% glycerol, 30 mM KCl, 30 mM HEPES (pH 7.6) (diluted from a 1 M stock), 0.01% NP-40, 1 mM DTT, and 1 mM EDTA] and stored at −80 °C.

Endonuclease Assay. Reactions were performed in buffer containing 30 mM HEPES (pH 7.6) (diluted from a 1 M

Table 1: Oligonucleotide Sequences (5′ to 3′)

	Length (nt)	
Downstream Primers[a]		
D_{amino}	58	TCGC**X**CGTTTCACGCCTGTTACTTAATTCACTGGCCGTCGTTTTACAACGTGACTGGG
$D_{2Ttdimer}$	64	GCAAG**TT**GGAGGCAAG**TT**GGAGCGTACGGACGTAGAGCTGTTTCCAAGTAAACGACGGCCAGTG
$D_{1Ttdimer}$	53	GCAAG**TT**GGAGCGTACGGACGTAGAGCTGTTTCCAAGTAAACGACGGCCAGTG
$D_{branch1}$	60	TCGC**A**CGTTTCACGCCTGTTACTTAATTCACTGGCCGTCGTTTTACAACGACGTGACTGG
$D_{branch2}$	71	TCGCACGTTTC**A**CGCCTGTTACTTAATTCACTGGCCGTCGTTTTACAACGACGTGACTGG
$D_{branch3}$	76	CCTAT**GG**CC**GG**TACATAACCTTATCTCTACTCCAATCAATTTAACTTCCACTTTCCTCACCCCA
D_{CDDP}	60	CCTAT**GG**CCAATAACCTTATCTCTACTCCAATCAATTTAACTTCCACTTTCCTCACCCCA
D_{2CDDP}	62	CCTAT**GG**CC**GG**TAATAACCTTATCTCTACTCCAATCAATTTAACTTCCACTTTCCTCACCCC
$D_{2CDDP.2}$	62	CCTAT**GG**CCAATAAT**GG**CCTTATCTCTACTCCAATCAATTTAACTTCCACTTTCCTCACCCC
Upstream Primers		
U_1	25	CGCCAGGGTTTTCCCAGTCACGACC
U_2	25	AAAAAAAACCCATTCACGACCGTGG
Templates		
T_{amino}	46	GCCCAGTCACGTTGTAAAACGGGTCGTGACTGGGAAAACCCTGGCG
T_{dimer}	43	GCACTGGCCGTCGTTTACGGTCGTGACTGGGAAAACCCTGGCG
$T_{branch1,2}$	48	GCCAGTCACGTCGTTGTAAAACGGGTCGTGACTGGGAAAACCCTGGCG
$T_{CDDP;branch3}$	50	CTGGGGTGAGGAAAGTGGAAGTTAACCACGGTCGTGAATGGGTTTTTTTT

[a] Bold nucleotides represent the location of modification. D_{AMINO} contains either guanosine, isopropyl amine, or 3-amino-thiocyano-1,2-propanediol at position X. $D_{2TTDIMER}$ and $D_{1TTDIMER}$ contain cis-syn thymine−thymine cyclobutane dimers. $D_{BRANCH1}$, $D_{BRANCH2}$, and $D_{BRANCH3}$ contain DNA side chains. D_{CDDP}, D_{2CDDP}, and $D_{2CDDP.2}$ are modified with *cis*-diamminedichloroplatinum. Underlined nucleotides indicate the first nucleotide of the unannealed 5′-tail. Downstream primers are annealed to their corresponding template to generate a flap substrate.

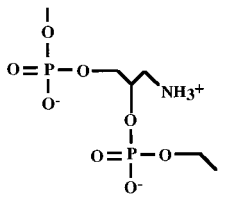
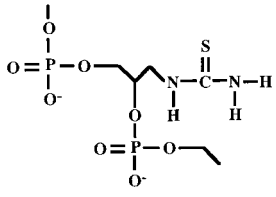
stock), 5% glycerol, 40 mM KCl, 0.1 mg/mL bovine serum albumin, and 8 mM $MgCl_2$ in a final reaction volume of 20 μL. Reaction mixtures were incubated at 37 °C for the periods of time indicated in the figure legends; the reactions were terminated with 10 μL of formamide dye [90% formamide (v/v) with bromophenol blue and xylene cyanol], and the mixtures were heated to 95 °C for 5 min. After separation on a 7 M urea, 12% polyacrylamide gel (*34*), products were detected by autoradiography or PhosphorImager (Molecular Dynamics) analysis.

RESULTS

Previous results suggest that FEN1 employs a tracking mechanism whereby it enters flap substrates at the 5′-end of the flap and then moves to the site of endonucleolytic cleavage at the base of the flap.

