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Human Exonuclease 1 Functionally Complements Its Yeast Homologues in DNA Recombination, RNA Primer Removal, and Mutation Avoidance*

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Yeast exonuclease 1 (Exo1) is induced during meiosis and plays an important role in DNA homologous recombination and mismatch correction pathways. The human homolog, an 803-amino acid protein, shares 55% similarity to the yeast Exo1. In this report, we show that the enzyme functionally complements *Saccharomyces cerevisiae* Exo1 in recombination of direct repeat DNA fragments, UV resistance, and mutation avoidance by *in vivo* assays. Furthermore, the human enzyme suppresses the conditional lethality of a rad27Δ mutant, symptomatic of defective RNA primer removal. The purified recombinant enzyme not only displays 5′-3′ double strand DNA exonuclease activity, but also shows an RNase H activity. This result indicates a back-up function of exonuclease 1 to flap endonuclease-1 in RNA primer removal during lagging strand DNA synthesis.

Mutations cause genomic instability and gene dysfunction, many of which affect cell growth and lead to tumorigenesis. Fortunately, in normal cells, mutation rate is low due to the existence of different DNA repair systems such as mismatch, excision, and recombinational DNA repair pathways. In humans, DNA mutation accumulation is a critical step in carcinogenesis. Dysfunctional mutations of DNA mismatch repair genes such as *MSH2*, *MLH1,* and *PMS2*, are the main cause of the hereditary non-polyposis colorectal cancer (1–6). A portion of sporadic cancers is due to acquired mutations in mismatch repair genes as well (7). Mutations of genes encoding nucleotide excision repair proteins including XPG nuclease are linked to xeroderma pigmentosum (8–17).

Nucleases play important roles in several pathways including DNA replication, repair, and recombination. DNA fragments containing a lesion are removed by the combined efforts of a helicase and a nuclease. For instance, in the DNA mismatch repair of *E. coli*, exonucleases, exo I (3′-5′ excision) and exo VII or Rec J (5′-3′ excision) are responsible for the bidirectional removal of DNA fragments containing mismatched lesions (18, 19). For DNA recombination or repair of double strand DNA breaks through recombination, an important step is to generate a 3′ single-stranded terminus for strand invasion. This step is accomplished by 5′-3′ exonucleases (20–22). During DNA replication the removal of RNA primers in the lagging strand also requires 5′-3′ nuclease activity (23). In *E. coli* and other bacteria, the removal of RNA primers is performed by the 5′-3′ exonuclease activity of DNA polymerase I (24–27). The *polA ex1* mutant, defective in 5′-3′ exonuclease activity, exhibits retarded joining of nascent DNA fragments. In eukaryotes, DNA polymerases lack an intrinsic 5′-nuclease. Removal of RNA primers is carried out by an independent enzyme, Rad27/FEN-1 nuclease, with both flap endonuclease and 5′-3′exonuclease activities (28–38). Disruption of the flap endonuclease gene *RAD27* in the yeast *Saccharomyces cerevisiae* resulted in DNA replication defective symptom including the conditional lethality (39–42). Survival of the null mutant at 30 °C suggests that other enzymes with 5′-3′ exonuclease activity could back up the function of Rad27/FEN-1 in DNA replication (29, 43).

A 5′-3′ exonuclease, called exonuclease 1 (Exo1) in *Schizosaccharomyces pombe* and *S. cerevisiae* and Tosca in *Drosophila melanogaster,* has recently been identified and partially characterized (44–48). The enzyme is a non-processive double-stranded DNA nuclease (44). Both the messenger RNA level and enzyme activity were dramatically induced during meiosis in *S. pombe* (44, 45). In *D. melanogaster,* the gene is specifically expressed in the early embryogenesis and female germline (46). The *S. cerevisiae* and human exonucleases 1 interact with mismatch repair protein Msh2 as demonstrated by the two-hybrid system and immuno-coprecipitation (48–50). Disruption of *EXO1* increased mutation rate in both *S. pombe* and *S. cerevisiae* cells, indicating that *EXO1* is a mutator gene (45, 48). Detailed analysis of the mutational spectrum of the *exo1*Δ cells suggested a role for the encoded protein in mismatch correction, most likely during homologous recombination. In addition, deletion of *yEXO1* significantly decreased both meiotic and mitotic recombination rates (45, 47, 48). One speculation is that the nuclease plays a role creating 3′ single-stranded complementary tails, thereby promoting joint molecule formation. Moreover, the null mutant displays a minor UV sensitivity unlikely due to a nucleotide excision repair deficiency but may be due to a defective DNA replication by-pass pathway (51). These findings indicate that the Exo1 homologs function as 5′-3′ exonucleases in mutation prevention via multiple DNA metabolic pathways. More recently, human homolog of the exonuclease 1 has been identified and the gene was named *HEX1* (52). It is specifically expressed in fetal liver and adult bone marrow, suggesting that the enzyme may operate prominently in processes specific to hemopoietic stem cell development. The gene has been mapped to 1q43, a region lost in some cases of acute leukemia and in several solid tumors (49, 52).

