Interaction Interface of Human Flap Endonuclease-1 with Its DNA Substrates

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Abstract

Flap endonuclease-1 or FEN-1 is a structure-specific and multifunctional nuclease critical for DNA replication, repair and recombination; however, its interaction with DNA substrates has not been fully understood. In the current study, we have defined the borders of the interaction between the FEN-1 protein and its DNA substrates and identified six clusters of conserved positively charged amino acid residues, which are in direct contact with DNA substrate. To further map the corresponding interactions between FEN-1 residues and DNA substrates, we performed biochemical assays employing a series of flap DNA substrates lacking some structural components and a series of binding deficient point mutants of FEN-1. It was revealed that R47, R70, and K326/R327 of FEN-1 interact with the upstream duplex of DNA substrates, whereas K244/R245 interact with the downstream duplex. This result indicates the orientation of the DNA/FEN-1 protein interaction. Moreover, R70 and R47 were determined to interact with the sites around the second nucleotide (R70) or the fifth/sixth nucleotide (R47) of the template strand in the upstream duplex portion counting from the nick point of the flap substrate. Together with previously published data and the crystallographic information from the FEN-1/DNA complex that we published recently (Chapados et al., 2004, Cell, 116, 39059), we are able to propose a reasonable model for how the human FEN-1 protein interacts with its DNA substrates.
Introduction

Flap endonuclease-1 is a critical structure-specific nuclease for α-segment processing during Okazaki fragment maturation and for DNA base excision repair (1–5). Deficiency of these pathways consequently results in genome instability, including significantly enhanced mutation frequency and micro-satellite instability (5–11). The enzyme is also involved in preventing illegitimate crossover activities such as short-repeated-sequence recombination and hence enhances genome stability (12). More recently, it has been demonstrated that FEN-1 has a novel function in promoting apoptotic DNA fragmentation (13).

Despite its multiple biochemical activities, protein-protein interaction partners and involvement in several DNA metabolic pathways, FEN-1 is a unique enzyme that solely recognizes abnormal DNA structures, typified by a flap DNA substrate. Initial motif analysis based on protein sequence comparison and biochemical assays identified two major conserved motifs, the N (N-terminal) and I (Intermediate) motifs, which are essential for the nuclease activities of FEN-1 proteins (4, 14). These two regions contain 7-8 conserved acidic amino acid residues that coordinate two magnesium ions and form an active center for catalysis (15). A third motif towards the C-terminus is involved in the interaction between FEN-1 and proliferating cell nuclear antigen (PCNA) (16, 17, 18). This interaction is required for the recruitment of FEN-1 to PCNA at sites of Okazaki fragment processing and DNA damage repair (4, 17, 19). In eukaryotic cells, FEN-1 also has a C-terminal motif with multiple clusters of positively charged amino acid residues, which are important for the localization of FEN-1 into the nucleus (20).

Crystal structures of FEN-1 enzymes have identified structural elements that may be important for interactions with DNA flap substrates. Structures of FEN-1 and its homologues, including three archaeal FEN-1 proteins, T4 RNase H, and T5 exonuclease (21–24) exhibit an arch element formed by
a helix-loop-helix motif located over the active center. This “helical arch” structure contains a number of positively charged and bulky amino acid residues on its inner side, which could be in the direct contact with the ssDNA flap of DNA flap substrates (23, 24). Corresponding residues in the 5’ nuclease of *Escherichia coli* DNA polymerase I have been shown to play a role in DNA binding (25). Furthermore, partial deletions of this region in *Methanococcus jannaschii* FEN-1 (mjFEN-1) abolish enzyme activity (24). This arch structure may allow FEN-1 to slide through the single stranded flap of flap DNA substrate to perform structure-specific cleavages - a sliding-through mechanism proposed for FEN-1 by Barnes, *et al.* (26). In addition, this structure was shown to be critical for catalysis in human FEN-1 (27). Recent evidence suggests that the helical arch region undergoes a conformational change upon binding to substrate DNA, resulting in increased helical content, which may aid in positioning the ssDNA flap near the catalytic site (28-29).

In addition to the helical arch region, analysis of the *Pyrococcus furiosus* FEN-1 (pfFEN-1) crystal structure revealed a positively-charged groove containing the active center and a H3TH motif, which were proposed to mediate binding of the double- or single-stranded regions of the flap DNA substrates to FEN-1 (23). The recent structure of *Archaeoglobus fulgidus* FEN-1 (afFEN-1) bound to DNA identified two additional helix-loop-helix motifs that contact the upstream portion of the DNA flap substrate (29). Consistent with this report, mutation of R47 and R70 in human FEN-1, which are both located in these regions, affects substrate-binding specificity (30). Furthermore, biochemical and mutational analysis of *Pyrococcus horikoshii* FEN-1 (phFEN-1) (31) revealed a total of five loop regions important for protein:DNA interactions, which include the helical arch, H3TH and other structurally-identified DNA-binding regions.