Basic Structural Requirements in the Flap. One possible means of traversing the flap would be to employ a "ratcheting mechanism", whereby the nuclease would recognize and interact with individual nucleotides on the flap as it moved to the point of annealing. While small substitutions on nucleotides allow FEN1 activity, the tracking mechanism might not be tolerant of complete replacement of a nucleotide with a chemical linker. We employed two substrates to test this idea. In the first, a nucleotide was replaced with a 3-amino-1,2-propanediol which forms an isopropyl amine adduct between adjacent phosphate residues (Table 2). In the second, the nucleotide was replaced by a guanidine isothiocyanate group, forming a 3-amino-thiocyano-1,2-propanediol adduct between the sugar phosphate residues (Table 2). Each substrate contains a 58-nucleotide downstream primer that forms a 38-nucleotide flap. Over time, FEN1 cleaves the 5′-tail, releasing the expected 38-nucleotide

Table 2: Non-Nucleotide Substitution Structures

Isopropyl amine adduct	3-amino-thiocyano-1,2-propanediol adduct
	

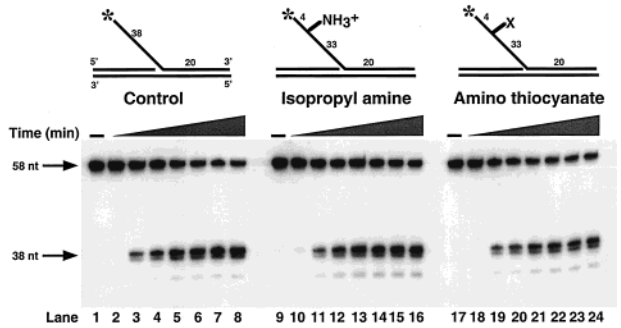


FIGURE 1: FEN1 is able to cleave non-nucleotide substitutions present in the tail. FEN1 was incubated with either unmodified or modified DNA substrates containing a non-nucleotide substitution. Reaction mixtures (179 μL) contained 90 fmol of DNA substrate and 80 fmol of FEN1, and reactions were performed as described in Experimental Procedures. Substrate oligonucleotide sequences are listed in Table 1. Reaction mixtures were incubated at 37 °C, and 20 μL aliquots were taken at 0, 1, 3, 5, 10, 15, 30, and 60 min as indicated by triangles. Substrate and cleavage product sizes are as indicated. Schematic representations of the substrates are depicted above the figure. The numbers represent the lengths of the adjacent segment in nucleotides, not including the site of modification. The asterisk shows the position of the radiolabel. The unmodified control substrate (lanes 1−8) is comprised of T_{AMINO}, U_1, and D_{AMINO} (Table 1) with the bold X residue replaced with a guanosine. The "isopropyl amine" substrate (lanes 9−16) is comprised of the same template−primer combination with the bold X residue replaced with a 3-amino-1,2-propanediol, forming an isopropyl amine adduct between two phosphate groups in place of a nucleotide base (Table 2). The "amino thiocyanate" substrate (lanes 17−24) is also comprised of the same template−primer combination with the substitution of a guanidine isothiocyanate group at the bold X residue, forming a 3-amino-thiocyano-1,2-propanediol adduct (Table 2). The modified residue is linked to two phosphates in place of a nucleotide base.

cleavage product (Figure 1). The rate of cleavage is similar for a control substrate (lanes 1−8) when compared to that of either the isopropyl amine (lanes 9−16) or amino thiocyanate (lanes 17−24) substrate. Efficient cleavage of both these substrates by FEN1 shows that successive recognition of some feature of nucleotide structure is not necessary for FEN1 to load onto its substrate.

Branched Flaps. Two types of flap modifications have previously been shown to inhibit FEN1. Platination within six residues of the cleavage site prevents cleavage (*22*). This is thought to interfere with the catalysis mechanism by distorting interactions with the active site. Biotin−streptavadin adducts inhibit by preventing tracking, or perhaps by a more general steric effect (*20*). To avoid this ambiguity, we attempted to block tracking with an adduct that was much smaller than a protein, but having a structure anticipated to frustrate threading the flap through a cleft or hole in the protein. We chose an oligodeoxynucleotide branch attached to the 2′-position of a ribonucleotide in the flap. The attachment is to a 3′-position on the first nucleotide of the branch, and the branch ends in a free 5′-end.

