The RAD2 Domain of Human Exonuclease 1 Exhibits 5' to 3'
Exonuclease and Flap Structure-specific Endonuclease Activities*

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The RAD2 family of nucleases includes human XPG (Class I), FEN1 (Class II), and HEX1/hEXO1 (Class III) proteins. These proteins exhibit a blend of substrate specific exo- and endonuclease activities and contribute to repair, recombination, and/or replication. To date, the substrate preferences of the EXO1-like Class III proteins have not been thoroughly defined. We report here that the RAD2 domain of human exonuclease 1 (HEX1-N2) exhibits both a robust 5' to 3' exonuclease activity on single- and double-stranded DNA substrates as well as a flap structure-specific endonuclease activity but does not show specific endonuclease activity at 10-base pair bubble-like structures, G/T mismatches, or uracil residues. Both the 5' to 3' exonuclease and flap endonuclease activities require a divalent metal cofactor, with Mg2+ being the preferred metal ion. HEX1-N2 is 3-fold less active in Mn2+-containing buffers and exhibits <5% activity in the presence of Co2+, Zn2+, or Ca2+. The optimal pH range for the nuclease activities of HEX1-N2 is 7.2–8.2. The specific activity of its 5'-to-3'exonuclease function is 2.5-7-fold higher on blunt end and 5'-recessed double-stranded DNA substrates compared with duplex 5'-overhang or single-stranded DNAs. The flap endonuclease activity of HEX1-N2 is similar to that of human flap endonuclease-1, both in terms of turnover efficiency (kcat) and site of incision, and is as efficient (kcat/Km) as its exonuclease function. The nuclease activities of HEX1-N2 described here indicate functions for the EXO1-like proteins in replication, repair, and/or recombination that may overlap with human flap endonuclease-1.

Genomic integrity is maintained by specific cellular responses to genetic damage, such as DNA repair and cell cycle checkpoint controls (1, 2). In the past several years, it has become increasingly evident that defects in repair can lead to increased genome instability and an elevated susceptibility to human disease, most notably cancer (3). Furthermore, many DNA repair systems share overlapping responsibilities in ameliorating DNA damage.

Central to most DNA repair processes is a nucleolytic step that is required for the immediate or eventual removal of the damage (1, 2). Nucleases are also essential for the successful and efficient execution of replication and recombination. Many of the nucleolytic activities act in a variety of structural frameworks, ranging from site-specific (e.g. abasic endonucleases) to structure-specific (e.g. flap endonuclease) to nonspecific (e.g. DNase I).

The RAD2 family of nucleases is conserved from phage to humans (4, 5). Many of these proteins feature a range of nuclease activities that contribute to DNA repair, replication, and recombination. The RAD2 family members maintain a core nuclease domain comprised of the conserved N-terminal and internal elements and are divided into three classifications based upon amino acid sequence identity, positioning of the N-terminal and internal domains, and their substrate specificity.

RAD2 Class I consists of the XPG1-like proteins that operate in nucleotide excision repair to incise the target strand to the 3'-side of the bubble-like, damage-containing structure formed as an intermediate during the repair event (6). This incision, coupled with incision to the 5'-side of the lesion by the excision repair cross-complementation group 1/XPF heterodimer, permits the removal of the damage-containing DNA segment. Mutations in the XPG gene have been found to lead to the human disorder xeroderma pigmentosum (XP), which is characterized by a hypersensitivity to sunlight and an increased likelihood of developing skin cancer (7). In some patients, XPG mutations have been associated with Cockayne’s syndrome, a disease stemming from a defect in transcription-coupled repair and characterized by neurodegeneration and a shortened life span (8).

The RAD2 Class II family is comprised of the FEN1-like proteins. These enzymes exhibit a flap-specific endonuclease activity for bifurcated DNA structures produced by DNA polymerase strand displacement or as intermediates during DNA recombination (9, 10). The 5' nuclease function of FEN1 is also responsible for the excision of Okazaki fragments, which are used to prime DNA synthesis (11, 12). Genetic studies have found that deletion of FEN1 (rad27) in yeast results in a marked sensitivity to the alkylating agent methylmethane sulfonate, modest sensitivity to ultraviolet light, increased spontaneous chromosome instability, and temperature-sensitive viability (13–15). The nuclease function of FEN1 also appears to be involved in preventing the expansion/contraction of DNA repeat elements, a phenomenon associated with cancer and the manifestation of neurodegenerative diseases (16–19).

