2036–2043 *Nucleic Acids Research, 1996, Vol. 24, No. 11*

Processing of branched DNA intermediates by a complex of human FEN-1 and PCNA

Xiantuo Wu[1,2], Jun Li[1,2], Xiangyang Li[2], Chih-Lin Hsieh[2], Peter M. J. Burgers[2] and Michael R. Lieber[1,2,*]

Departments of [1]Pathology and [2]Biochemistry and Molecular Biophysics, Division of Molecular Oncology, Washington University School of Medicine, 660 S. Euclid Avenue, Campus Box 8118, St Louis, MO 63110, USA

Received March 1, 1996; Revised and Accepted April 18, 1996

ABSTRACT

In eukaryotic cells, a 5′ flap DNA endonuclease activity and a ds DNA 5′-exonuclease activity exist within a single enzyme called FEN-1 [flap endo-nuclease and 5(five)′-exo-nuclease]. This 42 kDa endo-/exonuclease, FEN-1, is highly homologous to human XP-G, *Saccharomyces cerevisiae* RAD2 and *S.cerevisiae* RTH1. These structure-specific nucleases recognize and cleave a branched DNA structure called a DNA flap, and its derivative called a pseudo Y-structure. FEN-1 is essential for lagging strand DNA synthesis in Okazaki fragment joining. FEN-1 also appears to be important in mismatch repair. Here we find that human PCNA, the processivity factor for eukaryotic polymerases, physically associates with human FEN-1 and stimulates its endonucleolytic activity at branched DNA structures and its exonucleolytic activity at nick and gap structures. Structural requirements for FEN-1 and PCNA loading provide an interesting picture of this stimulation. PCNA loads on to substrates at double-stranded DNA ends. In contrast, FEN-1 requires a free single-stranded 5′ terminus and appears to load by tracking along the single-stranded DNA branch. These physical constraints define the range of DNA replication, recombination and repair processes in which this family of structure-specific nucleases participate. A model explaining the exonucleolytic activity of FEN-1 in terms of its endonucleolytic activity is proposed based on these observations.

INTRODUCTION

In all eukaryotic cells, an enzyme called FEN-1 [flap endo-nuclease and 5(five)′-exo-nuclease] appears to function as both a 5′ flap DNA endonuclease and a ds DNA 5′-exonuclease (1,2). This 42 kDa endo-/exonuclease, FEN-1, has been shown to be highly homologous to human XP-G, *Saccharomyces cerevisiae* RAD2 and *S.cerevisiae* RTH1 (3). These structure-specific nucleases recognize and cleave a branched DNA structure called a DNA flap, and its derivative called a pseudo Y-structure (4). FEN-1 and its corresponding *S.cerevisiae* homologue, RTH1, are important for DNA replication based on genetic studies (5,6), and FEN-1 is essential for DNA replication in cell-free systems (7,8). FEN-1 also appears to be important in mismatch repair (9). Other DNA repair and recombination pathways are being examined to determine the extent of FEN-1 involvement in branched DNA processing in other DNA transactions. Bambara and colleagues have published a study of calf thymus FEN-1 on various 5′ flap substrates (10) showing that double-stranded regions along an otherwise single-stranded 5′ flap block entry and sliding of FEN-1 to the branch point. In addition, the phosphorylation status at the 5′ flap terminus did not affect cleavage, though absence of a 5′-phosphate at a nick was inhibitory for exonucleolytic FEN-1 action.

The yeast proliferating cell nuclear antigen (PCNA) is the processivity factor for DNA polymerases δ and ε. Like FEN-1, PCNA is one of the 10 essential proteins for DNA replication (7). PCNA is also important in nucleotide excision repair (11). It is a homotrimer with a subunit molecular weight of 29 kDa and is highly conserved from yeast to mammalian cells. Based on the crystal structure, trimeric yeast PCNA forms a closed ring which appears to encircle double-stranded DNA (12). Processivity in DNA synthesis is achieved by PCNA binding to the polymerase, thereby tethering the DNA polymerase at the primer terminus (13). In addition to this structural function in DNA replication, mammalian PCNA, through its interactions with the cyclin-dependent protein kinase inhibitor p21 (CIP1/WAF1/SDI1), has also been implicated in cell cycle control (14).

Recently, we found that yeast PCNA and yeast FEN-1 interact (15). We were interested in verifying this interaction in the most distant multicellular eukaryote, human. Here we report that human PCNA binds to FEN-1 and stimulates the endonucleolytic cleavage of FEN-1 at flap structures and its exonucleolytic activity at nicks. In this ternary interaction between DNA substrate, FEN-1 and PCNA, it is interesting to consider how these three components assemble and interact. Does the mode of assembly influence the range of substrates cleaved? Here, we describe studies using recombinant human FEN-1 confirming that it requires a fully single-stranded 5′ terminus and flap for cleavage at the single- to double-stranded DNA junction. This is despite the insensitivity of FEN-1 to the phosphorylation state of the 5′ terminus and to the base in the 5′ terminal nucleotide in endonucleolytic assays. Deviations from single-stranded character anywhere between the 5′ terminus and the cleavage junction prevent cutting. Hence, heterologous loops and DNA bubbles,

* To whom correspondence should be addressed

potentially important intermediates in a variety of processes in DNA metabolism, are not recognized. These observations have important implications for the physiologic role of FEN-1 in DNA replication, DNA recombination, and DNA repair. These studies indicate that FEN-1 and PCNA may load onto different portions of branched DNA structures in assembly of the ternary complex of these two proteins with DNA. PCNA does not alter the substrate specificity at all. Based on the insensitivity of the endonucleolytic activity of FEN-1 to the 5′ phosphorylation status of the ss-DNA flap, but its sensitivity to the 5′ phosphorylation status at a DNA nick (where FEN-1 previously has been considered to act exonucleolytically), we describe a unified model that explains the exonucleolytic activity of FEN-1 in terms of its endonucleolytic activity.

MATERIALS AND METHODS

Two-hybrid analysis

Plasmid and strains. The plasmids, pJG4-5 and pEG202, have been described previously (16). The human PCNA acidic activation constructs, contain the entire human PCNA structural gene and is the result of ligation of an *Nde*I blunt fragment from p3038 2xT (17) into pEG202 digested with *Eco*RI and blunted. Lex A-Ku86 contains the carboxy-terminal 210 amino acids of human Ku86 protein, which was cloned as an *Eco*RI–*Xho*I fragment into pEG202 after polymerase chain reaction amplification. Lex A-bicoid was a gift of Roger Brent. The junctions of all constructs were sequenced. All strains were derived from the parent *S.cerevisiae* strain EGY48 (*MATa trp1 ura3 his3 leu2:: pLexAop6-leu2*). YPH499 (*MAT ura3-52 trp 1-63 his3-200 leu2-1 lys-801 ade 2-101*).