Although evidence for the DNA-protein interaction of FEN-1 is accumulating, it is still important to identify all the elements directly involved in substrate binding in order to understand how the enzyme positions its substrates in a way that optimizes cleavage. Here we present mutational and
biochemical analysis of human FEN-1 that define how FEN-1 interacts with DNA. We defined the borders of the interactions between the FEN-1 and its DNA substrates by Exo III footprinting. In addition, we identified 14 FEN-1 mutations that affect DNA-binding. Using FEN-1 substrates designed to test the interactions of these mutants with known specific regions of the flap substrate, we determined the substrate-binding orientation for human FEN-1 and implicated key structural elements in DNA-binding. Moreover, R70 and R47, which are known to be involved in FEN-1:DNA binding, were determined to interact with the sites around the second nucleotide (R70) and the 5th/6th nucleotide (R47) of the template strand in the upstream duplex portion counting from the nick point of the flap substrate. Together, these results and the protein/DNA complex structure (29) allowed us to propose a reasonable model for how the human FEN-1 protein interacts with its DNA substrates.
Experimental Procedures

Site-directed mutagenesis, protein overexpression, and purification - All human FEN-1 mutants used for this study were prepared using the QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA). Mutagenic primers were synthesized at the City of Hope DNA/RNA/peptide synthesis core facility. Mutations and corresponding oligo sequences for primers are listed in Table S1 for clarity as supplementary materials and is available upon request. Site-directed mutagenesis, overexpression, and purification of wild type and mutant FEN-1 enzymes were carried out based on our previously published procedures (30). Mutagenesis reactions were performed using pET-28 derived plasmids harboring the wild type human FEN-1 sequence as a template so that the isolated plasmids containing a mutation could be used directly for protein expression in E. coli.

Protein sequence alignment and three dimensional structure modeling of human FEN-1 and FEN-1:DNA complexes – The sequences of FEN-1 proteins were aligned using ClustalW 1.8 Multiple Sequence Alignment Algorithm at BCM Search Launcher, Baylor College of Medicine HGSC (http://searchlauncher.bcm.tmc.edu). The structure of human FEN-1 was modeled using the crystal structures of Methanococcus jannaschii and Pyrococcus furiosus FEN-1 proteins as templates (PDB files 1A76 and 1B43). Using the homology module within the molecular modeling program Insight II (Accelrys, San Diego, CA), the sequence of human FEN-1 was aligned with the archaeal FEN-1 sequences. Structural information was then used to modify the final alignment of the three FEN-1 sequences so that insertions and deletions fell between secondary structure elements. The model was built and refined using scripts provided with the program.

The hFEN-1:DNA model was constructed using initial DNA coordinates from the crystal structure of aFEN-1 bound to DNA (PDB code 1RXW) (REF). The upstream DNA substrate was initially positioned by aligning the hFEN-1 model with the aFEN-1 based on Cα positions. Close
contacts in the initial hFEN-1:DNA were eliminated by rigid body minimization based only on stereochemical parameters using CNS (32). The downstream DNA and 5’ flap regions were then added to the model, and positioned in a way that satisfied the greatest number of contacts to residues known to affect DNA binding. The steric clashes between side-chains and the DNA were resolved by minimizing only the side-chain atoms of hFEN-1 using CNS.

**DNA substrate preparation and FEN-1 nuclease activity assays** Protocols for DNA substrate preparation and nuclease activity assays were performed as previously published (30). Briefly, oligos as shown in the relevant figures were individually phosphorylated at the 5’ end. This was done by incubating 40 pmol of the oligo with 10 µCi of (γ-32P) ATP and 1µL (10 U/µL) of polynucleotide kinase (PNK) at 37 °C for 60 min. PNK was then inactivated by heating at 72 °C for 10 min. 80 pmol each of remaining oligos for individual substrates listed in the relevant figures were added to the labeled oligos, respectively. The samples were incubated at 70 °C for 5 min followed by slow cooling to 25 °C, thus allowing the oligos to anneal and form the flap and nick-duplex substrates. Substrates were precipitated at –20 °C overnight after adding 20 µL of 3M NaOAc and 1 mL of 100% ethanol. Substrates were collected by centrifugation and washed once with 70% ethanol and resuspended in sterile water.

Reactions were carried out with the indicated amount of hFEN-1 and 80nM of flap or nick duplex substrate in reaction buffer containing 50 mM Tris (pH 8.0) and 10 mM or 5 mM MgCl2. Each reaction was then brought to a total volume of 10 µl with water. All reactions were incubated at 30 °C for 15 minutes and terminated by adding an equal volume of stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). An aliquot of each reaction was then run on a 15% denaturing polyacrylamide gel at 1900 V for 1 hour. The gel was dried at 70°C for 50 minutes, and the bands were visualized by autoradiography.
**Kinetic analysis** - FEN-1 cleavage kinetic assays were performed using various concentrations of DNA substrates (30 to 500 nM) and constant amounts of FEN-1 (92 nM) following the procedures described in Hosfield, *et al.* (33). Briefly, reactions were initiated by combining standard reaction buffer, substrate, and enzyme in order. Samples were mixed and incubated for 2 min. The products and substrates were separated by denaturing gel electrophoresis. The initial velocity was calculated by measuring product and substrate intensity on the gel using the IP Lab Gel program and by using the equation
\[
\nu = \frac{I_1}{(I_0 + 0.5 I_1)t} \times [\text{substrate}],
\]
where \(t\) = time in seconds, \(I_1\) = product intensity, \(I_0\) = final substrate concentration. The substrate concentration was expressed in nM. \(V_{\text{max}}\) and \(K_m\) values were calculated by directly fitting the data into the Michaelis-Menten equation and then \(K_{\text{cat}}\) was calculated by \(V_{\text{max}}/[E_0]\). \(K_m\), \(K_{\text{cat}}\), and \(K_{\text{cat}}/K_m\) values were finally used for plotting.

