Mechanism Whereby Proliferating Cell Nuclear Antigen Stimulates Flap Endonuclease 1*

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Human flap endonuclease 1 (FEN1), an essential DNA replication protein, cleaves substrates with unannealed 5'-tails. FEN1 apparently tracks along the flap from the 5'-end to the cleavage site. Proliferating cell nuclear antigen (PCNA) stimulates FEN1 cleavage 5–50-fold. To determine whether tracking, binding, or cleavage is enhanced by PCNA, we tested a variety of flap substrates. Similar levels of PCNA stimulation occur on both a cleavage-sensitive nicked substrate and a less sensitive gapped substrate. PCNA stimulates FEN1 irrespective of the flap length. Stimulation occurs on a pseudo-Y substrate that exhibits upstream primer-independent cleavage. A pseudo-Y substrate with a sequence requiring an upstream primer for cleavage was not activated by PCNA, suggesting that PCNA does not compensate for substrate features that inhibit cleavage. A biotin-streptavidin conjugation at the 5'-end of a flap structure prevents FEN1 loading. The addition of PCNA does not restore FEN1 activity. These results indicate that PCNA does not direct FEN1 to the cleavage site from solution. Kinetic analyses reveal that PCNA can lower the \( k_{\text{cat}} \) for FEN1 by 11–12-fold. Overall, our results indicate that after FEN1 tracks to the cleavage site, PCNA enhances FEN1 binding stability, allowing for greater cleavage efficiency.

Flap endonuclease 1 (FEN1) is a member of the RAD2 superfamily of nucleases that play a critical role in DNA replication and repair in prokaryotes and eukaryotes (1–5). Biological and genetic studies support a role for FEN1 during these cellular processes. Reconstitution reactions in vitro with either calf or human FEN1 illustrate the need for this nuclease during Okazaki fragment processing (6, 7) and long patch base excision repair (8). In Saccharomyces cerevisiae, a null mutant of the FEN1 homolog (RAD27/RTH1) exhibits slow growth, excision repair (8), and pyrococcus furiosus FEN1 (17, 18). Methanococcus jannaschii FEN1 (19) indicate that this physical structure is critical for both the binding and cleaving of flap substrates (17).

Crystal structures of FEN1 homologs including T5 exonuclease (15), T4 RNase H (16), Methanococcus jannaschii FEN1 (17, 18), and Pyrococcus furiosus FEN1 (19) reveal a helical arch or loop. This arch may be utilized by the nuclease to recognize the 5'-end of an unannealed flap and to track along the flap structure. Mutational analyses of the loop region in the M. jannaschii FEN1 indicate that this physical structure is critical for both the binding and cleaving of flap substrates (17).

PCNA has been found to be a potent stimulator of FEN1 cleavage activity (13, 20). PCNA is the processivity factor for DNA polymerases \( \delta \) and \( \epsilon \) (21). It is a toroidal homotrimer that is assembled around double-stranded DNA to form a sliding clamp (22). Biochemical and genetic analyses have shown that FEN1 interacts with a hydrophobic cleft located on the front face of the PCNA toroid (13, 20, 23, 24). Interaction of PCNA with both polymerases and FEN1 suggests that it recruits FEN1 to the protein complex at the replication fork in vivo (25). PCNA also interacts with the regulatory protein p21Cip, which is induced when chromosomal DNA is damaged (26–28). Because p21Cip uses the same binding site on PCNA as replication proteins, it has been postulated that binding of this regulatory protein inactivates the replication complex while the chromosomal DNA is being repaired (23, 29, 30).

Reconstitution reactions have revealed more about how FEN1 is employed in DNA metabolism. Okazaki fragment processing involves the coordinated action of a DNA polymerase, RNase HI, FEN1, PCNA, and DNA ligase I (3). On the lagging strand of a replication fork, RNase HI removes the initiator RNA primer on an Okazaki fragment leaving a single 5'-ribonucleotide. FEN1 subsequently removes the 5'-ribonucleotide, and the resulting nick is sealed by DNA ligase I.