Modified two-hybrid screening. We developed a yeast liquid mating strategy to screen libraries with the yeast interaction trap of Brent (16). The prey strain, YPH499, was transformed with a HeLa cDNA expression library. The transformants, selected on tryptophan dropout plates, are harvested, aliquoted and then stored at –80°C in 32.5% glycerol, 12.5 mM Tris–HCl pH 8.0, 50 mM $MgSO_4$. The resulting library strain consists of 1.8×10^6 independent YPH499 transformants. The bait strain, EGY48, was transformed with a lacZ reportor plasmid pSH 18-34 and lexA-hPCNA (human PCNA) fusion plasmid pEG202-hPCNA. Screening is performed by mating of library and bait strains followed by selection of leucine prototrophy. In brief, 5×10^7 colony forming units of the library strain are combined with 5×10^7 cells from the bait strain. This mixture is pelleted, resuspended in 2 ml YPAD media, divided into 20 aliquots, and then incubated for 12–16 h at 30°C. Yeast were thoroughly washed with sterile water and resuspended in CM/-ura/-his/-trp/2%galactose/1% raffinose. Incubation in the latter medium for 3–4 h at 30°C permits induction of the galactose inducible promotor of the library plasmids. To screen the library for interaction, the mixture containing complete media/-ura/-his/-trp/-leu/2% galactose/1% raffinose. After 3–4 days of incubation at 30°C, the largest colonies were picked and further analyzed as described (16,18).

Protein purification

PCNA. Recombinant human PCNA was purified as described previously (17).

FEN-1. Recombinant human FEN-1 was purified as described for recombinant murine FEN-1 (3).

Physical interaction between FEN-1 and PCNA

Protein G (Pharmacia) beads (10 µl) were washed twice 0.4 ml Buffer A (40 mM HEPES pH 7.4; 2 mM $MgCl_2$) containing 150 mM NaCl (designated buffer B when it includes the 150 mM NaCl). Anti-human c-myc (anti-myc) or anti-human PCNA (2 µg) monoclonal antibodies (mouse IgG1) were added to the beads in 100 µl buffer B and incubated at 4°C for 14 h. The loaded beads were washed three times with 400 µl buffer B. Human FEN-1 (60 ng) was added to the beads in buffer B and incubated 2 h at 4°C. The beads were washed with 400 µl buffer B three times. Any bound protein (FEN-1) was eluted with two washes of 10 µl buffer A containing either 300 or 600 mM NaCl. This 20 µl was mixed with 20 µl 2× SDS loading buffer and fractionated on an 8% PAGE. Immunoblotting used rabbit polyclonal anti-human FEN-1 anti-sera at a 1:100 dilution. The secondary antibody was goat anti-rabbit coupled to horse radish peroxidase at a 1:400 dilution. Enhanced chemiluminescence (ECL) was used for detection.

FEN-1 endonuclease activity on flap substrates

Oligonucleotides are SC1, CAGCAACGCAAGCTTG (strand adjacent to the flap strand); SC3, GTCGACCTGCAGCCCAAGCTTGCGTTGCTG (bridge strand, which is annealed to the flap and the adjacent strand); and SC5, ATGTGGAAAATCTCTAGCAGGCTGCAGGTCGAC (flap strand, which is the one cleaved by FEN-1). Additional flap substrates were composed of HJ41 (bridge strand), 43 (flap adjacent strand), and 86 (flap strand). HJ41, GGACTCTGCCTCAAGACGGTAGTCAACGTG (30mer). HJ43, CACGTTGACTACCGTC (16mer). HJ86, GCCGCCGCCGCCGCCTTTTTTTTTTTTTTTTGAGGCAGAGTCC (44mer); note that upon Klenow fill-in, this 44mer becomes 46 nt long. The blocking oligo HJ87 is used in some experiments to anneal to the flap strand, HJ86. HJ87, GGCGGCGGCGGCGGC (15mer). Some structures use a different blocking oligo in place of HJ87. This is HJ88. HJ88, AAAAAAAGGCGGCGG (15mer). In cases where we wanted to label the flap strand at the 3′ end, we needed to make the bridge strand longer than HJ41. This longer version is HJ89 [TTGGACTCTGCCTCAAGACGGTAGTCAACGTG (32mer)].

FEN-1 activity on loop substrates. For heterologous loops, we paired MY2 with HJ90. MY2, GTATCTGCCGAAACTGATCCAGTTACAAGGCTGTGTCCTCAGAGGATC (48mer). HJ90, GATCCTCTGAGGACACAGATCAGTTTCGGCAGATAC (36mer). For a bubble structure, we paired MY2 with HJ91 [GATCCTCTGAGGACACTTTTTTCAGTTTCGGCAGATAC (38 mer)].

The endonuclease assay was done in a 15 µl total volume containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM NaCl, 0.5 mM β-mercaptoethanol, 500 µg/ml BSA, 10 fmol of flap substrate, FEN-1 at specified amounts (10 fmol = 0.4 ng = 20 U as defined in ref. 1), and, if present, PCNA trimer at specified amounts.

FEN-1 exonuclease activity on DNA nicks

Assays were as described for the flap substrates, except for adjustment to 10 mM Tris (pH 8), 5 mM $MgCl_2$ and 8 mM NaCl.

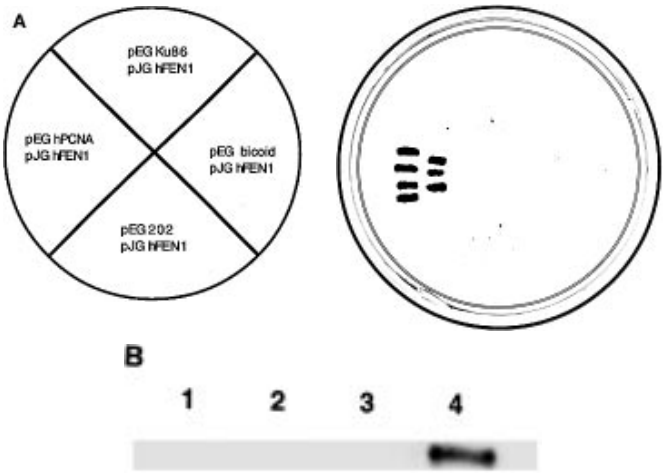

Figure 1. Genetic and physical interaction between human PCNA and human FEN-1. (**A**) Identity of matings (left), and growth of various matings (right) on agar plate of CM/-ura/-his/-leu/-trp/galactose/raffinose. (**B**) Binding of soluble recombinant human FEN-1 to protein G beads bearing anti-human c-myc (lanes 1 and 2) or anti-human PCNA (lanes 3 and 4) IgG_1 monoclonal antibodies. After the binding incubation, any binding was challenged with 300 mM NaCl (lanes 1 and 3) or 600 mM NaCl (lanes 2 and 4). Remaining protein was solubilized in denaturing SDS loading buffer, run on 8% PAGE, and immunoblotted to allow detection of human FEN-1 (see Materials and Methods).