**Direct enzyme/substrate binding analysis** - This assay was conducted using a method modified from one established by Harrington and Lieber (34). In brief, the indicated amounts of FEN-1 were mixed with 80nM of labeled DNA in a final volume of 10 µl containing 50 mM Tris (pH 8.0), 10 mM NaCl, 5 mM EDTA, 10% glycerol, and 50 µg/ml bovine serum albumin. After 15 min incubation at room temperature, each reaction was loaded onto a 5% polyacrylamide gel containing 0.5X TBE. The reactions were then electrophoresed for 45 min at 100 V at 4 °C. The gel was dried and then exposed to Kodak X-ray film for imaging.

**Exonuclease III footprinting** - For this experiment, we followed a procedure described by Hohl, *et al.* (35). Human FEN-1 mutant R100A or D181A was used to bind 0.27 pmol flap substrates with 5'-32P end labeling of the flap strand or template strand. They were incubated in 50 mM Tris (pH 6.8), 20 mM NaCl, 15% glycerol, and 50 µg/ml bovine serum albumin with 2 mM MgCl2 and 100-fold excess cold dsDNA competitor for 20 min. Subsequently, 20 units of ExoIII were added to allow 15 min
digestion at 15 °C. The reaction was stopped by the addition of EDTA to a final concentration of 50 mM and formamide loading buffer. After heating for 10 min at 75 °C, the reactions were analyzed on a 15% sequencing gel and the digestion patterns were visualized by autoradiography.

Circular dichroism (CD) measurements - CD measurements were performed using a J-600 spectropolarimeter (Japan Spectroscopic Co., Ltd, Tokyo, Japan) as previously described (36). Briefly, the far-UV (200-250 nM) CD spectrum was obtained at 25 °C using a solution containing 90 µg/ml enzyme, 20 mM sodium phosphate buffer (pH 7.6) in a 2-mm path length cuvette. CD data were analyzed using the K2D program (37) for the calculation of the relative ratio of α-helices, β-sheets and random coils.
Results

Defining the region of DNA substrates contacted by FEN-1 - To obtain an insight into how FEN-1 binds to DNA substrates, we used exonuclease III footprinting to determine the region of DNA protected by FEN-1. Exo III is an Mg^{2+}-dependent, 3’ to 5’ exonuclease that digests DNA in a progressive manner, but is blocked by the presence of proteins bound to DNA. This method has recently been used to define the protein-DNA borders of XPG endonuclease, a FEN-1 homologue, complexed with its DNA substrate (35). To prevent substrate cleavage by FEN-1, we used catalytically inactive FEN-1 mutants (R100A and D181A), which are still capable of binding to DNA substrates (15). While both mutants are catalytically inactive, the R100A mutant has a lower DNA-binding affinity than D181A, and was used to avoid non-specific binding to long DNA substrates. FEN-1 substrates were ^{32}P labeled at 5’ the end of either template strand (Fig. 1A) or the flap strand (Fig. 1B). Addition of R100A (Fig. 1A, lane 4 and 5) enhanced a band 34 nucleotides in length indicated by black arrows both on the schematic diagram of the substrates and on the right side of gel image. This result implies that the 3’ end border of the template strand is at the position of the 20th nucleotide relative to the nick site. The addition of D181A to the 5’ flap-labeled substrate produces a clear stalling band next to the 3’ end of the flap strand (Fig. 1B, lanes 8-9, black arrows). This result indicates that the flap strand forms a 3’ end border with FEN-1 at the position of 13th nucleotide relative to the nick site of flap substrate.

Effects of mutations of positively charged amino acids on hFEN-1 nuclease activities - In order to comprehensively investigate the key structural elements of FEN-1 that are involved in the interactions with DNA substrates, we mutated 29 positively charged amino acid residues to alanine, either individually or in combinations of two residues (Table 1, Fig. 2). For each FEN-1 mutant, we determined both the flap endonuclease (Fig. 3A) and exonuclease (Fig. 3B) activities. In addition to R47A and R70A, which were described previously (30), 13 mutants were determined to have

We also tested whether these mutations could affect the stimulation of nuclease activities by an additional 3’ flap on the substrate. As previously reported, FEN-1 activity is significantly stimulated by an extra nucleotide at the 3’ end of the upstream primer of the normal flap substrate (38). Such a double flap substrate was proposed to be an *in vivo* substrate for FEN-1 (39). If the corresponding amino acid residues are critical for interacting with the 3’ overlapping nucleotide of the upstream primer, we expect to see a loss or reduction of activity-stimulation in the mutants. However, our results indicate that these mutations had no effect on activity stimulation by the 3’ flap (data not shown), indicating that these positively charged amino acid residues are not involved in the interaction with the 3’ flap nucleotide of a double-flap DNA substrate.

*Residues that are critical for substrate binding* – Loss or reduction of the nuclease activities in the 15 mutants identified above could be due to conformational changes caused by mutations, deficiency in substrate binding or destruction of the catalytic center. Because CD spectra are good indicators of major protein conformational changes, we analyzed the conformation of the 15 mutants with decreased activities using CD spectroscopy. The CD spectrum profiles and calculated ratios of α-helices to β-sheets for the mutants were similar to those of wild-type FEN-1, indicating that the FEN-1 mutations did not cause significant conformational changes (data not shown).