Enzymatic footprinting of human FEN1 bound to the flap substrate shows that the nuclease protects a region of the flap less than 25 nucleotides from the cleavage site (*22*). Enzymatic footprints tend to be several nucleotides larger than the actual region of contact as determined by chemical footprinting techniques (*35*). On this basis, we felt that a modification 25 or more nucleotides from the annealing point of the flap would not interfere with FEN1 interaction with

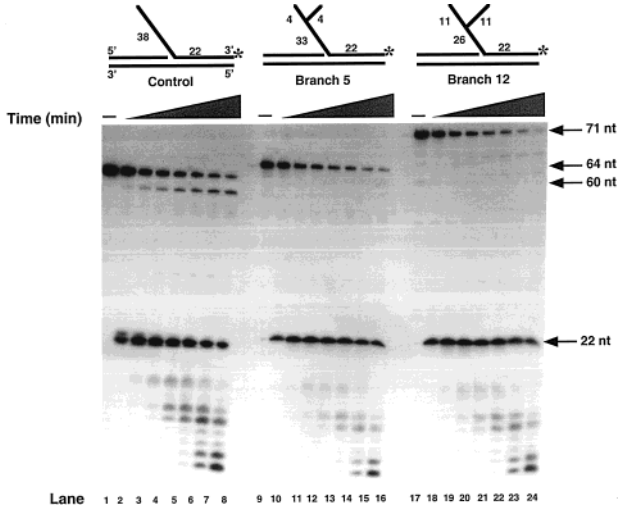


FIGURE 2: Branched DNA structure does not inhibit FEN1 nuclease activity. FEN1 was incubated with DNA substrates containing either a 4-nucleotide branch, an 11-nucleotide branch, or no branch near the 5′-end of the flap. Reaction mixtures (179 μL) contained 2 pmol of FEN1 and 90 fmol of 3′-end-labeled substrate, and reactions were performed as described in Experimental Procedures. Reaction mixtures were incubated at 37 °C, and 20 μL aliquots were taken at 0, 1, 3, 5, 10, 15, 30, and 60 min as indicated by triangles. Substrate and cleavage product sizes are as indicated. Schematic representations of the substrates are depicted above the figure. The numbers represent the lengths of the adjacent segment in nucleotides, not including the site of modification. The asterisk shows the position of the radiolabel. Lanes 1−8 contained the control substrate with no branch, which is comprised of $D_{BRANCH1}$, U_1, and $T_{BRANCH1,2}$. Lanes 9−16 contained the branch 5 substrate which is comprised of $D_{BRANCH1}$, U_1, and $T_{BRANCH1,2}$ with a 4-nucleotide branch. The branch point is located at the bold residue (at nucleotide 5) of $D_{BRANCH1}$ and consists of a sequence identical to the 5′-end region of the flap. Lanes 17−24 contained the branch 12 substrate, which is comprised of $D_{BRANCH2}$, U_1, and $T_{BRANCH1,2}$ with an 11-nucleotide branch. The branch point is located at the bold residue (at nucleotide 12) of $D_{BRANCH2}$ and consists of an 11-nucleotide sequence identical to the first 11 nucleotides of the 5′-end of the primer.

the site of cleavage. Consequently, the branches were placed either 33 or 26 nucleotides from the annealing point so that any interference with cleavage could be interpreted as a blockage of tracking.

Substrates containing branches 4 or 11 nucleotides long were placed either 5 or 12 nucleotides in from the 5′-end of the flap, respectively. The rate of cleavage was measured for each substrate and compared to that of a control substrate of the same sequence but lacking a branch structure (Figure 2). FEN1 was able to cleave both the 4 (lanes 9−16)- and 11-nucleotide branch (lanes 17−24) with efficiencies similar to that seen with an unmodified flap (lanes 1−8). In this experiment, the substrates were labeled at the 3′-end. Cleavage at the point of annealing results in a 22-nucleotide product. Smaller products appearing over time are the result of further exonucleolytic degradation of the downstream-annealed region. It was a distinct possibility that FEN1 would be able to first cleave off the protruding branch structure, followed by cleavage at the point of annealing. However, larger products consistent with cleavage within the flap were not observed. To verify the inability of FEN1 to recognize the branch point as a cleavage site, these experiments were repeated using substrates that were labeled at the 5′-end. Only a single cleavage product occurred in all cases, indicating

that FEN1 was not able to recognize the branch as a substrate (data not shown). It is a formal possibility that FEN1 can maneuver the flap into a configuration that can fit through a hole in the protein. However, we would expect such a process to at least have a noticeable effect on the rate of cleavage. A more reasonable explanation is that FEN1 employs a mechanism whereby the flap moves through a clamp-shaped cleft in the protein. Such a shape would allow branches of any length to slide through the open end of the clamp.

Another possibility is that the nuclease simply does not employ a tracking mechanism. As such, it would not be affected by any modification outside of the footprinted region. To test this, we altered the substrate to make the flap even more difficult to move through sites on the nuclease. A substrate with a 12-nucleotide branch was designed with two sets of adjacent deoxyguanosine (dG) residues separated by two nucleotides on each branch. The guanines are substrates for adduction by CDDP. Due to the positions of the dG residues, both intrastrand (between adjacent guanines) and interstrand (between opposite guanines) cross-linking is expected (*36*−*38*). No other adjacent dG residues are present in the substrate. The effect of CDDP adduction should be to make the region around the branch into a rigid structure.