Base excision repair pathways are responsible for replacing thousands of damaged nucleotides/mammalian cell/day (31). One of the pathways, long patch base excision repair, utilizes many of the components of Okazaki fragment processing including FEN1 (8, 32–36). Bases altered by ionizing radiation, oxidation, or alkylating agents are often targeted. During the repair process, the damaged base is removed by a DNA N-glycosylase to create an abasic site. An apurinic/apyrimidinic endonuclease then cleaves on the 5'-side of the abasic sugar by...
making a nick with a deoxyribose phosphate at the 5’-side. The damaged residue and additional downstream nucleotides are lifted to form a flap, and the abasic residue is endonucleolytically removed as part of an oligomer (8, 32, 34). The resulting short gap is filled and ligated to complete the repair process. PCNA has been found to facilitate excision in long patch base excision repair through interactions with FEN1 (37).

Hübsher and colleagues have shown that PCNA stimulation of FEN1 was inhibited on a linear flap substrate with biotin-streptavidin conjugated to the upstream region of the template (24). Conjugation of a biotin-streptavidin moiety to the downstream region did not result in any inhibition (24). This indicates that PCNA enters the template upstream of the flap for stimulation. The subsequent interaction with FEN1 and the stimulation reaction is the subject of our investigation. Given the complex mechanism of FEN1 cleavage, PCNA may affect the tracking, binding, or catalytic properties of FEN1. Determining how a protein on the double-stranded template upstream of the flap stimulates a protein thought to enter the substrate by tracking on the flap will be important for elucidating the biological relevance of this interaction.

**EXPERIMENTAL PROCEDURES**

**Materials—**All oligonucleotides were synthesized by Genosys Biotechnologies (The Woodlands, TX). Radio nucleotides [γ-32P]dCTP (6000 Ci/mmol) and [γ-32P]ATP (6000 Ci/mmol) were obtained from New England Nuclear. The acrylamide sequencing gel mix and T4 polynucleotide kinase were from Roche Diagnostics. Sequence (version 2.0) was obtained from Amersham Pharmacia Biotech. Micro Bio-Spin 30 chromatography columns were from Bio-Rad. All other reagents were the best available commercial grade.

Recombinant human FEN1 was expressed and purified from Escherichia coli utilizing the T7 expression plasmid pET-FCH (38). Recombinant ATP and T4 polynucleotide kinase were expressed in E. coli using the expression vector pT7/PCNA (39) or RG84A (28) and purified (39). Purified enzymes were dialyzed into a storage buffer (20% glycerol, 20 mM KCl, 30 mM HEPES (pH 7.6) (diluted from a 1 M stock), 0.01% Nonidet P-40, 1 mM dithiothreitol, and 1 mM EDTA) and stored at −80 °C.

**Oligonucleotide Substrates—**Oligomer sequences are listed in Table I, and the primer-template substrates were constructed as described in the figure legends. All substrates, the 3’-end regions of the downstream primers share homology with the 5’-ends of their respective templates. Once annealed, these primers create substrates with unannealed 5’-tails as illustrated in the figures. Each respective upstream primer was annealed to the proper template to create a nick or a gap at the base of the unannealed 5’-tail of the downstream primer. Prior to annealing, the 5’-radiolabeled primers were generated utilizing [γ-32P]ATP and T4 polynucleotide kinase according to the manufacturer’s instructions. Downstream primer D3 was annealed to T3 and radiolabeled at the 3’-end using [γ-32P]dCTP and Sequenase (version 2.0). Unincorporated radiolabeled nucleotides were removed with Micro Bio-Spin 30 chromatography columns. All radiolabeled primers were purified by gel isolation from either a 12 or 15% polyacrylamide, 7 M urea denaturing gel prior to annealing. Substrates were annealed by mixing 0.5 pmol of the respective downstream primer with 2 pmol of the corresponding template in annealing buffer (10 mM Tris base, 50 mM KCl, and 1 mM EDTA (pH 8.0)) to a final volume of 50 μl. The mixture was heated to 65 °C for 10 min and allowed to cool to room temperature. If present, a corresponding upstream primer (10 pmol) was added and annealed by incubating at 97 °C for 1 h.

**Enzyme Assay—**Reactions containing the indicated amounts of substrate, FEN1, and PCNA were performed in a buffer containing 30 mM HEPES (pH 7.6), 5% glycerol, 40 mM KCl, 0.1 mg/ml bovine serum albumin, and 8 mM MgCl2. Reactions were incubated at 37 or 25 °C as indicated in the figure legends, terminated with 20 μl of formamide dye (90% formamide (v/v) with bromphenol blue and xylene cyanol), and heated to 85 °C for 5 min. After separation on a 12% polyacrylamide, 7 M urea denaturing gel, products were detected by autoradiography or PhosphorImager (Molecular Dynamics) analysis.