To measure the DNA-binding capacity of the activity-deficient mutants, we performed gel shift assays. These assays are especially important for determining the substrate-binding capacity of the mutants that do not show any FEN-1 activity, since these mutants cannot be characterized by
conventional kinetic analysis. FEN-1 forms a complex with both the flap substrate (Fig. 4A) and the nicked duplex substrate (data not shown) in the presence of EDTA, which prevents the cleavage reaction (33). Overall, among the 15 mutants, almost all except for R100A have reduced affinity for DNA compared to wild-type FEN-1. Twelve mutants, R47A, R70A, R103A/R104A, K128A/R129A, K132A, R192A, K200A, K201A, K244A/R245A, K252A/K254A, K267A and K326A/R327A, had significantly lower affinity for both flap and nick duplex substrates compared to wild-type FEN-1, whereas K93A and K125A had binding affinities slightly lower than wild-type FEN-1. K132A, R192A K201A and K252A/K254A had no detectable affinity for either DNA substrate. Although the R100A mutant did not show any deficiency in binding to DNA, its enzyme activities were lost, indicating that R100 may play a critical role in enzyme catalysis. This might also be true for K93, because its mutation to alanine has only a slight effect on FEN-1 affinity for DNA, but completely abolishes FEN-1 enzyme activity.

To further address the roles of these positively charged amino acid residues in cleavage or substrate binding, we performed kinetic analysis. We measured cleavage activity with both the flap and nick duplex DNA substrates. Mutants with no detectable enzyme activity (6 out of 15) were excluded in this analysis since their kinetic parameters were not measurable. In this analysis, Michaelis-Menten kinetics was used to derive $V_{\text{max}}$ and $K_m$ values, and $K_{\text{cat}}$ and $K_{\text{cat}}/K_m$ values were then calculated (Fig. 5). Overall, the mutants R47A, R70A, R103A/R104A, K125A, K128A/R129A, K200A, K244A/K245A and K326A/R327A have increased $K_m$, decreased $K_{\text{cat}}$ and decreased $K_{\text{cat}}/K_m$ values (Fig. 5). The increased $K_m$ values of the mutants suggest a substrate binding deficiency. In particular, the mutants R103A/R104A, K128A/R129A, K200A, and K244A/K245A had significantly higher $K_m$ values compared to the wild type protein, whereas their $K_{\text{cat}}$ and $K_{\text{cat}}/K_m$ values were proportionally reduced. These results indicate that the decreased activity of these mutants is mainly due to a substrate binding deficiency and therefore, the corresponding amino acid residues of these
mutants are important for DNA substrate binding. These results are consistent with the gel shift assays (Fig. 4), supporting the role of these residues in DNA binding.

**Determination of the binding orientation between FEN-1 and its 5’ overhang substrate** – A typical flap DNA substrate can be designated into three portions: upstream duplex, single strand flap and downstream duplex (Fig. 1). To fully understand FEN-1:DNA interactions, it is critical to determine the identified protein structure elements (amino acid residues) that interact with each portion of the DNA substrate. Compared to the standard flap substrates, the 5’ overhang substrate has no upstream duplex portion. Therefore, it allows us to determine which amino acid residues of FEN-1 interact with the upstream duplex. If the amino acid residues interact with this component, we expect that mutations of these residues will significantly affect FEN-1 flap cleavage activity using a standard flap substrate. However, when a 5’ overhang substrate that lacks the upstream duplex is used, the mutations should have no effect.

A comparison of the substrate cleavage patterns of wild type and mutant FEN-1 enzymes using the 5’ overhang substrate identifies residues that may contact the upstream duplex (Fig. 6). Notably, three mutants, R47A, R70A, and K326A/R327A, have similar or even higher enzyme activity than wild type FEN-1 at the normal cleavage site near the flap-duplex junction. The same mutants have weaker enzyme activities than wild type FEN-1 in the presence of normal flap or nick duplex substrates (Fig. 3). The sharp contrast in activities of the mutants on different substrates suggests that R47, R70 and K326/R327 interact with the upstream duplex portion of FEN-1 DNA substrates.

In addition, we noticed that R47A, R70A, and K326A/R327A had a different cleavage pattern from wild type FEN-1 on cleaving the 5’ overhang substrate. The wild type FEN-1 appeared to cleave the 5’ overhang substrates at two main sites: one near the flap-duplex junction and a second site close to the 5’ end of the single stranded flap. Although the mutants R47A, R70A, and K326A/R327A
cleaved this substrate near the flap-duplex junction with similar or greater efficiency than wild type FEN-1, the mutants did not cleave the substrate at the second site near the 5’ end of the flap. This result may indicate a “pulling-back” mechanism of the single stranded flap: In the absence of the upstream duplex portion of the 5’ overhang substrate, the positively charged residues R47, R70, and K326/R327 in FEN-1 become free and exposed, thus they are able to interact with the single stranded flap. The interactions may pull the single stranded flap to form a structure, which leads its 5’ end portion to be close to the active center of FEN-1 and generates the additional cleavage sites. However, mutation of any of the residues R47, R70, and K326/R327 affects the stability of the unknown structure, which leads to the alteration of the cleavage sites close to the 5’ end of flap strand.

In contrast to R47A, R70A, and K326A/R327A mutants, K244A/R245A differs from the wild type FEN-1 in the opposite manner with regard to the two cleavage sites. The cleavage site of K244A/R245A mutant shifted from one near the flap-duplex junction to the one near the 5’ end of the single stranded flap (Fig. 6). This result suggests that K244/R245 interacts with downstream duplex such that the residues are close to the cleavage sites near the flap-duplex junction but distant from the cleavage sites towards the 5’ end of the single stranded flap. This explains why the mutation of K244A/R245A significantly affects cleavage near the flap-duplex junction, but has little effect on the cleavage near the 5’ end of the single stranded flap.