After platination, two forms of modified substrate were observed. The slower migrating species most likely represents occupation of all four platination sites on the branch strands by intrastrand cross-linking. In addition, a faster migrating species was observed, apparently representing constriction of the substrate through interstrand cross-linking between each arm of the branch by CDDP. Both substrates prevented FEN1 cleavage (Figure 3). This result supports the hypothesized tracking mechanism for FEN1. Overall, results with branched substrates lead to the conclusion that if FEN1 tracks the length of the flap, the mechanism is very tolerant of flap structure. Threading through a small hole appears to be unlikely.

Inhibition by CDDP. The means by which platinum adduction to the branched substrate inhibited cleavage remained unclear. Previous work has shown that a single platinum adduct beyond six nucleotides from the cleavage site allowed catalysis (*22*). So, platinum adducts are not always completely inhibitory. Possibly, the unique structure of the platinated branch stopped FEN1. Alternatively, multiple platinum adducts in the same region of the flap may stiffen the structure in a way that prevents tracking. To test this possibility, we made a substrate having two nearby platinum adducts on the same flap.

We compared the cleavage efficiencies of substrates with either no adducts or one to two CDDP adducts (Figure 4A). Each substrate was cleaved, resulting in products consistent with cleavage of the flap near the point of annealing. In this case, a single adduct (lanes 1−8) was moderately inhibitory when compared to the control (lanes 9−16). Two CDDP adducts (lanes 17−24) were much more inhibitory compared to its control substrate (lanes 25−32). Quantitation revealed an approximate reduction in cleavage efficiency of 30 or 68% for one or two adducts, respectively. Clearly, on these substrates, the platinum adduct was not an absolute block, but rather a structure that reduced the efficiency of cleavage. Inhibition has a number of possible sources. The platinum adduct carries a charge, alters the structure of the immediate

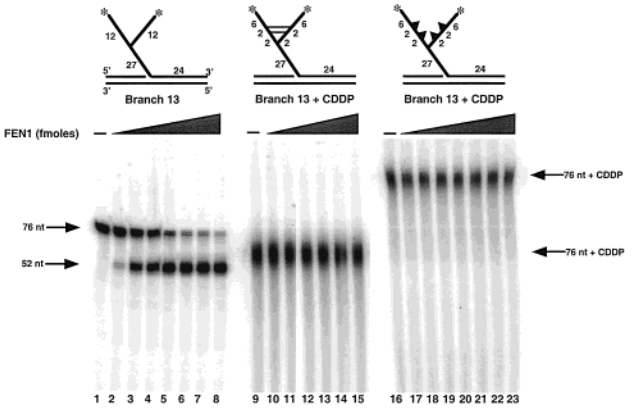


FIGURE 3: CDDP-adducted branched DNA substrates inhibit FEN1 activity. A 12-nucleotide branch substrate was modified with CDDP as described in Experimental Procedures and incubated with FEN1. Two CDDP-adducted branched substrates were gel purified and used in the FEN1 assay. Reaction mixtures contained 10 fmol of substrate with increasing amounts of FEN1 (1, 3, 5, 10, 30, 50, and 100 fmol) and were incubated at 37 °C for 15 min. The control reaction mixtures (−), containing substrate only, were incubated for 15 min. Substrate and cleavage product sizes are as indicated. Schematic representations of the substrates are depicted above the figure. The numbers represent the lengths of the adjacent segment in nucleotides, not including the site of modification. The asterisk shows the position of the radiolabel. Lanes 1−8 contained the unplatinated branch 13 substrate annealed to the template and upstream primer, comprised of T_{CDDP}, U_2, and $D_{BRANCH3}$ with a branch of 12 nucleotides, where the branch point is located at the bold X residue. The 12-nucleotide branch sequence is identical to the 5′-end region of the substrate $D_{BRANCH3}$. The two sets of G residues, present on either side of the branch point and indicated in bold on the downstream primer strand ($D_{BRANCH3}$), were platinated by CDDP. There are sites for two CDDP adducts on both the branch and the 5′-end of the tail, for a total of four adducts. Lanes 9−15 contained the CDDP-adducted substrate with the faster mobility. The result shown does not include the 5 fmol of FEN1 titration point. Lanes 16−23 contained the slower mobility platinated branch substrate.

region of the DNA strand, and should have an overall stiffening effect on single-stranded DNA (*39*). We considered the possibility that because the two platinum adducts were adjacent to each other in the double platinum substrate, they collaborate to create a structure that is particularly inhibitory to tracking. To test this possibility, we made a substrate where the platinums were placed 8 nucleotides apart (Figure 4B). When we compared the cleavage efficiency of FEN1 on substrates with two adducts having either 2 (lanes 8−14) or 8 nucleotides (lanes 15−21) between each adduct, the level of inhibition of cleavage versus control substrates (lanes 1−7 and 22−28) remained the same. The decrease in cleavage efficiency due to the two CDDP molecules occurred regardless of the proximity of the platinums. Multiple platinum adducts are clearly inhibitory, but the mechanism by which they collaborate is not obvious.