For the biotin-streptavidin assay, the initial reaction mixtures lacked Mg2+. The substrate was incubated with enzyme either before or after addition of streptavidin. Reactions contained 100 fmol of FEN1. Streptavidin, added in a 50-fold molar excess over substrate, was complexed with the biotinylated substrate by placing the reaction at 37 °C for 10 min. MgCl2 was then added to 8 mM, and enzyme activity was assayed as described above. Assays were performed at least in triplicate.

**Kinetic Analyses—**FEN1 cleavage kinetics were performed at 25 °C according to the standard conditions described in the enzyme assay. Various concentrations of DNA substrate (5, 10, 15, and 20 fmol) comprised of D3U3T3 and a constant amount of FEN1 (5 fmol) were utilized. Assays were performed at least in triplicate. Reactions in the absence of PCNA were initiated by sequentially combining standard reaction buffer, substrate, and enzyme. In the reactions with PCNA, 5 pmol of PCNA were added prior to the addition of FEN1. Samples were mixed, heated to 95 °C for 5 min, and allowed to cool to room temperature. The initial velocity was determined by measuring the substrate and product intensities on a 12% polyacrylamide, 7 M urea denaturing gel by means of PhosphorImager (Molecular Dynamics) analysis. The general expression for the velocity of the reaction is $v = d[\text{product}] / dt$, where $[\text{product}]$ = product concentration in nm. The product concentration was calculated using the equation $P = \text{IP} - \left( \text{IP} + \text{IP}_{\text{p}} \right) \times \text{substrate}$, where $\text{IP}$ = product intensity, $\text{IP}_{\text{p}}$ = starting material intensity, and substrate concentration is expressed in nm. Velocity is expressed as converted substrate concentration (nM/time (s)). $K_m$ and $V_{\text{max}}$ values were calculated by directly fitting the data to the Michaelis-Menten equation.

**Gel Mobility Shift Assay—**Reactions were performed in a buffer containing 30 mM HEPES (pH 7.6), 5% glycerol, 40 mM KCl, 0.1 mg/ml bovine serum albumin, and 8 mM CaCl2 in a final reaction volume of 20 μl. Reactions were incubated at 25 °C for 8 min. After separation on a 0.7% agarose gel in 0.1× TBE (8.9 mM Tris base, 8.9 mM boric acid, and 0.2 mM EDTA (pH 8.0)), products were visualized using a PhosphorImager (Molecular Dynamics).

**RESULTS**

**PCNA Stimulates FEN1 on a Gapped Substrate—**Previous studies have demonstrated that PCNA stimulates FEN1 cleavage activity on flap substrates (13, 20). These substrates commonly have a nick between the 3’-end of the upstream primer and the annealing point of the downstream primer. On many substrates, creating a short gap between the upstream primer and the base of the flap inhibits FEN1 cleavage (40). With these substrates, the upstream primer most likely helps the FEN1 nuclease to recognize the cleavage site. We considered that PCNA might overcome the inhibitory effects of a gap. If so, PCNA would stimulate FEN1 cleavage activity on a gapped substrate to the level observed for a nicked substrate with PCNA. Alternatively, PCNA might stimulate little, if at all, on the gapped substrate. Because Hübsher and colleagues proposed that PCNA enters a substrate upstream of the flap for stimulation of FEN1 (24), complete double-stranded structure upstream of the flap may be required for PCNA to affect FEN1. Fig. 1A is a time course showing the stimulation of FEN1 cleavage by PCNA on a 25-nucleotide flap substrate with a nick separating the upstream primer and the base of the flap. FEN1 cleavage yields 25- and 26-nucleotide products (lanes 1–6). The addition of PCNA resulted in an approximate 8-fold stimulation of cleavage (lanes 7–12).