Although the cleavage patterns of the R103A/R104A and K244A/K245A mutants look similar, they differ in the relative effects on substrate cleavage at each site. At the sites near the flap-duplex junction, the R103A/R104A mutant only makes slight cleavages, whereas K244A/K245A makes much stronger cleavages. Since R103 and R104 are conserved residues in the large loop region, mutation of these residues should alter the interaction between the large loop and the single stranded flap, which may affect cleavage at either site. Thus, this result is consistent with the previous proposals that the large loop region interacts with the single stranded flap (21-24, 40). As discussed
previously, K125 and K128/R129 might also interact with the single stranded flap. Therefore, the cleavage patterns of the two mutants K125A and K128A/R129A could be similarly explained. In addition, the K200A mutation totally abolished the FEN-1 activity on the 5’ overhang substrate. Since K200 is located near the active center (Fig. 2), its mutation could affect DNA-protein interactions near the active site and therefore prevent cleavage of the substrate.

**Mapping of site-specific interactions of FEN-1 with DNA**– Thus far, we have determined the orientation of DNA substrate binding to FEN-1, which shows that R47 and R70 of FEN-1 interact with the upstream duplex portion, K244/R245 interacts with the downstream duplex portion and the large loop region represented by R103/R104 and K128/R129 probably interacts with the single stranded flap. In this experiment, we attempted to map the specific interaction sites of R47 and R70 with the upstream duplex. Since FEN-1 activity is only partially affected by the absence of an upstream duplex in the DNA substrates, it is reasonable to map the interaction sites using substrates with gradual changes in the length of the upstream duplex (Fig. 7).

The R47A and R70A mutants cleave both a flap substrate (Fig. 7, substrate 1) and a pseudo-Y substrate (Fig. 7, substrate 6), generating products that are similar to wild type FEN-1 for each substrate. Since the substrate cleavage patterns are not affected by the absence of the upstream primer, it is not likely that R47 or R70 interact with the upstream primer. This interpretation is further supported by the cleavage of the mutants on other substrates with different gap lengths (Fig. 7, substrate 2 – 5), which show a proportional reduction in enzyme activities of the mutants compared to the wild type FEN-1.

Notably, the substrate cleavage-pattern resulting from both the wild type and mutant FEN-1 proteins is altered by the introduction of gaps into the DNA substrates. It appears that the 3’ portion of the upstream primer is critical, as the shortening of the primer at 3’end (substrate 2 to 4) reduces
activity and alters the cleavage pattern. While the exact reason for how the gap-length alteration (especially from substrate 2 to 4) causes such a significant change in the cleavage-pattern is unknown, we speculate that the single stranded flap might go through a conformational change, which leads to cleavage-site shifts. The conformational alteration could be triggered by the exposed amino acid residues due to the introduction of gaps at the 3’ end of the upstream primer or the interaction of the nucleotide residue at 3’ end of the upstream primer with the single stranded flap, or by both of the factors.

To identify the specific interaction sites of R47 and R70 on the complementary (template) stand of the upstream primer in the upstream duplex portion of flap substrates, we examined the substrate cleavage of the R47A and R70A mutants using pseudo-Y substrates with various 3’ overhang lengths. Shortening the length of the upstream template strand by up to 10 nucleotides from the end significantly reduced the flap endonuclease activity of R47A compared to wild type FEN-1 with respect to cleavage sites near the flap-duplex junction (Fig. 8, substrates 1-4). However, further reduction of the length of the upstream template strand does not affect R47A cleavage at the flap junction compared to wild type FEN-1 (Fig. 8, substrates 5-8). Since substrates 4 and 5 differ by only two nucleotides it is reasonable to propose that R47 interacts with nucleotide 5 or 6 on the upstream template.

For the mutant R70A, shortening of the upstream template strand reduced substrate cleavage efficiency at the flap-duplex junction compared to wild type FEN-1 (Fig. 8, substrates 1-6). However, in the presence of very short upstream template strands (Fig. 8, substrate 7-8), R70A activity was comparable to wild-type FEN-1. Since substrates 6 and 7 differ by one nucleotide (nucleotide 2 counting from the flap-duplex junction), R70 likely interacts with the second nucleotide after the flap junction. Surprisingly, pseudo-Y substrates with short upstream template strands were cleaved at additional sites by wild type FEN-1, generating products of 3, 6 or 10 nucleotides in length (Fig. 8,
substrates 5,7-8). We think the additional cleavage by wild type FEN-1 is due to the pulling-back of the single stranded flap as we discussed in the section of using the 5’ overhang substrates to determine the orientation of FEN-1 binding.
Discussion

To function properly, FEN-1 nuclease requires the appropriate interaction between the protein and its DNA substrates. It is important, therefore, to understand how FEN-1 recognizes, binds and cleaves its substrates. In the current study, we defined the region of substrate DNA protected by FEN-1 binding and identified a number of positively charged amino acids critical for FEN-1 binding to DNA.