Flaps with Thymine Dimers. To determine whether increased rigidity is the primary determinant for inhibition of FEN1 cleavage, we created substrates containing cis-syn thymine−thymine cyclobutane dimers. The thymine dimers were contained in an 11-nucleotide oligomer that was ligated to the 5′-end of a flap substrate. Either one oligomer, containing a single dimer, or two oligomers were used, to analyze the effects of either one or two dimers (Figure 5). FEN1 was able to efficiently cleave substrates containing either a single dimer (lanes 17−24) or two dimers (lanes

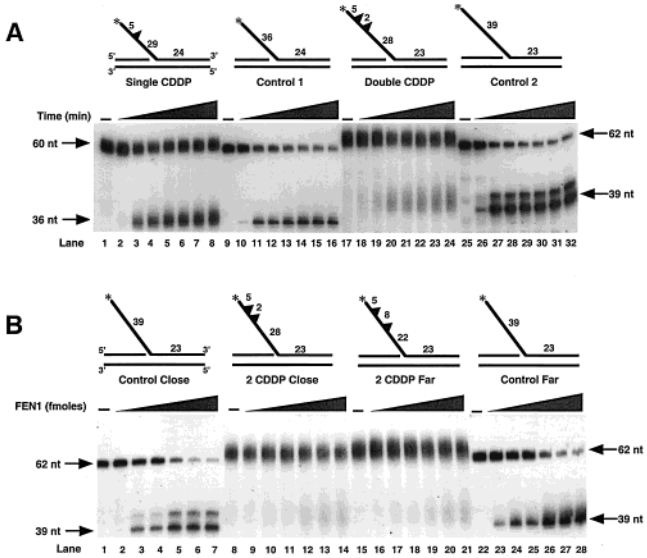


FIGURE 4: Inhibition of endonucleolytic cleavage of substrates containing two platinum residues. (A) FEN1 was incubated with DNA substrates containing zero, one, or two CDDP adducts. Reaction mixtures (179 μL) contained 400 fmol of FEN1 and 90 fmol of DNA substrate, and reactions were performed as described in Experimental Procedures. Reaction mixtures were incubated at 37 °C and 20 μL aliquots taken at 0, 1, 3, 5, 10, 15, 30, and 60 min as indicated by triangles. Substrate and cleavage product sizes are as indicated. Schematic representations of the substrates are depicted above the figure. The numbers represent the lengths of the adjacent segment in nucleotides, not including the site of modification. The asterisk shows the position of the radiolabel. Lanes 1−8 contained substrate comprised of T_{CDDP}, U_2, and D_{CDDP} (Table 1) with a single platinum located on the bold G-G nucleotides. Lanes 9−16 contained the control substrate for the single platinum comprised of unplatinated T_{CDDP}, U_2, and D_{CDDP} (Table 1). Lanes 17−24 contained a substrate that is comprised of T_{CDDP}, U_2, and D_{2CDDP} (Table 1) with two platinum residues on the two sets of bold G-G nucleotides. Lanes 25−32 contained the control substrate for the double platinum that is comprised of unplatinated T_{CDDP}, U_2, and D_{2CDDP} (Table 1). (B) FEN1 was incubated with DNA substrates containing two platinum residues separated by two nucleotides or by eight nucleotides and control substrates containing no modifications. Platination of substrates was performed as described in Experimental Procedures. Reaction mixtures contained 20 fmol of substrate with increasing amounts of FEN1 (10, 30, 50, 100, 300, and 500 fmol) and were incubated at 37 °C for 15 min as described in Experimental Procedures. Control reaction mixtures (−) containing substrate only were incubated for 60 min. Substrate and cleavage product sizes are as indicated. Schematic representations of the substrates are depicted above the figure. The numbers represent the lengths of the adjacent segment in nucleotides, not including the site of modification. The asterisk shows the position of the radiolabel. Lanes 1−7 contained the control substrate for 2 CDDP Close comprised of unplatinated T_{CDDP}, U_2, and D_{2CDDP} (Table 1). Lanes 8−14 contained the 2 CDDP Close substrate comprised of T_{CDDP}, U_2, and D_{2CDDP} with platinum residues on the two sets of bold G-G nucleotides (Table 1). Lanes 15−21 contained the 2 CDDP Far substrate comprised of T_{CDDP}, U_2, and $D_{2CDDP.2}$ with the platinum residues on the two sets of bold G−G nucleotides and separated by 8 nucleotides (Table 1). Lanes 22−28 contained the control substrate for 2 CDDP Far comprised of unplatinated T_{CDDP}, U_2, and $D_{2CDDP.2}$ (Table 1).