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The RAD2 Class III members consist of the EXO1-like enzymes found in yeast, fly, and mammals (20–26). To date, the substrate specificity of these proteins remains largely uncharacterized, and little is known about the in vivo function of the mammalian proteins. Based upon biochemical studies, the expression patterns of the EXO1 genes, the protein-protein interaction of EXO1 with MSH2, the defects of the yeast exo1 mutants, and functional complementation studies, the EXO1 proteins appear to play a role in DNA recombination, mismatch repair, and perhaps in replication.

Successful completion of DNA replication requires excision of the Okazaki RNA fragments used to prime DNA synthesis (27). In *Escherichia coli*, this processing activity is an intrinsic property of the 5′ nucleosome domain of DNA polymerase I (PolI) (28). *E. coli* polA (the gene encoding PolI) null mutants are inviable when grown on rich medium, a condition thought to promote rapid growth, cell division, and imposing chromosomal replication (29). However, these mutants are viable on minimal medium, presumably due to the reduced replication burden. The ability of *polA* mutants to grow on rich medium was restored by expressing either the 5′ nucleosome domain or the polymerase fragment of PolI independently, suggesting that either activity can provide sufficient function for cell division. A number of the viable *polA* mutants exhibit increased sensitivity to ultraviolet light and methylating agents, a temperature-sensitive growth phenotype, hyper-recombination, and/or synthetic lethality when combined with mutations in recombination genes, such as *recA* (30–32). In eukaryotes, the major 5′-processing activity for RNA and DNA bifurcated structures is the property of the flap endonuclease FEN1 (33).

As noted above, yeast FEN1 mutants display a similar array of defects to those observed for the *PolA* bacterial mutants. Moreover, the 5′ nucleosome function of FEN1 is not essential for viability (13, 14, 34, 35), as null mutations in *Saccharomyces cerevisiae rad27* or *Schizosaccharomyces pombe rad2* confer only conditional lethality (i.e. growth observed at 30 °C but not at 37 °C). This finding indicates that an alternative nuclease activity exists at permissive temperatures. The observation that *exo1* rad27 double mutants are inviable (21), unlike the corresponding single mutants, suggested that these two RAD2 family members maintained synergistic functions. Moreover, overexpression of the yeast EXO1 or human EXO1 protein was found to complement the defects of rad27 mutants (21, 26, 35), further suggesting a common role. Qi and colleagues (35) reported that EXO1 exhibits RNase H activity in vitro and propose that the lethality of the *exo1* rad27 double mutants is the result of the complete inability to remove initiator RNAs during lagging strand DNA synthesis. To explore further the relationship of these two proteins, we sought to define the biochemical properties and substrate preferences of the human EXO1 enzyme. Studies presented within indicate that, in addition to RNA primer removal activity (35), the EXO1-like proteins display a FEN1-like flap endonuclease activity, expanding the breadth of their potential functional complementarity and raising new issues regarding the biological synergy of these two RAD2 family members.