The Exo III footprinting analysis indicates that FEN-1 protects 13 nucleotides of the downstream duplex and 20 nucleotides of the upstream duplex. The significantly larger length protected length by FEN-1 in the upstream duplex portion may be explained by the existence of a C-terminal portion in FEN-1, which contains a nuclear localization signal (41) and was proposed to play a role in substrate binding (42). Although the structure of this C-terminal portion remains unknown, it may interact with the upstream duplex as we proposed in figure 9. Based on a previous footprinting analysis (26), the border of the single stranded flap is located near the 20th nucleotide counting from the flap duplex junction. Taken together, we now have comprehensive information on the borders of a DNA substrate bound to FEN-1. In addition, the current study provides the information on the orientation of DNA substrate bound to FEN-1. Our results indicate that FEN-1 residues R47 and R70 contact the template strand within upstream duplex, residues K244/R245 and K251/K254 interact with the downstream duplex, and that residues located in the large loop region of FEN-1 bind to the single stranded flap. Therefore, these data allow us to draw an overall picture of interaction of human FEN-1 with a DNA flap substrate (Fig. 9).

We further determined key elements of human FEN-1 in interaction with different portions of flap substrates. Although a number of archaean FEN-1 crystal structures were solved at high resolution, for human and other eukaryotic FEN-1s, no crystal structure is available. It becomes a
difficult task, hence, for researchers to establish an accurate model to address DNA-protein interaction of eukaryotic FEN-1 enzymes. In the current study, we performed activity, gel shift and kinetic assays, which enable us to determine 14 mutants, R47A, R70A, K93A, R103A/R104A, K125A, K128A/R129A, K132A, R192A, K200A, K201A, K244A/R245A, K252A/K254A, K267A and K326A/R327A with deficiency in the interaction with DNA substrates. These 14 mutants cover 18 positively charged surface and conserved amino acid residues that appear important for substrate binding. These amino acid residues can be assigned into different loop regions with reference to the archael FEN-1 (31) (Table 1). Among these residues, R47 is in small loop 1, which is a close neighbor to R70. Both of these residues were determined in our previous studies (30) to be important in substrate binding. R192, K200 and K201 are located in small loop 2. This loop is extremely critical as mutation of any of the three residues R192, R200 and K201 significantly affects both DNA binding and activity. Residues K244, R245, K252, K254 and K267 are in adjacent small loops 3 and 4, while R326 and R327 are located on the other side of FEN-1, closer to R70 (Fig. 2). Finally, K93, R104, K125, K128, R129 and K132 all belong to the large loop in the arch structure of FEN-1, which was proposed to be important in interaction with single stranded DNA flap (21 –24, 40).

For years, the large loop region has been recognized as the most critical element for DNA recognition and interaction. A thread-through or tracking-down model was proposed to account for the potential role of this region in recognizing the single stranded flap (40, 43). The corresponding residues of the large loop region in the 5’ nuclease of E. coli DNA polymerase I and archael FEN-1 (24) were also shown to have roles in DNA binding (24, 25). However, in a recent study some amino acid residues in the large loop region were revealed to be critical for catalysis (27). The mutations of K93 into arginine and S94, R100, L97, or L130 into proline in this region significantly affected enzyme activites of human FEN-1, but had little effect on DNA binding (27). These results imply that the large loop region has dual roles in both DNA interaction and catalysis. To address this possibility, we mutated almost all the positively charged amino acid residues located in the large loop into alanine.
Our results show that the large loop is indeed involved in DNA interactions, since the mutation of the majority of these amino acids into alanine either completely abolish (K132A) or significantly decrease (R104A, K125A, K128A, and R129A) FEN-1 enzyme activity and substrate binding affinity. In addition, our study identified two mutants, K93A and R100A, which retained an intact or slightly diminished substrate-binding capability but have no enzyme activity, suggesting that the large loop region could participate in catalysis.

It was proposed that the large loop region could be important for efficient cleavage by positioning the 5’ flap near the catalytic site (27). This hypothesis is supported by recent evidence that R94 in *Pyrococcus furiosus* FEN-1, which corresponds to R100 in human FEN-1, makes ionic contact with the phosphate bonds near the cleavage site (44). However, according to crystal structures of archaeal FEN-1 enzymes (23, 24) and the structural model of human FEN-1 (Fig. 2), R100 is located at the end of the large loop region, far away from the active site of FEN-1. Therefore, in order for R100 to play a role in catalysis, the large loop region must undergo a significant conformational change, which would position the residue near the active site. A conformation change would also explain why mutating large loop residues S94, L97, or L130 into proline, which would affect the flexibility of the large loop, significantly decreases human FEN-1 activity (27). Alternatively, the elements for catalysis in the large loop region of FEN-1, such as R100 and K93, might participate in a mechanism for maintaining an extensive catalytic solvent network within the active site. Such a mechanism was described by Chevalier *et al.* (45) to address DNA catalysis by a homing endonuclease (I-CreI). In this nuclease, residues with basic side-chains form a network surrounding the nucleophilic water molecules, extending around the scissile phosphate to the 3’ oxygen leaving group. The network supports a DNA cleavage mechanism in which the scissile phosphates contact two divalent cations that are extensively hydrated by several water molecules. The hydration is structured and polarized by interactions with a number of basic side chains. Disturbance of such a network is similar to mutations of metal ion ligands, which can abolish enzyme activities (15).
In order to establish a detailed model of FEN-1 interaction with DNA we mapped the specific interaction sites of residues R47 and R70 on the upstream duplex. Because the lack of an upstream duplex portion in flap DNA substrates does not completely abolish the activity of wild type FEN-1 or the R47A and R70A mutants, it is possible to map the DNA-protein interaction sites in the upstream duplex using flap substrates with gradual change of the upstream duplex lengths. This method is more precise than footprinting, since it uses substrates with well-defined sequence changes. Based on this method, we determined that R70 interacts with DNA near the second nucleotide and R47 interacts with either the fifth or sixth nucleotide of the template strand in the upstream duplex, counting from the nick point of the flap substrate (Fig. 8). Although we did not expect that R47 could interact with the template strand in the upstream duplex portion, we predicted that R70 interacts with the template strand in the upstream duplex portion based on previous results (29). Notably, R70 in human FEN-1 appears to interact with DNA at the same site as R64 in both pfFEN-1 and afFEN-1 (28, 44). This result indicates that human and archael FEN-1 enzymes may have a very similar DNA-protein interaction mechanism.