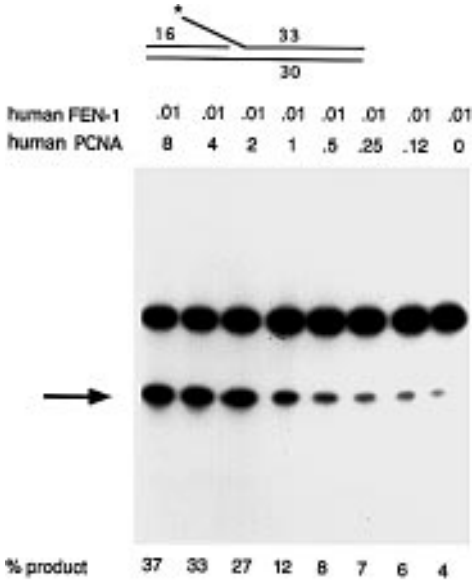

Figure 2. Human PCNA stimulates FEN-1 endonucleolytic cleavage at 5′ DNA flap structures. Flap substrate (0.01 pmol) [oligos SC 1(16mer), 3(30mer), 5(33mer)] was incubated with 0.01 pmol of human FEN-1 in a 15 µl total volume containing 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM NaCl, 0.5 mM β-mercaptoethanol, 500 µg/ml BSA. Human PCNA was added as indicated in pmol units of trimer. BSA is used to maintain the protein concentration constant. The arrow indicates the product, which is 20 nt in length. The top band is the flap strand (SC5), which is 33 nt in length. Controls in which PCNA was added in the absence of FEN-1 demonstrated the absence of any contaminating nuclease activities (not shown).

Oligonucleotides are CLH2 (24mer) GTAGGAGATGTCCCTTGATGAATT; CLH3 (16mer) CGAACCCAGATACGGC and AI4 (41mer) GGCCGTATCTGGGTTCGAATTCATCAAGGGACATCTCCTAC. In cases where we wanted to 3′ label the oligonucleotide downstream of a nick, we used HJ95, which is identical to CLH3 except that it is one nucleotide shorter at its 3′ end, permiting fill-in with the Klenow fragment of DNA pol I using labeled [α-^{32}P]dCTP. Control experiments showed no nuclease activity in all analogous experiments lacking FEN-1 (data not shown).

RESULTS

Isolation and identification of human FEN-1 cDNA in a two-hybrid analysis using the human PCNA gene

The modified yeast two-hybrid system was used to identify proteins encoded in the HeLa cell library that interact with human PCNA. 700 000 diploids were screened. The 86 largest colonies from galactose/-leu plates were gridded onto master plates and then tested on -ura/-his/-trp/X-gal/glucose plates and on galactose/raffinose plates to determine whether the leu+ phenotype is galactose-dependent and whether it correlates with galactose dependent β-galactosidase activity. Fifty-five colonies passed these tests. The plasmids were extracted by a rapid yeast minipreparation method. A polymerase chain reaction was used to amplify the cDNA with primers derived from the vector pJG4-5. The resulting PCR products were digested with *Hae*III and *Mbo*I to identify those that contain identical library plasmids. All of the plasmids sequenced were human FEN-1.

Specific interaction of human PCNA with human FEN-1

To prove that the human FEN1 specifically interacts with human PCNA, we transformed pJG-hFEN1 into the YPH499 strain and mated with human PCNA bait and other strains expressing unrelated baits, such as LexA-bicoid, LexA-Ku86 and the parent vector, pEG202. The diploid progeny were streaked onto a glucose/-ura/-his/-trp master plate, and then replica plated onto a galactose/-ura/-his/-trp/-leu plate. Interaction results in activation of the Lex A op-Leu2 reporter and growth in the absence of leucine. Human FEN-1 causes strong growth in the presence of the human PCNA bait, and weak growth in the presence of the Ku86 bait, bicoid bait, and pEG202 bait (Fig. 1A).

Physical interaction of human PCNA and human FEN-1

To test whether the strong genetic indication of an interaction could be documented physically, we bound human PCNA to protein G beads via an anti-human PCNA monoclonal antibody. As a control, we used anti-human c-myc antibodies. Soluble FEN-1 was then incubated with the beads and found to associate with the PCNA beads to a greater extent than to the anti-myc control beads (Fig. 1B). The interaction was stable at 300 mM NaCl and required 600 mM to elute. Hence, the physical interaction is detectable even at salt concentrations more than twice physiologic.

Human PCNA stimulates FEN-1 endonucleolytic activity at 5′ DNA flap structures

In order to test the functional significance of this interaction, we examined human FEN-1 cleavage at 5′-flap structures. In the various DNA metabolic transactions in which FEN-1 is known to function, DNA flap structures, nicks and gaps are involved. In the first structures examined, we tested for endonucleolytic activity. We found that human PCNA stimulates FEN-1 cleavage nearly 10-fold at 5′ DNA flaps (Fig. 2). Stimulation begins to be apparent at 12-fold molar excess of PCNA over FEN-1. The

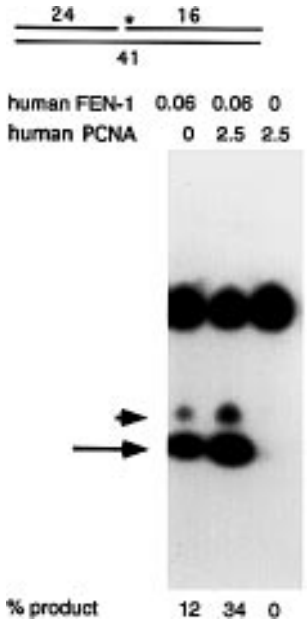

Figure 3. Human PCNA stimulates FEN-1 exonucleolytic activity at nicks. Assays were as described for the flap substrates (Fig. 2), except for adjustment to 10 mM Tris (pH 8), 5 mM $MgCl_2$ and 8 mM NaCl. In addition, the amount of human FEN-1 was 62.5 fmol, and the amount of human PCNA was 2.5 pmol. The oligonucleotides forming a nick configuration are CLH2, CLH3 and AI4. The long arrow shows the position of mononucleotides, and the short arrow shows the position of dinucleotides. BSA is used to maintain the protein concentration constant.