### EXPERIMENTAL PROCEDURES

#### Materials—-[γ-32P]ATP, [α-32P]dCTP, and Klenow-(exo)

*Oligonucleotide primers (5′-gga tgc cat ggg aat tca agg cct-3′) and various 3′-primers (see Table I for sequences and Fig. 1 for positioning).* The amplified fragment was purified, digested, and subcloned into the *Novo* and *BamHI* restriction sites of PET-11d (Novagen, Madison, WI). The resulting plasmids (pET11d-HEX1-N1, -N2, -N3, -N4, or -N5) were transformed into *E. coli* BL21 (DE3) for protein expression. Transformed colonies were harvested and inoculated into 2 liters of LB broth containing 50 μg/ml ampicillin and were grown at 37 °C. The culture was induced by adding IPTG to a final concentration of 0.5 mM when the absorbance of medium was 0.6 to 600 nm, and gene induction was carried out for 4 h at 37 °C. The cells were harvested by centrifugation at 3,000 × g for 10 min at 4 °C, washed with 30 ml of 1× phosphate-buffered saline, resuspended in 30 ml of buffer A (10 mM MOPS, pH 6.8, 100 mM KCl, 5% glycerol) containing 0.5% Triton X-100 and 1 mM DTT, and sonicated 3 times at 10 s with a Misonic XL sonicator (Farmington, NY). Debris was removed by centrifugation at 20,000 × g for 20 min, and the insoluble (pellet) and soluble (supernatant) materials were analyzed by SDS-polyacrylamide gel electrophoresis using either Coomassie Blue staining or Western blotting with polyclonal antibodies raised against a peptide region in the RAD2 domain (not shown).

*Purification of the Human Exonuclease 1 RAD2 Domain and Human FEN1*—To generate the RAD2 nuclease domain of HEX1, PCR was carried out using Pfu polymerase with a 5′-primer (5′Nco) and various 3′-primers (see Table I for sequences and Fig. 1 for positioning). The amplified fragment was purified, digested, and subcloned into the *Novo* and *BamHI* restriction sites of PET-11d (Novagen, Madison, WI). The resulting plasmids (pET11d-HEX1-N1, -N2, -N3, -N4, or -N5) were transformed into *E. coli* BL21 (DE3) for protein expression. Transformed colonies were harvested and inoculated into 2 liters of LB broth containing 50 μg/ml ampicillin and were grown at 37 °C. The culture was induced by adding IPTG to a final concentration of 0.5 mM when the absorbance of medium was 0.6 to 600 nm, and gene induction was carried out for 4 h at 37 °C. The cells were harvested by centrifugation at 3,000 × g for 10 min at 4 °C, washed with 30 ml of 1× phosphate-buffered saline, resuspended in 30 ml of buffer A (10 mM MOPS, pH 6.8, 100 mM KCl, 5% glycerol) containing 0.5% Triton X-100 and 1 mM DTT, and sonicated 3 times at 10 s with a Misonic XL sonicator (Farmington, NY). Debris was removed by centrifugation at 20,000 × g for 20 min, and the insoluble (pellet) and soluble (supernatant) materials were analyzed by SDS-polyacrylamide gel electrophoresis using either Coomassie Blue staining or Western blotting with polyclonal antibodies raised against a peptide region in the RAD2 domain (not shown).

For purification of HEX1-N2, the supernatant was applied to a cation exchange Bio-Rad S2 column attached to a BioLogic chromatography system (Bio-Rad). The protein was eluted with a 0.1 to 1.0 M KCl linear gradient in buffer A, concentrated with a Centriprep 30 filter device, and further separated by gel filtration (Bio-Silect SEC125–5 column) in 10 mM HEPES, pH 7.5, 100 mM KCl, 5% glycerol. Under these conditions HEX1-N2 fractionated as a monomer. Fractions containing HEX1-N2 were identified by Western blot analysis using HEX1-specific polyclonal antibodies (not shown), and the sample purity (>95%) was examined by SDS-polyacrylamide gel electrophoresis and Coomassie Blue staining (Fig. 1) or silver staining (not shown). Typically, 100 μg of HEX1-N2 protein was generated from a 2-liter bacterial culture.
PCR reactions were performed under standard conditions. Amplified cDNA collection at Lawrence Livermore National Laboratory.