Decreasing the size of the upstream primer to make a 1-nucleotide gap greatly decreased the susceptibility of the substrate to cleavage by FEN1. As seen in Fig. 1B, PCNA stimulated FEN1, and in fact, the approximate 16-fold stimulation is an even greater percentage than that observed on the flap substrate with a nick. However, the overall amount of substrate converted to product was significantly reduced when compared with the cleavage of the substrate containing a nick. FEN1 cleavage results in 22- and 26-nucleotide products for the 25-nucleotide flap substrate with a gap. The 22-nucleotide product is likely caused by the flap transiently annealing in the gap region to form an alternate flap structure.

Surprisingly, the addition of PCNA does not lead to a specific increase in the formation of the cleavage product that results from cleavage at the base of the flap. Therefore, the addition of PCNA does not alter the specificity of FEN1 cleavage of the flap. The interesting implication of this observation is that
Mechanism Whereby PCNA Stimulates FEN1

Table I
Oligonucleotide sequences (5'-3')

| Downstream Primers* | Upstream Primers | Templates |
|---------------------|------------------|-----------|
| D1 (18-mer)         | U1 (20-mer)      | T1 (44-mer) |
| D2 (19-mer)         | U2 (24-mer)      | T2 (49-mer) |
| D3 (22-mer)         | U3 (25-mer)      | T3 (58-mer) |
| D4 (43-mer)         |                 |           |
| D5 (51-mer)         |                 |           |
| D6 (76-mer)         |                 |           |

* The underlined nucleotide represents the first position annealed to a template. The boldface nucleotide indicates a biotin modification.

PCNA is not required to slide to a position directly over the gap before interacting with FEN1. If PCNA must move to the base of the flap prior to cleavage, the structure required to generate the 22-nucleotide product would be blocked from forming. These results show that the PCNA stimulation mechanism is operative on a gapped substrate, but PCNA cannot compensate for the decreased cleavage efficiency caused by the absence of a nick.

**PCNA Stimulates FEN1 on Substrates with Varying Flap Lengths**—We considered that the flap structure itself might participate in the stimulation reaction. If so, stimulation would be more effective when the FEN1-PCNA complex interacts with the appropriate flap length. Substrates with varying flap lengths were analyzed to determine whether the length of the flap affects PCNA stimulation of FEN1. Fig. 2 illustrates PCNA stimulation of FEN1 cleavage of substrates with 25-, 4-, and 1-nucleotide flap lengths. In addition, a substrate with a fully annealed downstream primer was analyzed. The addition of PCNA to the reactions led to an increase in product formation. The level of stimulation ranged from 12- to 16-fold with no noticeable trend among the substrates. This analysis of substrates with long or short flaps and one without a flap shows that PCNA stimulates FEN1 cleavage irrespective of the flap length. In fact, the stimulation seen with the fully annealed substrate shows that the portion of the FEN1 reaction cycle that involves tracking on the flap is probably not the point of stimulation.

**PCNA Cannot Substitute for an Upstream Primer**—Pseudo-Y substrates are flap structures lacking an upstream primer. In certain contexts, removal of the upstream primer virtually eliminates the ability to serve as a substrate for FEN1-directed cleavage (2, 11, 40). Such a substrate, called upstream primer-dependent, is shown in Fig. 3A. The pseudo-Y structure could not be activated by PCNA (lanes 3–5). In the presence of the upstream primer, FEN1 experienced efficient PCNA stimulation (lanes 8–10). The stimulatory effect seen for PCNA in lanes 8–10 is much greater than the effect observed in lanes 3–5. This shows that PCNA cannot efficiently activate an inert substrate. It also demonstrates that PCNA cannot substitute for an upstream primer.

In the case of the upstream primer-independent substrate (41), PCNA stimulates cleavage product formation regardless of the presence of an upstream primer. However, removal of the upstream primer alters the cleavage specificity (Fig. 3B). Examination of the sequence at the 3'-end of the template reveals the absence of secondary structures. This interesting observation shows that entry of PCNA onto the upstream primer template does not require that this short region be double-stranded DNA. Furthermore, a double-stranded structure does not appear to be necessary to orient PCNA so that it can interact with the FEN1 nuclease for stimulation.