In comparison to the model proposed for archael FEN-1 (44), our results experimentally confirmed proposed roles of a number of positively charged amino acid residues in substrate binding and identified several new residues such as R192, K200 and K201 that are critical for substrate binding in human FEN-1. In addition, we determined the specific interaction sites of R47 and R70 with DNA. The new information supports the proposal that the DNA substrate might be kinked upon binding to FEN-1 (28). Upon binding, there could be a significant conformational change in FEN-1, especially in the large loop region, which positions amino acid residues, such as R100, closer to the active site to participate in catalysis. In addition, a conformational change in the region of small loop 1 may allow R47 to shift its orientation from that shown in Fig. 9 towards favoring the interaction with nucleotide 5 or 6 on the upstream template as revealed by biochemical analysis.
Acknowledgement

We are grateful for the contributions or efforts made by David N. Bimston, Arthur Partikian, and Paul Luu in the initial stage of this work. Thanks also goes to Steve Alas for critical reading on the manuscripts and valuable comments, and to Li Zheng and other members of the Shen laboratory for technical assistance and stimulating discussions. This work was supported by an NCI/NIH grant R01CA073764 to BHS and R01CA081967 to JAT.
References


Figure Legends

Fig. 1. Exo III footprinting analysis reveals the borders of flap DNA substrate bound to FEN-1.
A. Footprinting of flap DNA substrate with the 5’ end labeling of the template strand. Lane 1, mark DNA only; lanes 2-5, added with indicated DNA substrate and proteins (Exo III: 20 units and R100A: 200, 400 or 600 nM). (…), represents a DNA sequence 5’ TGCAACTGATGGCAG 3’. B. Footprinting of flap DNA substrate with the 5’ end labeling of the flap DNA strand. Lane 6, mark DNA only; lanes 7-10, added with indicated DNA substrate and proteins (Exo III: 20 units and R181A: 200, 400 or 600 nM). C. A schematic diagram indicating the borders of the flap substrate bound to FEN-1. Filled circle represents FEN-1 and lines with short bars represent DNA substrate. The border of the single stranded flap bound to FEN-1 was determined by Barnes, et al. (42). The arrow represents the stalled cutting site by addition of R100A or D181A mutant protein, indicating the border of DNA substrate bound to FEN-1. ss-flap, the single stranded flap; Up-D, upstream duplex; Dn-D, downstream duplex and nt, nucleotides; +, the designated material is added; -, negative control; *, p32 labeled.

Fig. 2. Three-dimensional view of the human FEN-1 positively charged amino acid residues selected for site-directed mutagenesis. The human FEN-1 structure was modeled based on the crystal structures of Methanococcus jannaschii and Pyrococcus furiosus FEN-1 proteins. The residues emphasized in this study are indicated in yellow.

Fig. 3. Enzyme activities of wild type and mutant FEN-1 proteins. A. The flap endonuclease activity of FEN-1 proteins. B. The exonuclease activity of FEN-1 proteins. For both A and B, the top bands represent the uncut substrates and the bottom bands are the cleavage products. The oligo size is indicated in nucleotides (nt). Reactions were carried out with 133 nM hFEN-1 protein and 80nM of
flap or nick duplex substrate in reaction buffer containing 50 mM Tris (pH 8.0) and 10 mM or 5 mM MgCl₂ in a total volume of 10 µl with water. Reactions were incubated at 30 °C for 15 minutes.

**Fig. 4. Substrate binding assay of hFEN-1 proteins.** A. Binding assay based on flap substrate. B. Binding assay based on nick duplex substrate. FEN-1 (666 nM), DNA substrate (80nM), and binding buffer were mixed and incubated for 15 min at room temperature. The reactions were then loaded onto a 5% native polyacrylamide gel for electrophoresis and radio-imaging. The substrates are the same as those shown in Fig.3A (flap substrate) and B (nick duplex substrate).

**Fig. 5. Kinetic assays of FEN-1 mutants.** Relative $K_m$, $K_{cat}$, $K_{cat}/K_m$ values of FEN-1 mutants with partial endonuclease (A) and exonuclease (B) activity are shown. The assay conditions were described under “Experimental Procedures”. The protein concentration for WT and mutants was 92nM, and the substrate concentrations were 30nM-500nM. The substrates are the same as those shown in Fig. 3A (flap substrate) and B (nick duplex substrate).

**Fig. 6. FEN-1 nuclease activities based on 5’ overhang substrate.** The top bands represent the uncut substrates and the bottom bands are the cleavage products. The oligo size is indicated in nucleotides (nt). Reactions were carried out with 400 nM hFEN-1 protein and 80nM of flap or nick duplex substrate in reaction buffer containing 50 mM Tris (pH 8.0) and 10 mM or 5 mM MgCl₂ in a total volume of 10 µl with water. Reactions were incubated at 30 °C for 15 minutes.