**TABLE II**

DNAs employed for HEX1-N2 substrate-specificity studies

All substrates were generated as described under “Experimental Procedures.” The asterisk indicates the position of the $^{32}$P label. Oligonucleotide that was labeled in the experiment is underlined, and the nucleotide sequences are shown in Table I.

| Type of DNA | Sequence 1 | Sequence 2 |
|-------------|------------|------------|
| 5' labeled double stranded | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| Single stranded | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| Overhang double stranded | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| Recessed double stranded | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| 5' flap | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| 5' pseudo flap | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| 3' flap | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| 3' pseudo flap | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| Gapped double stranded | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |
| Nicked double stranded | 5'-ggg aat ttt tcc ctt ttt aca-3' | 5'-aag cgg aag ctt tta ttt tcc cct ttt aaa-3' |

5'-ggg aat ttt tcc ctt ttt aca-3’ were synthesized to encode the N- and C-terminal ends of hFEN1 and for PCR amplification of its coding region. Template plasmid DNA (clone 531968) was obtained from the IMAGE cDNA collection at Lawrence Livermore National Laboratory. PCR reactions were performed under standard conditions. Amplified DNA product was digested with NcoI and HindIII and subcloned into these restriction sites of pET28a (Novagen) to generate pET28FEN1.

**FIG. 1.** Expression and purification of HEX1-N2. A position of the HEX1/hEXO1 RAD2 domain truncations (HEX1-N1, -N2, -N3, -N4, and -N5). The underlined residues indicate the amino acids included in the oligonucleotide primer used for PCR amplification; a stop codon immediately proceeds. Shown is the amino acid sequence of the full-length hEXO1 protein (846 amino acids), and the arrow indicates the position of the C-terminal end (a phenylalanine in place of the second lysine) of HEX1 (803 amino acids). The dashed line depicts the location of the proposed degradation-susceptible connecting region. B, comparison of solubility and stability of each truncated HEX1/hEXO1 protein. Solubility: + indicates >95% pure; not shown and biochemical assays measuring flap endonuclease activity.

**Preparation of DNA Substrates**—The nucleotide (nt) sequences of the oligonucleotides used in these studies for DNA substrate generation are shown in Table I. For gel purification, DNAs were electrophoresed on a 20% denaturing polyacrylamide gel, visualized by UV shadowing, and cell debris removed by centrifugation for 20 min at 20,000 × g. The clarified protein extract was applied to an anion exchange column (Q20, Bio-Rad) pre-equilibrated with buffer B containing 200 mM KCl.

**Nuclease Analysis**—Nuclease assays were typically performed using 0.05 ng of protein (HEX1-N2 or hFEN1) and 0.5 pmol of end-labeled DNA substrate in a final volume of 10 μl containing 20 mM HEPES, pH 7.5, 50 mM KCl, 0.5 mM DTT, 5 mM MgCl₂, 0.05% Triton X-100, 100 μg/ml, 5% glycerol. Reactions were carried out at 37 °C for 20 min (unless otherwise indicated) and stopped immediately by adding 4 μl of formamide loading buffer and heating at 90 °C for 5 min. An aliquot was subsequently analyzed on a 16% polyacrylamide, 8 μm urea-denaturing gel. Visualization and quantitation of labeled DNAs was achieved using a Molecular Dynamics (Sunnyvale, CA) STORM 860 PhosphorImager and Molecular Dynamic ImageQuant version 1.11 software. For the pH titration experiment, the appropriate pH was achieved using a Molecular Dynamics (Sunnyvale, CA) STORM 860 PhosphorImager and Molecular Dynamic ImageQuant version 1.11 software. For the pH titration experiment, the appropriate pH was achieved using a Molecular Dynamics (Sunnyvale, CA) STORM 860 PhosphorImager and Molecular Dynamic ImageQuant version 1.11 software.
Purification of the RAD2 Nuclease Domain of HEX1/hEXO1—Attempts to express and purify the full-length human exonuclease 1 protein from bacteria have proven difficult, largely due to rapid degradation (24, 35). Based on the fact that the yeast proteins exhibit similar instability (37, 38), it seems likely that the C- and N-terminal domains of the EXO1-like proteins exist as distinct entities, separated by a degradation-susceptible connecting region. Thus, we elected to define the substrate preferences of a RAD2 Class III member by characterizing the biochemical properties and substrate preferences of the nuclease domain of human exonuclease 1 (HEX1/hEXO1), as the active site (recognition and catalytic) components likely reside in this domain alone. It should be noted that we have adopted the nomenclature where hEXO1 refers to the longer human exonuclease 1 protein of 846 amino acids and HEX1 refers to the shorter 803 amino acid version; these proteins arise from alternatively spliced RNA transcripts and differ only in their C-terminal content outside of the RAD2 domain (25, 26). The nuclease domain is common among these two proteins (Fig. 1A).