**Increasing FEN1 Concentration Reduces PCNA Stimulation**—To further analyze the mechanism involved in PCNA stimulation of FEN1, two of the previously tested flap substrates were utilized in an enzyme titration assay. A molar excess of PCNA over FEN1 was maintained for each FEN1 concentration. At very high concentrations of FEN1, the level of PCNA stimulation is less than that observed at lower concentrations of nuclease (Fig. 4). For example, when 100 fmol of FEN1 are utilized in the reactions with either substrate, PCNA stimulation is approximately 2-fold. This is a greatly reduced percentage of stimulation as compared with the 5–20-fold val-
ues measured at lower FEN1 concentrations. This result shows that the mechanism of PCNA-directed stimulation involves making the FEN1 molecules act as if they were present at a higher concentration. This observation is consistent with PCNA improving the binding of FEN1 to the substrate.

**PCNA Alters the $K_m$ and $V_{max}$ of the FEN1 Cleavage Reaction—Quantitative analysis of the effects of PCNA on FEN1 reaction parameters can be achieved by measuring the $K_m$ (Michaelis constant) and $V_{max}$ (limiting velocity) with respect to the flap substrate concentration. A large change in the $K_m$ value would indicate that the sites of cleavage are occupied by nuclease molecules for a greater portion of time. At any point in the reaction, a larger amount of FEN1 is bound, providing more opportunity to generate the cleavage product. Alternatively, a large change in the $V_{max}$ value would suggest that PCNA directly improves the efficiency of the FEN1 cleavage reaction.

Table II gives the $K_m$ and $V_{max}$ values that were determined from the Michaelis-Menten equation. The addition of PCNA leads to an approximate 2-fold increase in $V_{max}$ and an 11–12-fold decrease in $K_m$. The moderate increase in $V_{max}$ suggests...
that PCNA alters the conformation or orientation of FEN1, resulting in a small improvement in the rate of catalysis. The large decrease in $K_{\text{m}}$ suggests that the major effect of PCNA is to stabilize FEN1 binding to the substrate. This could be accomplished by binding FEN1 molecules after they have tracked to the cleavage site, preventing them from reversing direction on the flap. Alternatively, PCNA could have created a new mechanism of binding that augments the tracking mechanism. For example, PCNA might have allowed FEN1 to bind the cleavage site directly from solution, bypassing tracking steps. Evidence that PCNA Stabilizes FEN1 Binding to Its Cleavage Site—Previous studies have shown that placing a biotin-streptavidin moiety at the 5'-end of a flap structure inhibits FEN1 cleavage (12). These results led to suggestions that the nuclease tracks along the flap before arriving at the site of cleavage. For current experiments, we employed a biotinylated substrate with a 53-nucleotide flap length. The enzymatic footprint of FEN1 was found to cover ~25 nucleotides at the base of the flap (42). The streptavidin conjugation should then be beyond the site of FEN1 binding and cleavage. Nevertheless, when streptavidin was conjugated to the biotinylated 5'-end of this flap substrate prior to the addition of any enzyme, FEN1 activity was greatly reduced (Fig. 5). This result is consistent with blockage of the tracking reaction. The addition of PCNA did not relieve the inhibitory effect of this blocking protein. We conclude that interaction with PCNA cannot circumvent the tracking mechanism by binding FEN1 to the cleavage site directly from solution. The addition of streptavidin to a biotinylated substrate did not result in an inhibition of FEN1 cleavage activity (data not shown).

When FEN1 was prebound before the addition of streptavidin, there was essentially no increase in cleavage product formation. Apparently, very little FEN1 was bound to the cleavage site at the time that tracking was blocked. The addition of PCNA after the 5'-end was blocked did not lead to any substantial increase in product formation, presumably because FEN1 could no longer load onto the flap. However, when FEN1 and PCNA were prebound before the addition of streptavidin, there was an observed increase in the conversion of substrate to product. This would occur if the presence of PCNA increased the amount of FEN1 bound to the cleavage site. The level of product formation was still diminished as compared with the control substrate because FEN1 could no longer recycle and load onto other flap substrate molecules that were already blocked. Observed differences in product formation depending on the order of addition verify that products are not being formed from a low level of a contaminating nonbiotinylated substrate. A high concentration of FEN1 was utilized to increase the amount of nuclease molecules available to bind the substrate. The results with the streptavidin-blocked substrates are consistent with the conclusions of the kinetic analyses, which show that PCNA improves the binding stability of FEN1 at the cleavage site.