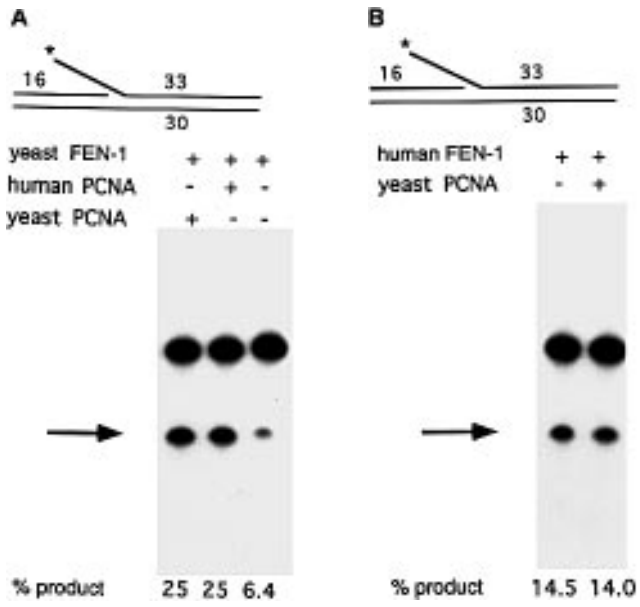

Figure 4. Human PCNA stimulates yeast FEN-1 activity but yeast PCNA does not stimulate human FEN-1 activity. (**A**) Ten fmol of substrate were treated with 50 fmol of yFEN-1 under the same conditions as in Figure 2, except in the presence or absence of yeast or human PCNA (5 pmol). BSA is used to maintain the protein concentration constant in the absence of PCNA. Other experiments show that the amount of product increases with increasing FEN-1 addition above what is used here (not shown). The arrow shows the position of the 20mer flap cleavage product. (**B**) Same as in (A), except with 50 fmol human FEN-1 in the presence or absence of 5 pmol yPCNA.

highest levels of stimulation occur at the highest stoichiometric ratios of the PCNA trimer to FEN-1 (800-fold). Equal concentrations of BSA in place of PCNA had absolutely no effect.

Human PCNA stimulates FEN-1 exonucleolytic activity at DNA nicks

FEN-1 is also a ds DNA exonuclease that is most active at nicks. Its activity and binding at gaps decreases with increasing gap size. We were interested in whether PCNA stimulates FEN-1 exonucleolytic activity at a DNA nick. We find that it does. However, the magnitude of the stimulation is somewhat smaller than for the endonucleolytic activity (Fig. 3). The products are predominantly mononucleotides but dinucleotides exonucleolytic products are also generated.

Human PCNA stimulates yeast FEN-1 activity but yeast PCNA does not stimulate human FEN-1 activity

Yeast PCNA stimulates yeast FEN-1. FEN-1 is 61% identical and 78% similar between yeast and human (3). PCNA is 30% identical between yeast and human. Hence, we were interested in the extent to which these were interchangeable. We find that human PCNA can stimulate yeast FEN-1 to an extent that is equivalent to human FEN-1 (Fig. 4A). However, yeast PCNA does not stimulate human FEN-1 (Fig. 4B).

Does PCNA broaden the substrate specificity of FEN-1?

FEN-1 acts on substrates with 5′ flaps as an endonuclease and at nicks or recessed 5′ DNA ends as a 5′→3′ exonuclease. We were interested in whether substrates that lack a 5′ flap or a recessed 5′ end could function as FEN-1 substrates when PCNA is provided. We tested heterologous DNA loops (Fig. 5). At the site where the loop departs from double-stranded DNA conformation, these substrates share some features with 5′ flap structures and pseudo-Y branched DNA structures. Specifically, if the heterologous loop were nicked at its upstream attachment point to the double-stranded DNA, then it would become the 5′ flap structure optimal for FEN-1 cutting. The only difference then is that there is no free 5′ flap terminus. When tested, FEN-1 is able to distinguish the heterologous loop from the 5′ flap structure, and it does not cut the loop. We reasoned that in the cell, FEN-1 is acting in the presence of PCNA. We wondered if this failure to cut such a similar structure could be overcome by PCNA stimulation. We find that PCNA does not help FEN-1 to cleave heterologous loops. Hence, a free 5′ terminus is necessary for FEN-1 cleavage.

The bubble structure (right side of Fig. 5) is similar to a branched pseudo-Y structure but with the two single-stranded arms annealed at there most distal points. We find that FEN-1 is sensitive to this difference also and does not cleave it. As was the case for heterologous loops, PCNA is unable to stimulate FEN-1 to overcome this structural requirement for a free 5′ terminus (Fig. 5).

Do FEN-1 and PCNA load from different portions of branched DNA substrates?

PCNA loads onto linear DNA substrates by diffusion onto the double-stranded DNA termini (19). For FEN-1 loading, we wondered if the 5′ flap had to be single-stranded over its entire length, only near the site of cleavage, or only at its most 5′ terminal end. *Escherichia coli* polymerase I has a 5′→3′ nuclease domain that has very similar endonucleolytic and exonucleolytic

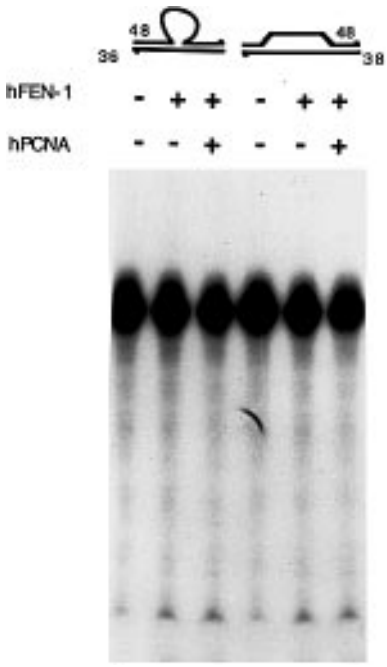

Figure 5. Human FEN-1 fails to cleave at heterologous loops and DNA bubble structures with or without PCNA. The left three lanes show the analysis of a heterologous loop and the right three lanes show analysis of DNA bubble. The reaction total volume was 15 µl, the amount of substrate was 10 fmol, the amount of FEN-1 where used was 50 fmol, and the amount of PCNA trimer where used was 2.8 pmol. The label was at the 5′-P of the 48mer. The oligonucleotides were gel purified. The 48mer is MY2, the 36mer is HJ90 and the 38mer is HJ91. Human FEN-1 and PCNA are abbreviated as hFEN-1 and hPCNA in this and the remaining figures. Controls done in parallel demonstrate that the FEN-1 preparation is active on standard flap substrates (see Fig. 6). Except for a small amount of liberated 5′-P label release to generate mononucleotide, there are no lower molecular weight cleavage products, indicating that there is no cleavage of the heterologous loop and DNA bubble structures.