1−8) when compared to the control substrates (lanes 9−16 and 24−32). This shows that not all adducts that reduce the flexibility of the flaps, or covalently attach two adjacent nucleotides, can affect FEN1 cleavage.

Additionally, FEN1 cleaves the control substrate for two dimers (lanes 9−16) with a slightly reduced efficiency and fails to cleave specifically at the base of the flap. Sequence

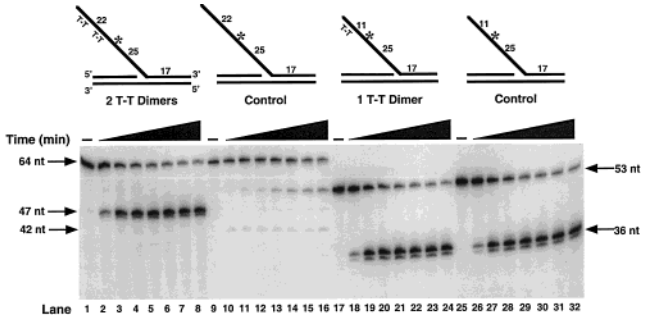


FIGURE 5: FEN1 can cleave flap substrates containing thymine−thymine dimers. FEN1 was incubated with substrates containing zero, one, or two cis-syn cyclobutane thymine−thymine dimers. Creation of substrates is described in Experimental Procedures. Reaction mixtures (160 μL) contained 80 fmol of substrate and 200 fmol of FEN1, and reactions were performed as described in Experimental Procedures. Reaction mixtures were incubated at 37 °C and 20 μL aliquots taken at 0, 1, 3, 5, 10, 15, 30, and 60 min as indicated by triangles. Substrate and cleavage product sizes are as indicated. Schematic representations of the substrates are depicted above the figure. The numbers represent the lengths of the adjacent segment in nucleotides, not including the site of modification. The asterisk shows the position of the radiolabel. Lanes 1−8 contained substrate comprised of T_{DIMER}, U_1, and $D_{2TTDIMER}$ (Table 1). The bold T residues are dimerized thymines. Lanes 9−16 contained the same substrate as lanes 1−8 except that the thymines were not dimerized. Lanes 17−25 contained the substrate comprised of T_{DIMER}, U_1, and $D_{1TTDIMER}$ (Table 1), where the bold T residues were dimerized. Lanes 26−32 contained the same substrate as lanes 17−25 except that the thymine residues were not dimerized.

analysis reveals that this downstream primer forms a hairpin loop structure within the 5′-flap due to interactions between the 11-nucleotide oligomers. This creates an alternate cleavage site for FEN1. Upon dimerization of the thymine residues, the secondary structure is eliminated. FEN1 can then cleave more effectively at the base of the flap. This result suggests strongly that rigidity of the flap alone is not sufficient for inhibition of FEN1 activity.

DISCUSSION

FEN1 is a central component of eukaryotic lagging strand DNA replication and long-patch base excision repair (*4*−*6*). Current models of these processes involve FEN1-directed cleavage of a substrate consisting of a template with a primer having an unannealed 5′-tail or flap structure (*3*, *6*, *18*). To fully understand how FEN1 performs its biological functions, it is important to define how it interacts with the flap substrate and removes the flap. Results of prior investigations suggest that FEN1 employs a complex loading process in which it tracks down the flap to the site of cleavage (*20*, *22*). Apparently, the 5′-end of the flap must be free, since bubble structures and flaps having bound primers are poor substrates (*19*, *20*). Proteins and primers bound at positions along the flap are also inhibitory, suggesting the need to traverse the flap (*20*, *22*). Crystal structures of FEN1 homologues uniformly exhibit a loop structure, making a hole in the protein. In *M. jannaschii*, the dimensions of this hole have been calculated to be 8 Å $\times$ 25 Å. This would be large enough to accommodate only a single strand of DNA, as the approximate diameter of double-stranded B-form DNA is 20 Å (*25*). The above information has prompted suggestions that the flap threads through the hole during tracking (*23*).