**Fig. 7. Enzyme activities and patterns of R47A, R70A and wild type FEN-1 based on flap substrates with variant upstream gap lengths.** The top panel lists all the substrate used for the analysis. The arrow indicates the sequences of the changed upstream primers or template strand in the following substrate (s). For the bottom panel, the top band represents the uncut substrates and the
bottom bands are the cleavage products. Lane numbers correspond to the numbers of substrates used for the assay. The oligo size is indicated in nucleotides (nt). Reactions were carried out with 90 ng hFEN-1 protein and 80nM of flap or nick duplex substrate in reaction buffer containing 50 mM Tris (pH 8.0) and 10 mM or 5 mM MgCl₂ in a total volume of 10 µl with water. Reactions were incubated at 30 °C for 15 minutes.

**Fig. 8. Enzyme activities and cleavage patterns of R47A, R70A and wild type FEN-1 on pseudo-Y substrates with variant 3’ overhang lengths.** The top panel lists all the substrate used for the analysis. The arrow indicates the sequences of the changed template strands in the following substrates. For the bottom panel, the top band represents the uncut substrates and the bottom bands are the cleavage products. Lane numbers correspond to the numbers of substrates used for the assay. The oligo size is indicated in nucleotides (nt). Reaction condition is the same as in Fig. 6 except for the different substrate were used.

**Fig. 9. A model showing the general orientation, borders and some specific interaction sites in the human FEN-1/DNA substrate complex.** A structural model of human FEN-1 bound to a flap DNA substrate (ribbon representation) was created based on the crystal structure of aFEN-1 bound to DNA (PDB ID: 1RXW). Residues determined to affect DNA binding (white residues) interact with either the upstream DNA (20 bp, brown), the downstream DNA (13 bp, green), or the 5’ flap (10 nt, green), defining the substrate-binding orientation. Residues from two key α-loop-α regions (red) interact with the upstream DNA duplex. Several different regions of FEN-1, including the H3TH motif (small loop 2, cyan), along with other loops (small loop 3, cyan; small loop 4, magenta) interact with the downstream duplex. Residues in the helical arch (large loop, blue) likely interact with the 5’ flap. The C-terminal domain (gray circle), which follows the PCNA binding domain (purple) could interact with the upstream DNA, thus protecting the full 20 base-pairs.
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Notes: +++: Wide type enzyme activity, ++: decreased activity, +: minimal activity, -: no activity, nd: not defined, *: with binding deficiency; up-D: upstream duplex; down-D: downstream duplex; ss-Flap: single stranded DNA Flap.
A.

Flap substrate

WT

R29A

R47A

R70A

R73A

K93A

K99A

R100A

R103A

R103A/R104A

K125A

K128A/R129A

K132A

R192A

K200A

K201A

R239A

K244A/K254A

K252A/K254A

K261A/R262A

K267A

R320A

K326A/R327A


B.

Flap substrate

WT

R29A/K80A

R47A

R70A

R73A

K93A

K99A

R100A

R103A

R103A/R104A

K125A

K128A/R129A

K132A

K192A

K200A

K201A

K239A

K244A/K254A

K252A/K254A

R261A/R262A

K267A

R320A

K326A/R327A


5' *GATGTCAGCAGCTTGTAGGCAGAGTCC
3' GTGCAACTGATGGCAAGAATTCCGTCTCAGG
5' CACGTTGACTACGTC

5' TTGAGGCAGAGGCC
3' GTGCAACTTGATGGCAAGAATTCCGTCTCAGG
5' CACGTTGACTACGTC

16nt

3nt

2nt

1nt
A.

- K326A/R327A
- K252A/K254A
- K244A/R245A
- K200A
- K128A/R129A
- K125A
- R103A/R104A
- R70A
- R47A
- WT

B.

- K326A/R327A
- K252A/K254A
- K244A/R245A
- K200A
- K128A/R129A
- K125A
- R103A/R104A
- R70A
- R47A
- WT
5’ GATGTCAGCAGCTCATAAGTTAGGCCAGAGCTCC 3’ AACTCCGTCTCAGG

Substrate
WT
R47A
R70A
R103A/R104A
K125A
K128A/R129A
K200A
K244A/R245A
K267A
K326A/R327A

34nt
21nt
20nt
6nt
Enzyme               No protein  wtFEN-1            R47A                  R70A
Substrate 1   2   3   4   5   6  7   1   2   3 4 5   6   7   1   2   3 4   5 6   7 1   2   3 4 5   6 7

3’ TTGAGGCAGAGTCC
5’ GTGCAACTGATGGCAGAACTCCGTCTCAGG

3’ CACGTTGACTACC
5’ CACGTTGACTAC
5’ CACGTTGACT
5’ CACGTTGACT
5’ CTACCGAC

(1)
(2)
(3)
(4)
(5)

3’ AACTCCGTCTCAGG

(6)
(7)
Enzyme | No protein | wtFEN-1 | R47A | R70A
--- | --- | --- | --- | ---
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Note: Bold letters indicate nucleotides mutated, K93/R103A/R104A was created based on R103A/ R104A mutant DNA using K93Af and K93Ar primers, and R47A and R70A were generated as previously (Qiu, et al., 2002).
Interaction Interface of human flap endonuclease-1 with Its DNA substrates
Junzhuan Qiu, Ren Liu, Brian R. Chapados, Mark Sherman, John A. Tainer and Binghui Shen

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