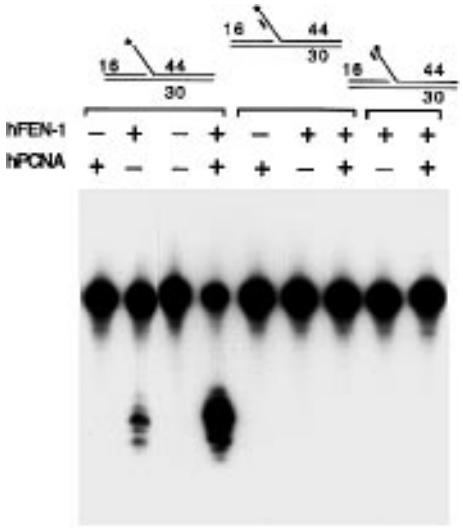

Figure 6. Human FEN-1 cleavage is prevented by double-stranded regions along the otherwise single-stranded DNA flap. In all lanes, the 44mer is HJ86 (labeled at its 5′-P), the 16mer is HJ43 and the 30mer is HJ41. A fourth oligonucleotide, shown in bold, is a 15mer; it is HJ87 in the case where it is positioned at the most distal end of the DNA flap (lanes 3 and 4 from the left), and it is HJ88 where it is annealed in the middle of the DNA flap (lanes 5 and 6 from the left). The total reaction volume was 15 µl, the amount of substrate was 10 fmol, the amount of FEN-1 was 50 fmol, the amount of PCNA was 2.8 pmol. The substrate used in Figures 2 and 4 generates one predominant labeled cleavage product that begins one nucleotide into the double-stranded region adjacent to the elbow of the flap strand; in contrast, this substrate generates one predominant but several larger and smaller cleavage products, and overall, this substrate cleaves with lower efficiency. These qualitative and quantitative variations appear to be a function of the sequence at the elbow of the flap strand (i.e., at the junction of the single-stranded and the double-stranded portions of the flap strand), as we have described previously (1).

properties to FEN-1. In fact, FEN-1 is the counterpart of this domain for eukaryotic polymerases (1,7,20,21). There is significant amino acid homology between the FEN-1 and *E.coli* pol I 5′ nuclease domain (24% overall and 52% in a selected 63 aa region) (4). The 5′ nuclease of *E.coli* pol I appears to slide down the single-stranded flap until it reaches the branch point where it cleaves. Double-stranded character along this 5′ flap is known to prevent *E.coli* pol I from acting (22). One might assume that FEN-1 would function in a similar fashion. However, *S.cerevisiae* RAD2 is a member of the highly conserved FEN-1 family of nucleases (3), and it does not require a free 5′ terminus to cut at the corresponding position as FEN-1 (23).

We wondered which mechanism of loading FEN-1 uses and whether the manner of loading is modified by PCNA. In order to examine this issue, we generated 5′ flap structures as we have described previously, and we tested the ability of oligonucleotides annealed to various locations along this flap to prevent FEN-1 action (Fig. 6). In these cases, the flap is 30 nt long. The oligonucleotide being annealed to the flap is 15 nt. When we anneal the 15 nt oligonucleotide to the most distal portion of the flap, cleavage is entirely blocked. When we anneal in the middle of the flap 7 nt from the 5′ terminus and leaving 8 nt of single-stranded character before the branch point, cleavage is also entirely blocked. We have done the same experiments with a 65 nt flap and a 15 nt oligonucleotide annealed in the middle of its length. This leaves 25 nt of single-stranded DNA on each side, with the duplex portion located in the middle of the flap. We find that cleavage is still blocked (not shown). Hence, FEN-1, like the 5′ nuclease domain of *E.coli* pol I (22) and unlike *S.cerevisiae* Rad2 (23), requires fully single-stranded character between the 5′ terminus and the branch point.

Rad2 is able to circumvent the requirement for a fully single-stranded region 5′ of the cleavage site, perhaps by binding more tightly to the branch point (23). If PCNA were to stabilize FEN-1 sufficiently at its cleavage site, we considered that it might convert FEN-1 to a Rad2-type of loading, thereby allowing it to circumvent the requirement for a fully single-stranded 5′ flap. However, we find that this is not the case (Fig. 6). Hence, the physiologic mechanism of FEN-1 loading onto its substrates appears to require an energy-independent (hence, nondirectional) tracking along the entire length of the single-stranded region. This also means that FEN-1 and PCNA load from different portions of the substrate and meet at the branch point.

Interactions between FEN-1 and the 5′ terminus of DNA strands at flaps and nicks

The data above indicate that FEN-1 loads at DNA flaps by energy-independent tracking from the 5′ terminus to the branch point rather than by binding to the terminus and relying on collisional interaction with the DNA branch point. Given that FEN-1 loads from the 5′ terminus, we considered what structural features are critical for FEN-1 recognition there. We know that