Five expression constructs were generated to produce the RAD2 nuclease domain of HEX1/hEXO1 (Fig. 1A). Of these, three constructs expressed the nuclease domain predominantly as insoluble material (Fig. 1B), independent of temperature (at 14, 28, or 37 °C) or induction method (using either an IPTG or salt-inducible system (39)). Two of the truncated HEX1/hEXO1 proteins were expressed predominantly (>50%) as soluble material. Of the soluble proteins, HEX1-N4 exhibited significant susceptibility to degradation (Fig. 1B). Based on the stability of HEX1-N2 (i.e. resistance to degradation), this fragment was selected for further analysis and purified to >95% homogeneity using a combination of cation exchange and gel filtration chromatography (Fig. 1C). Purification of HEX1-N2 was monitored by following both nuclease activity and HEX1-specific antibody cross-reactivity, which co-fractionated on the gel filtration column (not shown). It is worth noting that during purification of HEX1-N4 (which is 55 amino acids longer at the C-terminal end than HEX1-N2), we observed two prominent protein forms, one “full”-length (49.8 kDa) and one ~4 kDa shorter that is similar in size to HEX1-N2 (not shown), suggesting that the susceptible hinge region exists in the 55-amino acid segment between the C-terminal ends of N2 and N4 (Fig. 1A).

The 5' to 3' Exonuclease Activity of HEX1-N2—Purified HEX1-N2 was found to exhibit an exonuclease activity on 5'-end-labeled double-stranded (ds) DNA substrates, as determined by the release of mononucleotides (Fig. 2, left). Incubation of HEX1-N2 with a 45-base pair duplex DNA substrate labeled at the 3'-end resulted in the generation of a 6-nucleotide product, likely representing the minimal size required for protein-DNA complex formation after an initial 5'-to 3' degradation (Fig. 2, right). Other DNA fragments were also observed with the 3'-labeled substrates reflecting either site-specific endonuclease activity or limited processivity. The finding that...
mononucleotides were released only with a 5'-labeled substrate is evidence for an intrinsic 5' to 3' exonuclease activity, as seen previously with full-length HEX1 (24) and the other Class III members (37, 38).

Determination of the enzymatic requirements of HEX1-N2 revealed that the pH range for its exonuclease activity is 7.2–8.2 (Fig. 3A), with maximum activity at pH 7.8. Furthermore, the nuclease function of HEX1-N2 was dependent on the presence of a divalent metal ion, preferring Mg$^{2+}$ (Fig. 3B) at concentrations of $\geq$1 mM, while showing $<0.01\%$ activity in 4 mM EDTA. Exonuclease activity was also observed in Mn$^{2+}$-containing buffer but at $\sim$3-fold reduced efficiency relative to Mg$^{2+}$-containing reactions; $<5\%$ activity was detected in Co$^{2+}$, Zn$^{2+}$, or Ca$^{2+}$ (Fig. 3B). The nuclease activity of HEX1-N2 was stimulated $\sim$60-fold by the simultaneous addition of 0.05% Triton X-100 and 0.5 mM DTT and was sensitive to repeat freeze-thaw cycles. The addition of bovine serum albumin at a final concentration of 100 ng/ml helped stabilize HEX1-N2 (when at concentrations of $<100$ ng/ml), increasing its enzymatic activity 5–10-fold.