Human and *S. cerevisiae* exonuclease sequences have high similarity. This similarity leads us to predict that functions of these two enzymes are also similar and the human gene may complement the Exo1 functions in yeast. In addition, when

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TABLE I
S. cerevisiae strains used in the study
Differences in the genotype from the parental strains are indicated in the table.

Strain	Deletion	Genotype	Source
W1021–7c		a, *ade2–1, can1–100, his3–11, 17, leu2–3, ura3–1*	R. Rothstein
W1089–6c		α, *ade2–1, can1–100, his3–11, 17, trp1–1, ura3–1*	R. Rothstein
AB15B[a]		a, *ade2–1, can1–100, his3–11, 17, leu2–1, trp1–1, ura3–1*	This work
AB15Ba[a]		Genotype of AB15B + PDB	This work
AB15Bb[a]		Genotype of AB15B + PDB-ScEXO1	This work
AB15Bc[a]		Genotype of AB15B + PDB-hEXO1	This work
HUWT[a]		α, *his3-Δhis3::URA3::Δhis3*	This work
HUWTa[a]		Genotype of HUWT + PRS314-ADH1	This work
HUWTb[a]		Genotype of HUWT + PRS314-ADH1-ScEXO1	This work
HUWTc[a]		Genotype of HUWT + PRS314-ADH1-hEXO1	This work
AB15D[a]	EXO1	a, *exo1::URA3*	This work
AB15Da[a]	EXO1	Genotype of AB15D + PRS314-ADH1	This work
AB15Db[a]	EXO1	Genotype of AB15D + PRS314-ADH1-ScEXO1	This work
AB15Dc[a]	EXO1	Genotype of AB15D + PRS314-ADH1-hEXO1	This work
HUWT1[a]	EXO1	a, *his3-Δhis3::URA3::Δhis3, exo1:hisG*	This work
HUWT1a[a]	EXO1	Genotype of HUWT1 + PRS314-ADH1	This work
HUWT1b[a]	EXO1	Genotype of HUWT1 + PRS314-ADH1-ScEXO1	This work
HUWT1c[a]	EXO1	Genotype of HUWT1 + PRS314-ADH1-hEXO1	This work
FDER[a]	EXO1&RAD51	a, *exo1::URA3, rad51::LEU2*	This work
FDERa[a]	EXO1&RAD51	Genotype of FDER + PRS314-ADH1	This work
FDERb[a]	EXO1&RAD51	Genotype of FDER + PRS314-ADH1-ScEXO1	This work
FDERc[a]	EXO1&RAD51	Genotype of FDER + PRS314-ADH1-hEXO1	This work
IC2–1[a]	RAD27	α, *rad27::LEU2*	This work
IC2–1a[a]	RAD27	Genotype of IC1–2 + PDB	This work
IC2–1b[a]	RAD27	Genotype of IC1–2 + PDB-ScEXO1	This work
IC2–1c[a]	RAD27	Genotype of IC1–2 + PDB-hEXO1	This work
U687[a]	RAD51	a, *rad51::LEU2*	A. Bailis
U687a[a]	RAD51	Genotype of U687 + PRS314-ADH1	This work
U687b[a]	RAD51	Genotype of U687 + PRS314-ADH1-ScEXO1	This work
U687c[a]	RAD51	Genotype of U687 + PRS314-ADH1-hEXO1	This work
RKY2672		a, *ade2–1, ade8, hom3–10, his3–200, 17, leu2–1, lys2ΔBgl, trp1–63, ura3–52*	R. D. Kolodner
RKY2672a		Genotype of RKY2672 + PRS314-ADH1	This work
RKY2672b		Genotype of RKY2672 + PRS314-ADH1-ScEXO1	This work
RKY2672c		Genotype of RKY2672 + PRS314-ADH1-hEXO1	This work
MEXO1[b]	EXO1	a, *exo1::LEU2*	This work
MEXO1a[b]	EXO1	Genotype of MEXO1 + PRS314-ADH1	This work
MEXO1b[b]	EXO1	Genotype of MEXO1 + PRS314-ADH1-ScEXO1	This work
MEXO1c[b]	EXO1	Genotype of MEXO1 + PRS314-ADH1-hEXO1	This work

[a] The strains are derivatives of W1021–7c and W1089–6c.
[b] The strains are derivatives of RKY2672.