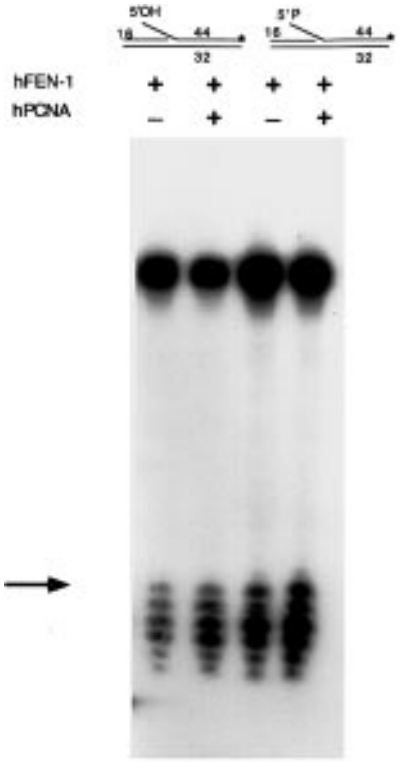

Figure 7. Human FEN-1 activity shows only limited sensitivitiy to the phosphorylation status of the single-stranded DNA flap with or without PCNA. The total reaction volume was 15 µl, the amount of substrate was 10 fmol, the amount of FEN-1 was 25 fmol, and the amount of PCNA trimer where used was 5.6 pmol. The oligonucleotides were as follows: 44mer, HJ86; 16mer, HJ43; and 32mer, HJ89. The 44mer (flap strand) was labeled at the 3′ end by Klenow fill-in of two nucleotides, making it a 46mer after labeling. The 5′ end of this flap strand was either left unphosphorylated or was phosphorylated with unlabeled ATP and polynucleotide kinase. It is important to note that FEN-1, in addition to its endonucleolytic activity, has 5′→3′ exonucleolytic activity, [as illustrated in Fig. 3 and previously by us (1) and others (2,8,20,21)]. Therefore, with 3′-end-labeling of the flap strand, it is expected that one will see not only an initial set of endonucleolytic cleavage products (as in Fig. 6), but also the secondary products due to exonucleolytic shortening of these 3′-end-labeled strands. The exonucleolytic shortening can progress all the way until the flap strand oligonucleotide dissociates because it becomes so short that it melts off at the temperature of these enzyme reactions. The arrow indicates the position of the largest of the range of cleavage products (15mer). The ratio of product (integrated over the entire range of product bands) divided by (substrate + product) was determined by phosphorimager quantitation; these are 2.1, 10.2, 3.3 and 12.4% from left to right.

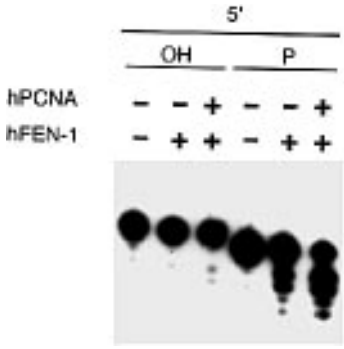

Figure 8. Human FEN-1 exonucleolytic activity demonstrates limited sensitivity to the phosphorylation status at a DNA nick. The total reaction volume was 15 µl, the amount of substrate was 10 fmol, the amount of FEN-1 was 0.1 pmol, and the amount of PCNA trimer where used was 5.6 pmol. The oligonucleotides were as follows: 24mer, CLH2; 15mer, HJ95; and 41mer, AI4. The 15mer was labeled at the 3′ end by Klenow fill-in of one nucleotide. The 5′ end of this now 16mer was either left unphosphorylated or was phosphorylated with unlabeled ATP and polynucleotide kinase prior to being annealed. Reaction conditions were 30°C for 30 min in 10 mM Tris (pH 8), 5 mM $MgCl_2$, 0.5 mM β-mercaptoethanol. The top band is substrate and cleavage products migrate at the faster mobilities. The phosphorylated substrate has a slightly faster mobility than the unphosphorylated substrate. The ratio of product divided by (substrate + product) was determined by phosphorimager quantitation; these are 0, 5, 8; 0, 16 and 66% from left to right.

FEN-1 is insensitive to the base at the 5′ terminal flap position (1). We wondered if FEN-1 is sensitive to the phosphorylation status of the 5′ terminus. To test this, we used a flap substrate similar to that described in Figure 6. We radiolabeled the 3′ end of the flap strand and then phosphorylated the 5′ flap terminus with cold phosphate or left it as a 5′-OH (Fig. 7). We find that the percentage of substrate converted to product is similar regardless of the 5′ flap terminus phosphorylation status, though the phosphorylated form shows ~1.2–1.5-fold greater cleavage. The arrow indicates cleavage at the branch point, one nucleotide into the double-stranded region. The lower cleavage bands represent FEN-1 exonucleolytic processing of the gapped product left after the flap cleavage. One can see from the exonucleolytic processing that the endonucleolytic and exonucleolytic activities are not dramatically dissimilar under these ionic conditions. [If the endonucleolytic activity were much greater, then one would see accummulation of the 15 nt flap cleavage product. If the exonucleolytic activity were much greater, then one would see this 15 nt product rapidly chased predominantly to mononucleotides. The ratio of the endonucleolytic and exonucleolytic activities can vary dramatically, depending on the ionic conditions (1).] Therefore, FEN-1 recognizes the 5′ terminus and requires it for loading, yet it has only limited sensitivity to the base and the 5′ phosphate regions of the terminus.

In considering the similarities and differences in how FEN-1 loads onto substrates in its endo- and exonucleolytic modes, we wondered if the exonucleolytic activity is also insensitive to the 5′ phosphorylation status. We used a substrate identical to that described in Figure 3, except that we replaced the 16mer used in that study with a 15mer (HJ95) and then used the Klenow fragment of DNA pol I to label the 3′ end with radioactive [α-^{32}P]dNTP. (Prior to this step, we either kinased the 5′ terminus with cold phosphate or left it as a 5′-OH.) We find that the phosphorylated form is cleaved ~2.7–8-fold more efficiently under the reaction conditions of this study (Fig. 8). (As indicated in Figure 3, PCNA stimulates FEN-1 exonucleolytic activity to some extent.) Hence, FEN-1 requires a free 5′ terminus for branched DNA substrate loading. However, it shows only limited sensitivity to the phosphorylation status of the 5′ terminus at which it loads. This limited sensitivity is greater for FEN-1 loading at a DNA nick. This may have its basis in isomerization of the nicked substrate to a flap configuration (see Discussion).

DISCUSSION

Human FEN-1 and PCNA functional interaction in DNA metabolism

In a two-hybrid search using human PCNA, we found that human FEN-1 is detected as the predominant interactor. However, does

this binding reflect a functional interaction in the nucleus? Based on the enzymatic stimulation of FEN-1 by PCNA, we infer that it does. PCNA stimulates the endonucleolytic activity at 5′ DNA flap structures. It also stimulates the exonucleolytic activity of FEN-1 at DNA nicks.

Human FEN-1 in the absence of PCNA is markedly inhibited by increasing concentrations of monovalent salt. The binding between FEN-1 and PCNA results in substantially more endonucleolytic and exonucleolytic activity than would otherwise occur at these salt concentrations (1). Therefore, the functional binding with PCNA explains how FEN-1 can be active at higher salt concentrations.

In DNA replication, PCNA is localized to the 3′-OH of the primer DNA strand by RF-C (19). At the replication fork, there is no free ds DNA end onto which PCNA can diffuse. Therefore, RF-C binds to the 3′-OH and then catalyzes PCNA assembly from monomer to trimer around the axis of the ds DNA. It is reasonable that PCNA would then bind FEN-1 because this nuclease is required for Okazaki fragment processing. Specifically, RNase H degrades the RNA primer of each lagging strand down to a point where the last ribonucleotide remains (20,21). Pol δ extends the upstream strand to a nick. At that point, either of two pathways achieve the same result. In one pathway, the polymerase extends further to displace the downstream strand. This generates a 5′ flap structure, which FEN-1 can then cleave endonucleolytically. In the second pathway, FEN-1 functions exonucleolytically, cleaving the last ribo- and deoxyribonucleotide off exonucleolytically as a dinucleotide. The localization of FEN-1 to the replication fork by PCNA and the stimulation of FEN-1 activity by PCNA are consistent with their functions in replication.