A more detailed look at the substrate preferences of HEX1-N2 revealed that this protein degrades blunt (S.A. = (1.3 ± 0.2)$\times$10$^5$) or 5'-recessed (S.A. = (1.4 ± 0.5)$\times$10$^5$) duplex substrates at a rate roughly 2.5–7-fold faster than single-stranded (S.A. = (0.5 ± 0.2)$\times$10$^5$) or 5'-overhang (S.A. = (0.2 ± 0.2)$\times$10$^5$) dsDNAs (Fig. 4). Mononucleotides were the predominant product released from all substrates, except for the 4-nt, 5'-recessed dsDNA (Table II), where the main product released was a trimer (not shown). A trimer product was not released when a 10-nt 5'-recessed dsDNA was employed, but instead mononucleotides were released, suggesting a yet unidentified DNA structural element or sequence-specific effect in recognition and/or catalysis. The exonuclease activity of HEX1-N2 at nick (S.A. = (1.0 ± 0.3)$\times$10$^5$) and gap (S.A. = (2.5 ± 0.4)$\times$10$^5$) duplex substrates was similar or slightly higher than that observed for blunt end duplex DNAs.

**HEX1-N2 Exhibits Flap-specific Endonuclease Activity** —In light of the broad structure-specific nuclease activities associated with the RAD2 protein family, we examined the substrate preferences of HEX1-N2 using a series of DNA substrates (Table II and Fig. 5). This analysis revealed that HEX1-N2 has 5'-flap (bifurcated) and pseudo flap-like structure-specific endonuclease activities analogous to hFEN1 (Fig. 5A). Neither HEX1-N2 nor hFEN1 were active on 3'-flap substrates, yet HEX1-N2 exhibited a significant 5' to 3' exonuclease activity on these DNAs that was not observed with hFEN1 (Fig. 5A). Flap endonuclease activity was also detected with partially purified full-length HEX1 material from Sf9 protein extracts and with HEX1-N4 (not shown), indicating that this activity is an intrinsic property of the HEX1/hEXO1 protein. The pH profile and metal dependence of flap endonuclease activity of HEX1-N2 are similar to its exonuclease parameters. HEX1-N2 did not incise endonucleolytically at bubble-like structures (i.e., a non-complementary 10-bp region), G:T mismatches, or uracil residues (a U:G mismatch) but did degrade these duplex substrates exonucleolytically (Fig. 5B). Triplet repeat (CCG and CAG)-containing (12 or 18 nt) 5'-flaps, structures thought to promote genetic instability (40), were cleaved with a similar efficiency to the non-repeat-containing 5'-flap substrates (Fig. 5) by either HEX1-N2 or hFEN1 (not shown).

**Comparison of the Nuclease Functions of HEX1-N2 and substrates. The minus represents samples without protein. The asterisk indicates the position of the $^{32}$P label, and the flap size is indicated (10 nt). The size of the products was confirmed with a 2-nt marker (Life Technologies, Inc.) on a high resolution 58-cm polyacrylamide gel.
**Table III**

Kinetic parameters of HEX1-N2 and hFEN1 enzymes for flap and blunt end dsDNA substrates

| Enzyme      | Specific activity | \( k_{cat} \) \( \text{pmol min}^{-1} \text{mg}^{-1} \) | \( k_{cat} \) \( \text{min}^{-1} \) | \( K_m \) \( \mu M \) | \( k_{cat}/K_m \) | \( \text{min}^{-1} \text{mu}^{-1} \) |
|-------------|------------------|--------------------------|-----------------|-----------------|-----------------|------------------|
| HEX1-N2     |                  |                          |                 |                 |                 |                  |
| Exo         | (1.30 ± 0.1) \( \times 10^6 \) | 5.6 ± 0.7 | 5.86 ± 0.6 | 18.4 ± 1.9 | 0.32 |
| Endo        | (1.13 ± 0.2) \( \times 10^6 \) | 4.74 ± 1.2 | 2.73 ± 0.7 | 8.0 ± 1.2 | 0.34 |
| hFEN1       |                  |                          |                 |                 |                 |                  |
| Exo         | (5.76 ± 0.8) \( \times 10^2 \) | 0.024 ± 0.003 | ND \(^b\) | ND \(^b\) | ND \(^b\) |
| Endo        | (3.68 ± 0.2) \( \times 10^5 \) | 14.8 ± 2.1 | 23.4 ± 4.8 | 39 ± 6.0 | 0.60 |

\(^a\) Values are derived from specific activity.