Our current results remain consistent with a model for FEN1 action that involves tracking along the flap to the site of cleavage. Both platinum adducts and platinated branch structures on the flap inhibited the cleavage reaction. Since these structures were placed beyond the region protected by FEN1, it is unlikely that they could have interfered with catalysis if FEN1 directly binds the cleavage site from solution. Together with previous results, it appears that the nuclease must interact with a free 5′-end and the entire length of the flap, as a prerequisite for cleavage. A tracking process is a logical explanation based on this information.

However, our observations are not consistent with a threading model. FEN1 was tested on a substrate with a large branch emanating from the flap. The presence of the branch had no apparent effect on the rate of cleavage. Even if the branch could fold in a way that the flap could be pulled through a hole in the protein, the process would be expected to reduce the cleavage rate. We consider this result to be evidence that the nuclease can move past the branch using a tracking process that is tolerant of large base adducts. The invariable presence of the loop or arch in the FEN1 family of proteins suggests a role in substrate interaction (*26*, *27*). Possibly, the loop actually functions in much the same way as the thumb subdomain of reverse transcriptases (*40*). These proteins have the general shape of a human right hand, such that the thumb subdomain closes down upon the primer template, allowing it to slide through the active site. The flexible nature of the FEN1 loop would be consistent with a role in folding over the flap for entrapping it during the tracking process. Since such a structure does not fully enclose the flap, it could be designed to allow even large modifications to slide past the loop region.

We have considered the cleavage behavior of FEN1 on many different flap substrates in an attempt to define the underlying principles that control its ability to track to the site of catalysis. The first principle is that flap modifications are either intrinsically inhibitory or not. Thymine dimers, even when present twice, appear not to be inhibitory. Recently, FEN1 was shown to endonucleolytically remove a single-thymine dimer present on the 5′-terminus of a four-nucleotide flap (*41*). The authors suggest a role for FEN1 in an alternate repair pathway for the processing of ultraviolet damage, including cis-syn cyclobutane pyrimidine dimers (*41*). The presence of a single streptavidin is inhibitory (*20*). A modification this large might interfere with the proposed clamping of the nuclease or simply may obstruct FEN1 from recognizing the substrate.

The second principle is that modifications that affect only a single nucleotide may not be inhibitory, whereas the same modification made over a longer region will slow the reaction. Chemical linkers, single platinum adducts, and even the branch structures have minimal effects on the efficiency of cleavage. However, two platinum residues either adjacent or separated by several nucleotides are considerably more inhibitory. In this manner, the dual platinum adducts may act like an annealed primer in affecting a longer segment of the flap.

The reason for a tracking mechanism with the observed characteristics remains mysterious. We had hypothesized earlier that it protects the replication fork from cleavage (*20*). Alternatively, it may allow the nuclease to find and bind the cleavage site more efficiently. But, why does it allow such a large variety of modifications and succumb to others? Possibly, some long-patch base excision repair intermediates have flap modifications that require this degree of tracking flexibility. Additional understanding awaits the creation of FEN1 mutants that have altered tracking specificity, and the crystal structure of FEN1 bound to a flap substrate.

ACKNOWLEDGMENT

We thank Dr. Min S. Park for the generous gift of the FEN1 expression plasmid. We thank Dr. Christopher Lawrence for the generous gift of the thymine dimer oligomers. We thank members of the Bambara Laboratory for useful discussions, especially Dr. Richard S. Murante, Dr. Mesut Muyan, and Samson Tom. We also thank Dr. Michael A. Leffak for insightful advice. We thank Eric Sechman for assistance in the purification of recombinant human FEN1.