Further support for the conclusion that PCNA stabilizes the interaction of FEN1 with the substrate came from a gel mobility shift assay (Fig. 6). The addition of progressively higher concentrations of PCNA increased the observed amount of the DNA-FEN1 complex (lanes 4–6). Lane 7 is a control lane with a higher concentration of FEN1 in the absence of PCNA. This lane clearly shows the DNA-FEN1 complex. Also, the lane with PCNA alone (lane 2) does not reveal the complex.

**DISCUSSION**

FEN1 is an essential component of the DNA replication and repair systems in eukaryotic cells. This nuclease prefers a flap substrate, removing the flap at the point of annealing. It has been postulated to enter the flap from the unannealed 5'-end, track to the annealing point, and then endonucleolytically cleave (2, 12, 13). PCNA is a potent stimulator of the cleavage reaction. However, monomeric PCNA does not stimulate FEN1 (13, 20). Previous results indicate that the PCNA trimer loads by diffusion onto the double-stranded primer template upstream of the flap on a linear substrate (24). The toroid could then move to the base of the flap to effect stimulation. Because this is a diffusion-limited process, FEN1 stimulation requires a large stoichiometric excess of PCNA (13, 43). In addition, all experiments were performed with excess bovine serum albumin to reduce nonspecific protein effects. We have designed experiments to test alternative potential mechanisms of stimulation. We conclude that PCNA acts largely by stabilizing the interaction of FEN1 with its cleavage site.

Because PCNA binds to FEN1 and the sites of interaction have been characterized (24, 44), we anticipated that the stimulation process would involve direct FEN1-PCNA interaction. Recent genetic studies have emphasized the importance of the role of PCNA binding to FEN1 in vivo (45). Binding of the two

**TABLE II**

| $K_{\text{m}}$ (nt) | $V_{\text{max}}$ (nt/s) |
|---------------------|------------------------|
| FEN1 only           | 2.18 ± 0.29            |
| FEN1 + PCNA         | 0.19 ± 0.03            |

**FIG. 4.** The level of PCNA stimulation decreases at high concentrations of FEN1. Reactions were incubated at 37 °C for 15 min. Substrate and cleavage product sizes are as indicated. Schematic representations of the substrates are depicted above each panel. The asterisk indicates the position of the radiolabel. Reactions of 20 μl containing 5 fmol of DNA substrate and 10, 50, or 100 fmol of FEN1 were performed as described under “Experimental Procedures.” The reactions in the presence of PCNA contained 5, 25, or 50 pmol of PCNA corresponding to increasing FEN1 concentrations. A, product analysis of a substrate with a 25-nucleotide flap (D$_9$U$_9$T$_{11}$). B, product analysis of a substrate with a 1-nucleotide flap (D$_9$U$_9$T$_{10}$).
proteins might alter the structure of the FEN1 nuclease to allow it to react more efficiently with its substrate. Our kinetic analyses showed a moderate increase in $V_{\text{max}}$ with respect to the substrate concentration in the presence of PCNA, which is consistent with a minor improvement in the basic cleavage efficiency. The more striking effect on reaction kinetics is the 11–12-fold reduction in $K_m$ that occurred in the presence of PCNA. The reduction in $K_m$ indicates that PCNA increased the reaction rate with a subsaturating substrate concentration. A likely interpretation is that PCNA increases the time that the nuclease spends bound to the cleavage site, improving the probability that the cleavage reaction will occur. Alternative mechanisms whereby a FEN1-PCNA interaction could augment binding stability at the cleavage site are presented in Fig. 7. Current models suggest that FEN1 enters the flap at the 5′-end and moves to the cleavage site. We recently proposed that FEN1 tracks along the flap using a clamp-shaped cleft in the protein (38). Once the nuclease is bound to the cleavage site, it could cleave or track away from the intact site. We postulate that PCNA holds the nuclease at the cleavage site for a longer time than it would ordinarily remain. By one potential mechanism, PCNA initially slides to the base of the flap (Fig. 7A). When FEN1 arrives at the cleavage site, it binds both the point of cleavage and PCNA. In a second mechanism, PCNA and FEN1 interact before binding the flap substrate (Fig. 7B). The complex is held on the substrate by PCNA, effectively increasing the local concentration of FEN1. Both tracking and binding of the cleavage site could then be enhanced.