We start to see endonucleolytic stimulation of FEN-1 by PCNA at a molar ratio of 12 PCNA trimer molecules per each FEN-1 molecule. The stimulation continues for as far as we carried out the titration, which was a ratio of 800 PCNA trimers per FEN-1. In the absence of RF-C, PCNA loads onto DNA as a toroid diffuses onto the end of a rod. This is a diffusion-limited process that has obvious steric requirements. This type of PCNA loading is much less efficient than that catalyzed by RF-C. The requirement for stoichiometric excess of PCNA under conditions for diffusional loading has been extensively documented (19). Hence, it is not surprising that FEN-1 stimulation by PCNA would require a large stoichiometric excess and that it would increase progressively with increasing PCNA.

It is noteworthy that this PCNA–FEN-1 interaction extends from yeast (15) to humans. The features of the stimulation are similar overall. However, there is one interesting difference that we have noted. We did not detect stimulation of yeast FEN-1 exonucleolytic activity at linear ds nick sites by yeast PCNA even in the presence of yeast RF-C. We could only detect stimulation of yeast FEN-1 by PCNA and RF-C at nick sites on circular M13 molecules. We reasoned that this was because the PCNA molecules diffused off of the linear DNA molecules too quickly. For human FEN-1 and PCNA, we do detect some stimulation of FEN-1 exonucleolytic activity at simple nicked ds linear DNA molecules (this expands the nick to a gap). Hence, the residence time of the human PCNA may be longer, allowing for stimulation of FEN-1. This could be due to a difference between yeast and human PCNA or a difference between yeast and human FEN-1. The human PCNA may diffuse across the nicked linear DNA slower or the human FEN-1 may bind more tightly to the nick site than the yeast FEN-1.

In addition to replication, the interaction between FEN-1 and PCNA may have broader implications in DNA metabolism. FEN-1 mutants are adversely affected in mismatch DNA repair (9). The specific enzymatic steps in eukaryotic mismatch repair are not yet sufficiently well-defined to permit specification of the precise step for FEN-1 activity or the involvement of PCNA. However, the result is marked instability of dinucleotide repeats, just as is the case for the other mismatch repair components. In another DNA repair process, nucleotide excision repair, PCNA is known to be important (24,25). Although FEN-1 itself has not been shown to be involved in this reaction, RAD2 (XP-G in higher eukaryotes) is absolutely required (26). Based on the involvement of PCNA in nucleotide excision repair and the presence of homology between FEN-1 and RAD2, it is possible that PCNA may also interact with RAD2 to facilitate its loading and thereby excision of damaged nucleotides. Though RAD2 has not specifically been shown to be the target of PCNA stimulation in nucleotide excision repair, the work of Nichols and Sancar (1992) makes it clear that PCNA is stimulating some form of nucleolytic activity in excision repair. Thus, there may be a common theme in various aspects of DNA metabolism, in addition to DNA replication, in which a processivity factor stimulates a structure-specific nuclease in processing nicked and branched DNA intermediates. We are currently investigating the role of FEN-1 in DNA end joining (double-strand break repair). In this case, PCNA could diffuse onto the free ds DNA end and stimulate FEN-1 action at a nick, gap or flap. The fact that PCNA stimulation of FEN-1 exonucleolytic activity can occur on linear substrates in the absence of RF-C in human cells is potentially important in DNA end joining. We did not find such stimulation in yeast for this particular substrate configuration. It is interesting that in yeast the predominant mode of DNA end joining is different from mammalian cells and procedes by homologous recombination involving the homologous chromosome as a template.

Substrate structural features and the human FEN-1–PCNA interaction

PCNA trimer loads diffusionally onto the end of double-stranded DNA (19). Monomeric PCNA is unable to stimulate FEN-1 (15). Therefore, the assembled trimer is important for FEN-1 stimulation. In the absence of a DNA terminus, RF-C catalyzes the assembly of PCNA monomers into trimers at a 3′-OH (19).

Based on the failure of FEN-1 to cleave at DNA bubbles and heterologous loops, we inferred that a free 5′ terminus is important for FEN-1 substrate recognition. We confirmed this by annealing oligonucleotides at various positions along the single-stranded DNA flap. Whether annealed to the most 5′ portion of the flap or along the middle of the flap, FEN-1 endonucleolytic activity was eliminated. Therefore, it appears that FEN-1 tracks along the single-stranded DNA from the 5′ terminus to the cleavage point.

Given these observations, it appears that FEN-1 and PCNA may load from different points of a branched DNA substrate (Fig. 9A), with PCNA loading from one end and FEN-1 loading along the single-stranded 5′ flap. The simplest model is that PCNA stabilizes FEN-1 at the branch point, increasing its residence time there, this being the basis for PCNA stimulation of FEN-1 activity.

Within the *E.coli* pol I/FEN-1 nuclease family, there appear to be two mechanims of loading. One mechanism is by energy-independent diffusion down the single-stranded flap (Fig. 9A). The second is direct binding to a DNA branch point. The first mechanism appears to be used by *E.coli* pol I and by FEN-1. The

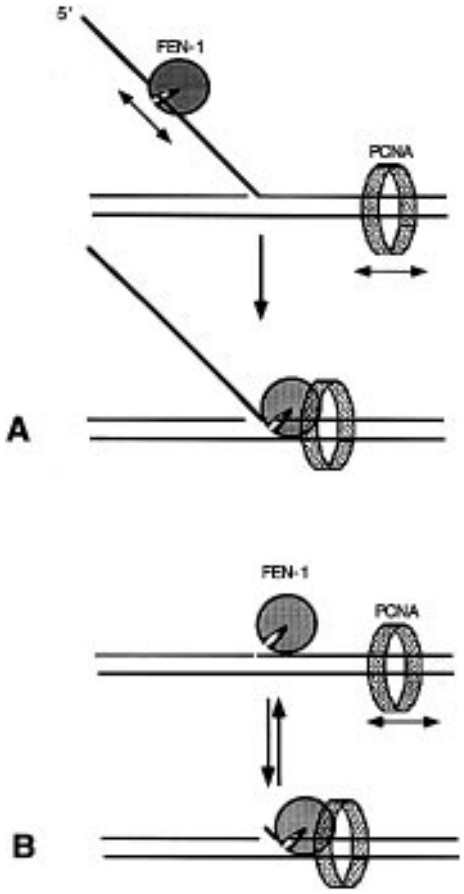

Figure 9. Model for FEN-1 and PCNA loading onto DNA substrates. (**A**) FEN-1 tracks along the single-stranded 5′ flap of a branched DNA structure. Without this 5′ single-stranded DNA entry point, FEN-1 is unable to localize to the DNA branch point. Double-stranded interruptions of the single-stranded character at any point along the flap prevent FEN-1 from reaching the branch point and cleavage is prevented. PCNA loads onto double-stranded DNA termini by diffusion. (PCNA is also able to load onto long linear DNAs and circular DNA by PCNA monomer assembly into trimers catalyzed by RF-C and ATP.) PCNA stimulates FEN-1 by increasing the residence time at the branch point. (**B**) FEN-1 action at a nick may occur by initial recognition and binding of the breathing pseudo-flap form of the nick. By this model, cleavage at nicks would actually be flap cleavage that occurs as the nick breathes, forming a transient 5′ flap.