Szankasi and Smith (45) identified the *S. pombe* gene (*SpEXO1*), they noticed that the encoded protein displays significant similarity to the proteins of rad13 (XPG/Rad2) and rad2 (FEN-1/Rad27) nuclease families. However, the similarities were limited to regions of N-terminal putative nuclease domains and the major part at C terminus was largely divergent (45). We hypothesize that the human exonuclease 1 may further complement the Rad27 nuclease functions in RNA primer removal during lagging strand DNA synthesis based on the observations of the sequence conservation between these two proteins, their enzymological properties, and phenotypical characteristics (*i.e.* conditional lethality) of the rad27 null mutants. Our *in vitro* and *in vivo* data demonstrate that the human exonuclease can functionally complement the yeast homologues (yExo 1 and yRad27) in recombination, UV resistance, RNA primer removal during the Okazaki fragment processing, and mutation avoidance.

EXPERIMENTAL PROCEDURES

Materials—A cDNA clone (number 843301) harboring a putative human exonuclease 1 was obtained from ATCC (Manassas, VA). Oligonucleotides used in this study were synthesized in the City of Hope Cancer Center core facility. The vector pET-28a and *E. coli* strains BL21 and BL21 (DE3) were from Novagen (Madison, WI) and *E. coli* strain XL2 blue and pBSK vector were from Stratagene (La Jolla, CA). Yeast expression vector pDB20 was a gift from D. Becker, California Institute of Technology. Restriction enzymes were obtained from New England Biolabs (Beverly, MA). Yeast culture media including YPD, synthetic complete (SC), minimal sporulation, and synthetic dextrose minimal (SD) media were prepared according to Sherman *et al*. (53). Amino acids and all other medium components and chemicals were purchased from Sigma. Isopropyl β-D-thiogalactoside was purchased from Roche Molecular Biochemicals (Indianapolis, IN).

Yeast Gene Disruption—All of the yeast strains constructed and used in this study are listed in Table I. For construction of the *EXO1* null mutant strain, the *EXO1* gene was amplified by PCR[1] using primers with *Hin*dIII and *Nco*I sites (exolpl (AGAAAGCCATGGGTATCCAAGGT) and exolp2 (CTCCGAAAGCTTTCAGTGATGATGGTGGTGGTGTTTACCTTTATAAACAAATTGGGAA) and cloned into the vector pET-28b (Novagen). The marker gene *URA*3 was obtained from plasmid YEp24 and inserted into *Pst*I sites, replacing the *EXO1* coding sequence between positions 170 and 828. The fragment containing the *exo1::URA*3 construct was removed from the plasmid with restriction enzymes *Xba*I and *Not*I. The *exo1::URA3* fragment was then transformed into yeast strains W1021-7c and W1089-6c (Table I). Ura3$^+$ transformants were analyzed by PCR using primers exolp3 (CGAACAAACTGAAAGGCGTAG) and exolp4 (GTCTTGAGGCATTTCGACGAG) and Southern blotting to verify disruption of *EXO1*. The *exo1 rad51* double mutant was obtained by crossing *exo1* (FDAB15D) with *rad51* (U687, a gift from Adam Bailis, City of Hope). *RAD27* gene was deleted by transforming the wild type strains W1021-7c and W1089-6c with a PCR-generated DNA fragment. The PCR product was generated using YEp13 containing *LEU2* marker gene as a template and two primers KNRTH1 (CATCGATGAAAAGCGTTGACAGCATACATTGGAAAGAAATAGGAAACGGACACCGGAAGTTAACTGTGGGAATACTCA) and KNRTH2 (AGCTGTTCCTTTGTCTTAGGCACCACTTGGAAGAACCCATCTAACCTACCCTGACTACGTCGTAAGGCCGTTT) (underlined sequences were designed to amplify *LEU2* marker gene, the remaining sequences are from *RAD27*). Another pair of the primers was

[1] The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s).

used to confirm the deletion of *RAD27* gene by PCR: RAD27F (AAACGCGACGCGTAACATCG) and RAD27R (ATATGCCAAGGTGAAGGACC). Null mutant strains of *RAD51* and *RAD51/EXO1* were from laboratory stock (51).