\(^b\) ND, not determined.

hFEN1—To compare further the nuclease functions of HEX1-N2 and hFEN1, we determined the relative activities of these enzymes on ds blunt end DNA substrates and 5'-flap DNA structures. The flap endonuclease activities (both S.A. and specificity constants \( k_{cat}/K_m \)) of HEX1-N2 and hFEN1 were similar, whereas the S.A. values of the exonuclease activities were >200-fold different, with hFEN1 exhibiting only a minor 5'- to 3'-degradation activity (Table III). The kinetic parameters of the exo- and endonuclease activities of HEX1-N2 are similar (Table III).

**DISCUSSION**

Previous studies have shown that exo1 mutants exhibit defects in mitotic and meiotic recombination (38) and display a mutator phenotype characteristic of a mismatch repair defect (20, 21). Furthermore, an interaction has been identified between EXO1 and MSH2 (21, 25), a factor that recognizes mis-paired nucleotides (41) and Holliday junctions (42), supporting the idea that the EXO1 proteins participate in repair and recombination. The finding that the EXO1 proteins maintain an RNase H activity in vitro (35) suggests that these enzymes may also be involved in DNA replication. Our observation that HEX1/hEXO1 exhibits a powerful 5' to 3' exonuclease activity and a comparable flap endonuclease function not only supports the notion that these proteins operate in the aforementioned DNA metabolic pathways but begins to define the specific enzymatic activities of these factors.

The 5' to 3' exonuclease activity of HEX1/hEXO1 likely operates to excise mispaired nucleotides during mismatch repair or to generate 5'-resected ends (i.e. 3'-ss tails) needed for strand exchange and recombination (43). The flap endonuclease activity presumably functions to resolve DNA intermediates formed during recombination and replication. This endonuclease activity may also contribute to the excision of ultraviolet-induced damage through a repair mechanism similar to that executed by the UVDE and FEN1 proteins of S. pombe (44), perhaps explaining the modest sensitivity of the exo1 yeast mutants to ultraviolet light (45). It is noteworthy that HEX1-N2 exhibits a ~4-fold higher affinity (i.e. a lower \( K_m \)) for flap structures relative to hFEN1 in the in vitro reaction conditions employed here. Whether the mammalian EXO1 proteins participate in other pathways of repair, such as "long patch" base excision repair (46), through its nuclease activities will need to be determined.

Despite the similar biochemical properties of EXO1 and FEN1, the corresponding yeast mutants display very different phenotypic profiles, indicating distinct biological functions for these two enzymes. For instance, the rad27 mutants of S. cerevisiae are hypersensitive to methylmethane sulfonate (14, 34), whereas the exo1 mutants display no notable increase in alklylation sensitivity (38). This observation suggests a more prominent role for FEN1 in the repair of alklylation-induced DNA damage, probably during base excision repair (47). Furthermore, rad27 mutants exhibit a temperature-sensitive growth phenotype, not displayed by the exo1 mutants, apparently due to a prominent role in removing mature Okazaki fragments or other bifurcated structures during chromosome replication. Yet, EXO1 does appear to contribute to replication, since human EXO1 displays RNase H activity (35), the yeast and human EXO1 proteins can functionally complement the replication defects of the rad27 mutants when overexpressed (35), and rad27exo1 double mutants are inviable (21). However, the inviability of the double mutants can also be explained by the recombination defect of an exo1 deletion (35, 38), as previous studies have found that simultaneous mutations in rad27 with seemingly any recombination gene lead to synthetic lethality (16, 21, 48). The biochemical activities reported here are consistent with EXO1 operating in processes of genetic recombination.

We note that there exist conflicting reports on the requirement of both FEN1 and EXO1 for yeast viability, as Johnson and colleagues (47) found that rad27 (equivalent to rth1) exo1 double mutants are viable. Strain or genetic differences may explain this discrepancy.