second appears to be used by *S.cerevisiae* Rad2. The only substantial sequence difference between yeast FEN-1 and Rad2 is what we have previously termed the S region, which is located between two of the remaining three highly conserved regions. It may be that this region increases the affinity of Rad2 for the branch point, making tracking down the single-stranded tail unnecessary.

Separability of the endonucleolytic and exonucleolytic activities of FEN-1

Are the endonucleolytic and exonucleolytic activities of FEN-1 related or distinct activities? One way of answering this question is by considering the similarities and differences in binding and cleavage of exo- versus endonucleolytic substrates. Both activities show little sensitivity to the base at the 5′ most position at the flap or nick. Both FEN-1 endo- and exonucleolytic substrate binding and cutting are stimulated by an upstream oligonucleotide (flap adjacent strand or primer) (27). This is also the case for *E.coli* pol I (28).

Although a 5′ OH terminus is a good substrate for FEN-1 loading onto a 5′ flap substrate, it serves as a poor substrate when part of a double-stranded DNA nick. The electrostatic repulsion by the terminal phosphate may allow increased breathing of the substrate into a pseudo-flap configuration, providing the active form of the substrate for FEN-1 (Fig. 9B). Such an explanation would indicate a single active site and a single mechanism of loading of FEN-1 onto the 5′ ss-DNA terminus of the flap or pseudo-flap configuration of the nick. Consistent with this model are our observations that optimal activity at a nick requires very low Mg^{2+} and monovalent salt, which destabilize base-pairing, whereas flap cleavage is optimal at moderate Mg^{2+} and monovalent salt concentrations. Furthermore, we have previously shown that one nucleotide flaps are efficient substrates (1).

ACKNOWLEDGEMENTS

This work was supported by NIH grants CA51105 and GM43236 to M.R.L. and GM32431 to P.M.J.B. M.R.L. is a Leukemia Society of America Scholar.

REFERENCES

1 Harrington,J.J. and Lieber,M.R. (1994) *EMBO J.* **13**, 1235–1246.
2 Murante,R., Huang,L. Turchi,J. and Bambara,R. (1994) *J. Biol. Chem.* **269**, 1191–1196.
3 Harrington,J.J. and Lieber,M.R. (1994) *Genes Dev.* **8**, 1344–1355.
4 Robins,P., Pappin,D., Wood,R.D. and Lindahl,T. (1994) *J. Biol. Chem.* **269**, 28535–28538.
5 Reagan,M.S., Pittenberger,C., Siede,W. and Friedberg,E.C. (1995) *J. Bacteriol.* **177**, 364–371.
6 Sommers,C., Miller,E., Dujon,B., Prakash,S. and Prakash,L. (1995) *J. Biol. Chem.* **270**, 4193–4196.
7 Waga,S., Bauer,G. and Stillman,B. (1994) *J. Biol. Chem.* **269**, 10923–10934.
8 Ishimi,Y., Claude,A., Bullock,P. and Hurwitz,J. (1988) *J. Biol. Chem.* **263**, 19723–19733.
9 Johnson,R., Kovvali,G., Prakash,L. and Prakash,S. (1995) *Science* **269**, 238–240.
10 Murante,R.S., Rust,L. and Bambara,R.A. (1995) *J. Biol. Chem.* **270**, 30377–30383.
11 Aboussekhra,A. and Wood,R.D. (1994) *Curr. Opin. Genet. Dev.* **4**, 1–24.
12 Krishna,T., Kong,X., Gary,S., Burgers,P.M. and Kuriyan,J. (1994) *Cell* **79**, 1233–1243.
13 Bauer,G. and Burgers,P.M.J. (1988) *Biochim. Biophys. Acta* **951**, 274–279.
14 Li,R., Waga,S., Hannnon,G., Beach,D. and Stillman,B. (1994) *Nature* **371**, 534–537.
15 Li,X., Li,J., Harrington,J., Lieber,M.R. and Burgers,P.M.J. (1995) *J. Biol. Chem.* **270**, 22109–22112.
16 Zervos,A., Gyuris,J. and Brent,R. (1993) *Cell* **72**, 223–232.
17 Fien,K. and Stillman,B.(1992) *Mol. Cell Biol.* **12**, 155–163.
18 Bendixen,C., Gangloff,S. and Rothstein,R. (1994) *Nucleic Acids Res.* **22**, 1778–1779.
19 Burgers,P.M. and Yoder,B.L. (1993) *J. Biol. Chem.* **268**, 19923–19936.
20 Turchi,J.J. and Bambara,R.A. (1993) *J. Biol. Chem.* **268**, 15136–15141.
21 Goulian,M., Richards,S.H., Heard,C.J. and Bigsby,B.M. (1990) *J. Biol. Chem.* **265**, 18461–18571.
22 Lyamichev,V., Brow,M. and Dahlberg,J.E. (1993) *Science* **260**, 778–783.
23 Habraken,Y., Sung,P., Prakash,L. and Prakash,S. (1995) *J. Biol. Chem.* **270**, 30194–30198.
24 Shivji,K., Kenny,M. and Wood,R. (1992) *Cell* **69**, 367–374.
25 Nichols,A.F. and Sancar,A. (1992) *Nucleic Acids Res.* **20**, 2441–2446.
26 Guzder,S.N., Habraken,Y., Sung,P., Prakash,L. and Prakash,S. (1995) *J. Biol. Chem.* **270**, 12973–12976.
27 Harrington,J.J. and Lieber,M.R. (1995) *J. Biol. Chem.* **270**, 4503–4508.
28 Cozzarelli,N.R., Kelly,R.B. and Kornberg,A. (1969) *J. Mol. Biol.* **45**, 513–531.