We do not yet know whether tracking on the flap is an obligatory step in the FEN1 reaction. FEN1 might be able to bind the cleavage site directly. In a third possible mechanism, the PCNA molecule near the cleavage site attracts FEN1 directly from solution (Fig. 7C). The added stability of interacting with PCNA allows FEN1 to interact with the cleavage site without the need to enter the site by tracking.

If the second model were correct, we would expect PCNA to alter the tracking process. A likely outcome is that the amount of stimulation would be influenced by the length of the flap. However, we found similar stimulation of cleavage on long and short flaps and on pseudo-Y structures. Because cleavage activity was stimulated on substrates with short flaps, which are presumed to be too short for tracking, the unannealed 5′-tail does not appear to be related to the stimulation. These results argue against the second mechanism but are not sufficient to completely discount this possibility. Furthermore, a substrate that required an upstream primer could not be activated by PCNA. Overall, these results suggest that PCNA does not alter either the loading mechanism of FEN1 or the structural requirements of the substrate.

Blocking the flap with a biotin-streptavidin moiety is very effective at preventing FEN1-directed cleavage. The observation that the blockage cannot be bypassed by the addition of PCNA discounts the third model. This result also strongly supports the conclusion that the tracking step on flap substrates is obligatory.

We have acquired considerable evidence in support of the first mechanism. When FEN1 is prebound to a flap substrate before the flap is blocked, cleavage is inhibited even with the addition of PCNA. However, there is a significant increase in cleavage product formation when FEN1 and PCNA are prebound prior to modifying the 5′-tail. These results are consistent with FEN1 tracking on the flap to the point of cleavage and...
binding that site with moderate affinity. On each flap substrate, the nuclease appears to track bidirectionally. At any point in time, some nucleases are bound to cleavage sites. After blocking the 5′-tails, only nucleases present at cleavage sites can remove the flaps. However, upon addition of PCNA, more substrates have FEN1 trapped at cleavage sites following blockage. This produces more cleavage product in the reaction performed after the flap is blocked. Genetic studies have shown that a mutant of PCNA with reduced binding to FEN1 was defective in FEN1 stimulation (24), which is in agreement with our conclusions.

Another important observation is that increasing the concentration of FEN1 has the same effect as adding PCNA. In fact, at high concentrations of FEN1, PCNA can no longer augment the rate of reaction. This result implies that PCNA does not change the basic reaction mechanism. It is also consistent with two earlier conclusions. First, PCNA cannot compensate for characteristics that make a substrate insensitive to cleavage by FEN1. Second, the percentage of stimulation is generally independent of flap length and other structural characteristics of cleavage-sensitive substrates.

These results are fully consistent with the lowering of $K_m$ with respect to substrate by PCNA, indicating that the affinity for the cleavage site is being enhanced. Also, the gel mobility shift assay shows that PCNA greatly increases the amount of FEN1 bound to DNA. Curiously, PCNA is not present in the gel mobility shift complex. Possibly, PCNA dissociates during the electrophoresis step, and the FEN1 dissociation rate could be sufficiently slower so that the nuclease is retained during the movement of the complex on the gel. Another interesting possibility is that PCNA induces a conformation in the nuclease that increases its binding stability to the cleavage site. Subsequently, PCNA can dissociate. The remaining FEN1 molecules would then dissociate more slowly than those that had not undergone any conformational change.

The ability of PCNA to stimulate cleavage of a pseudo-Y substrate shows that PCNA does not require double-stranded DNA to assume the orientation necessary to interact with FEN1. It also shows that PCNA can enter the substrate on a short single strand. Previous studies have shown that the β-subunit of DNA polymerase III holoenzyme, the prokaryotic homolog of PCNA, does not slide over long stretches of naked single-stranded DNA (46). This constraint is most likely the result of secondary structures in the single strand, which impede the movement of the PCNA homolog. The single-stranded region in our substrate is not expected to form secondary structures.

In summary, we present considerable evidence that human PCNA stimulates the cleavage activity of human FEN1 by stabilizing binding of the nuclease to the site of cleavage on its polymer substrate. Stimulation occurs on a variety of FEN1 substrates as long as they have the appropriate structural features to allow cleavage. FEN1 has a complex reaction cycle, apparently involving tracking on the tail of flap substrates. Nevertheless, the mechanism of stimulation can be explained solely by a binding interaction between a PCNA molecule surrounding the template and a FEN1 molecule at the cleavage site as illustrated in Fig. 7A.

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