There are significant differences in the mutational spectrum of the rad27 and exo1 single mutants that provide additional clues into their biological activities. rad27 mutants exhibit a significant increase in genetic duplications, which likely arise from replication errors (16), although a contribution from inefficient mismatch repair cannot be excluded (13). exo1 mutants, on the other hand, display a mutator phenotype that appears to stem from a mismatch repair defect (21). The rad27 and exo1 mutants do exhibit some similarities, e.g., a mild sensitivity to ultraviolet radiation, mismatch repair and recombination defects, and genetic instability, offering evidence for complementary roles as well. Additional studies aimed at teasing out the synergistic nature of these two enzymes are required. Finally, it is worth mentioning that mammalian EXO1 mutants may exhibit phenotypic characteristics that differ from the corresponding yeast mutants, since mammals appear to possess only a single EXO1-like gene (23–26), whereas S. cerevisiae maintain three EXO1-like homologs (EXO1, YEN1, and DIN7; see Refs. 21 and 38).

The cellular responsibilities of the mammalian EXO1 and FEN1 proteins will likely be determined by their expression specificities, protein-protein interactions, and substrate preferences. The high level of EXO1 gene expression observed in testis and lymphoid tissues of mice and human may suggest a role for the EXO1 proteins in meiotic recombination and antibody gene rearrangement events (23–26). There are no published reports on the expression specificity of FEN1, providing
The protein-protein interaction specificities of HEX1/hEXO1 and hFEN1 appear to be dictated by their divergent C-terminal domains. Human MSH2 associates with human EXO1 through a C-terminal interaction, and such a physical association may direct EXO1 to sites of mispaired nucleotides or recombining chromosomes. The C-terminal region of hFEN1 maintains a consensus PCNA-interaction sequence (not present in the EXO1 proteins), which presumably directs FEN1 to sites of PCNA-dependent repair and replication (49). Provocatively, the MSH2-MSH6 protein complex may serve as a sliding clamp (50) for the non-progressive EXO1 nucleases (37, 38, and data within), much like PCNA operates as an accessory factor for hFEN1 (51).

It is worth emphasizing that whereas the C-terminal domain of HEX1/hEXO1 may determine its protein-protein interaction specificity, this domain will likely have little effect on the intrinsic in vitro nuclease properties of the protein. More specifically, the nucleosome core, including the catalytic and DNA binding residues, is retained in HEX1-N2. How particular protein-protein interactions may affect the nucleosome function(s) of HEX1/hEXO1 in vivo or how the C-terminal domain may influence the kinetics of the nucleosome reaction steps needs to be determined.

It is interesting that both FEN1 and HEX1/hEXO1 display flap endonuclease activity, yet only the latter exhibits a powerful 5’ to 3’ exonuclease function. Whether coupling of these nuclease activities would serve a biological advantage is presently unclear, and the precise protein elements that determine the varying degrees of nucleosome potential are not obvious. Studies performed to date indicate that the physical nature of the different loop structures found among the RAD2 family members (termed the flexible loop or helical arch) is a major contributor to specificity (52). It is this loop structure that is believed to target the ssDNA region of the bifurcated substrate into the active site of the enzyme (49, 53), and there are notable amino acid sequence differences between HEX1/hEXO1 and hFEN1 in this region. There is also evidence that the metal ion plays a role in DNA recognition and protein-DNA complex stability for the RAD2 proteins (54). Whether the loop-specific differences or metal ion preferences (Mg$^{2+}$ for HEX1/hEXO1 and Mn$^{2+}$ for hFEN1) of HEX1/hEXO1 and hFEN1 influence substrate specificity is presently unknown. The similar pH range of HEX1/hEXO1 and hFEN1 (9) does suggest that these proteins execute a similar catalytic reaction, which interestingly has yet to be defined for any RAD2 member (5). Future biochemical and structural studies involving the RAD2 proteins are critical for addressing these issues.

Finally, the 5’ nuclease function of FEN1 has been shown to play an important role in maintaining genomic integrity and in regulating nt repeat expansion/contraction (40). Whether human FEN1 and, in light of the results presented within, human exonuclease 1 are involved in protecting against genetic instability and disease, most notably cancer and neurodegenerative syndromes, merits further investigation.

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2 B.-I. Lee and D. M. Wilson III, unpublished observations.

3 L. Rasmussen, personal communication.