Complementation of ScEXO1 and HEX1 in S. cerevisiae—Two vectors, pDB20 and pRS314, were used for the expression of *EXO1* in *S. cerevisiae*. pDB20 is a *URA3* and *ADH1* promoter-based yeast expression vector. Two plasmids, PDB-ScE and PDB-hE, were constructed to express *Sc*- and h- exonuclease 1 in *S. cerevisiae*. PDB-ScE has an insertion of *S. cerevisiae EXO1* (*ScEXO1*) at the *Hin*dIII site of pDB20 while PDB-hE has an insertion of human *EXO1* (h*EXO1*) at the *Not*I site of pDB20. pRS314 is a yeast subclone vector using TRP 1 as a selection marker. PRS-ScE and PRS-hE were constructed to complement the exonuclease 1 function in the *rad27::URA3* background. PRS-ScE has an insertion of *Padh*-ScEXO1-*Tadh* (Padh, ADH 1 promoter; Tadh, ADH1 terminator) at *Sac*I and *Apa*I sites. PRS-hE has an insertion of Padh-hEXO1-Tadh at *Bam*HI site. The plasmids were transformed into different *S. cerevisiae* strains for exonuclease 1 functional complementation as listed in Table I.

Overexpression and Purification of hExo1—Full-length human *EXO1* coding sequence was subcloned into pET-28a vector (Novagen) using the cDNA clone harboring h*EXO1* and PCR primers 5exo1 and 3exo1 containing *Nhe*I and *Sal*I sites (5exo1, GACTGTGCTAGCATGGGGATACAGGGATTG and 3exo1, TGTCACTGTCGACAATCCAAAGTTTTTCCAG), respectively, yielding pET-HEX. Both pET-28a and pET-HEX were transformed into BL21 (DE3) cells (Novagen). Colonies were inoculated into 50 ml of LB broth supplemented with 30 μg/ml kanamycin and cultured overnight. The culture was pelleted and transferred in 4 $\times$ 1-liter of sorbitol medium (54). Cultures were grown at 37 °C to OD 0.6 followed by induction at 25 °C with 1 mM isopropyl-β-D-thiogalactoside for 4 h. The cells were harvested and stored at -80 °C until use. All of the purification steps were performed at 4 °C. The cells was thawed in a mixture of ice and water and resuspended in buffer A (20 mM Na_2HPO_4, pH 7.9, 0.5 NaCl) with 10 mM imidazole. Cells were sonicated and the lysate was then centrifuged at 17,000 rpm for 45 min to remove cell debris. A 5-ml HiTrap Chelating Ni^{2+} column was equilibrated with buffer A using Fast Protein Liquid Chromatography system (Pharmacia). After loading, the column was washed with 25 ml of buffer A with 10 mM imidazole, 25 ml of buffer A with 60 mM imidazole, and then eluted with a 50-ml linear gradient from 60 to 500 mM imidazole in buffer A at 2 ml/min. The fractions were run on the 10% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R-250 to visualize the purity of protein. Buffers of the fractions were exchanged to 10 mM Tris, 150 mM NaCl, pH 8.0, by HiTrap desalting column (Pharmacia). Protein concentrations were determined using the Bio-Rad protein assay.