REFERENCES

1. Lieber, M. R. (1997) *BioEssays 19*, 233−240.
2. Henricksen, L. A., and Bambara, R. A. (1998) *Leuk. Res. 22*, 1−5.
3. Bambara, R. A., Murante, R. S., and Henricksen, L. A. (1997) *J. Biol. Chem. 272*, 4647−4650.
4. Turchi, J. J., Huang, L., Murante, R. S., Kim, Y., and Bambara, R. A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 9803−9807.
5. Waga, S., Bauer, G., and Stillman, B. (1994) *J. Biol. Chem. 269*, 10923−10934.
6. Kim, K., Biade, S., and Matsumoto, Y. (1998) *J. Biol. Chem. 273*, 8842−8848.
7. Sommers, C. H., Miller, E. J., Dujon, B., Prakash, S., and Prakash, L. (1995) *J. Biol. Chem. 270*, 4193−4196.
8. Wu, X., Li, J., Li, X., Hsieh, C. L., Burgers, P. M., and Lieber, M. R. (1996) *Nucleic Acids Res. 24*, 2036−2043.
9. Budd, M. E., Choe, W. C., and Campbell, J. L. (1995) *J. Biol. Chem. 270*, 26766−26769.
10. Budd, M. E., and Campbell, J. L. (1997) *Mol. Cell. Biol. 17*, 2136−2142.
11. Dianov, G., and Lindahl, T. (1994) *Curr. Biol. 4*, 1069−1076.
12. Frosina, G., Fortini, P., Rossi, O., Carrozzino, F., Raspaglio, G., Cox, L. S., Lane, D. P., Abbondandolo, A., and Dogliotti, E. (1996) *J. Biol. Chem. 271*, 9573−9578.
13. Frosina, G., Fortini, P., Rossi, O., Carrozzino, F., Abbondandolo, A., and Dogliotti, E. (1994) *Biochem. J. 304*, 699−705.
14. Biade, S., Sobol, R. W., Wilson, S. H., and Matsumoto, Y. (1998) *J. Biol. Chem. 273*, 898−902.
15. Fortini, P., Pascucci, B., Parlanti, E., Sobol, R. W., Wilson, S. H., and Dogliotti, E. (1998) *Biochemistry 37*, 3575−3580.
16. Gary, R., Kim, K., Cornelius, H. L., Park, M. S., and Matsumoto, Y. (1999) *J. Biol. Chem. 274*, 4354−4363.
17. Klungland, A., and Lindahl, T. (1997) *EMBO J. 16*, 3341−3348.
18. DeMott, M. S., Shen, B., Park, M. S., Bambara, R. A., and Zigman, S. (1996) *J. Biol. Chem. 271*, 30068−30076.
19. Robins, P., Pappin, D. J., Wood, R. D., and Lindahl, T. (1994) *J. Biol. Chem. 269*, 28535−28538.
20. Murante, R. S., Rust, L., and Bambara, R. A. (1995) *J. Biol. Chem. 270*, 30377−30383.
21. Wu, W., Blumberg, B. M., Fay, P. J., and Bambara, R. A. (1995) *J. Biol. Chem. 270*, 325−332.
22. Barnes, C. J., Wahl, A. F., Shen, B., Park, M. S., and Bambara, R. A. (1996) *J. Biol. Chem. 271*, 29624−29631.
23. Ceska, T. A., Sayers, J. R., Stier, G., and Suck, D. (1996) *Nature 382*, 90−93.
24. Mueser, T. C., Nossal, N. G., and Hyde, C. C. (1996) *Cell 85*, 1101−1112.
25. Hwang, K. Y., Baek, K., Kim, H. Y., and Cho, Y. (1998) *Nat. Struct. Biol. 5*, 707−713.

26. Sayers, J. R., and Artymiuk, P. J. (1998) *Nat. Struct. Biol. 5*, 668−670.
27. Hosfield, D. J., Mol, C. D., Shen, B., and Tainer, J. A. (1998) *Cell 95*, 135−146.
28. Banerjee, S. K., Christensen, R. B., Lawrence, C. W., and LeClerc, J. E. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8141−8145.
29. Huang, L., Turchi, J. J., Wahl, A. F., and Bambara, R. A. (1993) *Biochemistry 32*, 841−848.
30. Marrot, L., and Leng, M. (1989) *Biochemistry 28*, 1454−1461.
31. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol. 185*, 60−89.
32. Shen, B., Nolan, J. P., Sklar, L. A., and Park, M. S. (1996) *J. Biol. Chem. 271*, 9173−9176.
33. Murante, R. S., Huang, L., Turchi, J. J., and Bambara, R. A. (1994) *J. Biol. Chem. 269*, 1191−1196.
34. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1991) *Molecular Cloning: A Laboratory Manual*, 3rd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
35. Hayes, J. J., Clark, D. J., and Wolffe, A. P. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 6829−6833.
36. Lemaire, M. A., Schwartz, A., Rahmouni, A. R., and Leng, M. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 1982−1985.
37. Huang, H., Zhu, L., Reid, B. R., Drobny, G. P., and Hopkins, P. B. (1995) *Science 270*, 1842−1845.
38. Huang, H., Woo, J., Alley, S. C., and Hopkins, P. B. (1995) *Bioorg. Med. Chem. 3*, 659−669.
39. Coste, F., Malinge, J. M., Serre, L., Shepard, W., Roth, M., Leng, M., and Zelwer, C. (1999) *Nucleic Acids Res. 27*, 1837−1846.
40. Patel, P. H., Jacobo-Molina, A., Ding, J., Tantillo, C., Clark, A. D., Jr., Raag, R., Nanni, R. G., Hughes, S. H., and Arnold, E. (1995) *Biochemistry 34*, 5351−5363.
41. Yoon, J. H., Swiderski, P. M., Kaplan, B. E., Takao, M., Yasui, A., Shen, B. H., and Pfeifer, G. P. (1999) *Biochemistry 38*, 4809−4817.

BI991321U