Nuclease Activity Assay—To prepare substrates for ribonuclease activity assays, an upstream primer (5′-**TTTT**ATAACTCGGAGAGCCGTAATG-3′, the bold nucleotides are the 5′ single-stranded portion of exonuclease 1 substrates) and a 5′-labeled 21-ribonucleotide/30-deoxyribonucleotide junction oligo (5′-gggaacaaaagcuugcaugccTGCAGGTCGACTCTAGAGGATCCCCGGGTA-3′, the lowercase indicates the ribonucleotides) were annealed to the 76-mer template (5′-**TTTT**TACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTTTGTTCCCCATTACGGCTCTCCGAGTTAT-3′) resulting in a Okazaki fragment-like substrate (ribo-substrate-1). To test the ability of human exonuclease 1 in removal of the last ribonucleotide attached to DNA, 5′-labeled mono-ribonucleotide/30-deoxynucleotide junction oligo (5′-cTGCAGGTCGACTCTAGAGGATCCCCGGGTA-3′) were annealed to a 55-mer complementary strand (5′-**TTTT**TACCCGGGGATCCTCTAGAGTCGACCTGCAGTAGACGTCTGACACAGCCGT-3′) to form a nicked duplex substrate (ribo-substrate-2). An upstream primer (5′-**TTTT**ACGGCTGTGTCAGACGTCTA-3′) has also been added to the above substrate (ribo-substrate-2) to form the ribo-substrate-3. Furthermore, a 5′ end-labeled oligo (5′-ACGGCTGTGTCAGACGTCTACTGAGGTCGACTCTAGAGGATCCCCGGGTA-3′) was annealed to the template strand of the ribo-substrate-2 to form blunt end double-stranded DNA as a substrate for a 5′ DNA exonuclease activity assay. Based on the property that hExo 1 has a negligible activity on a single-stranded DNA, all unlabeled 5′ ends of the substrates have four additional nucleotides to form the single-stranded ends and block the enzyme activity. The ribonuclease reactions were performed as described previously (55). Briefly, a standard reaction mixture contained 0.8 pmol of [γ-^{32}P]nucleic acid substrate, 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, and 10 ng of enzyme in 13 μl and were incubated at 33 °C for 30 min. The labeled substrates were prepared in a concentration ratio of 1 labeled oligonucleotide to 10^3-10^6 unlabeled oligonucleotide. The reactions were stopped by adding an equal volume of stop solution (U. S. Biochemical Corp.), mixed, boiled for 3 min, and cooled in ice. 3 μl of each reaction product was run on a 15% denaturing polyacrylamide gel and exposed to Kodak x-ray film for an image. For time-dependent kinetic analysis, the cleavage reaction was stopped in different time points. The products were resolved on a denaturing gel. Quantification of the band intensities was done with a PhosphorImager (Stratagene, CA) and the computer program IPLab Gel assay (Signal Analytics, VA).

Recombination Assay—The recombination assay was based on a construct of *URA3* gene flanked at both ends by the same DNA fragment (413 bp) from the *HIS3* gene direct repeats (see Fig. 2 and Ref. 56). The construct was inserted into the *HIS3* gene in the wild type *S. cerevisiae* cells. These cells were hybridized with *exo1* or *rad27* null mutants. They were then sporulated and dissected to produce the exo1 or *rad27* null mutant strains with the assay construct. The strains were cultured in the SD-URA medium to saturation. They were then plated onto histidine minus SD (SD-HIS) medium. The optimal concentration for plating was tested based on three different concentrations (5 $\times$ concentrated, saturated, and 10 $\times$ diluted). For the wild type and *exo1* null mutants, 100 μl of saturated culture cells was optimal and plated onto SD-HIS medium. After 3–4 days, $HIS3^+$ recombinant colonies were counted and recombination rate was calculated by the number of recombinants divided by the total number of viable cells.

UV Sensitivity Analysis—Analysis was carried out as described previously (51) except that the strains in this study were transformed with plasmids, pRS314, pRS-ScE, or pRS-hE, respectively (Table I). Strains were grown in tryptophan minus SD (SD-TRP) liquid to saturation at 30 °C and cell density was measured with a spectrophotometer (600 nm), diluted in water, and plated on SD-TRP agar plate. The plates were exposed to different UV dosages (254 nm germicidal lamp) and incubated in the dark for 3–4 days at 30 °C before colonies were counted. Survival rate was determined based on the ratio between colony counts with and without UV treatment. Survival curves represent the average from at least six independent experiments.

Suppression Analysis—The pDB overexpression vector with or without insertion of either *HEX1* or *ScEXO1* was transformed into the *rad27* null mutant and wild type strain of *S. cerevisiae*. To observe colony growth, the transformants were streaked onto the SD-URA medium side by side with duplications. They were incubated at 37 and 30 °C, respectively. The transformants were also cultured in the SD-URA liquid medium to saturation to measure the growth curve. The saturated cultures were then normalized and inoculated in 10 ml of SD-URA liquid medium. The growth was determined using a spectrophotometer in different time intervals for the curve.

Mutator Phenotype Analysis—To observe the role of *EXO1* in mutation avoidance, the mutator phenotype of *exo1* deletion mutant (*exo1*) and the ones transformed with *ScEXO1* and *HEX1* were determined using an assay based on cycloheximide resistance (57). The strains were cultured to saturation in 2.5 ml of SD-TRP liquid medium. For control, 0.2 μl of the culture was diluted by 1 ml of distilled water and 50 μl of the diluted samples was plated onto the SD-TRP agar plate for cell number counting. The remaining samples were spun down and plated onto the SD-TRP agar plate with 10 μg/liter cycloheximide. Mutation rate for each strain was calculated from six independent experiments.

RESULTS

Protein Sequence Comparison Predicted That Human Exonuclease-1 Is a Yeast Functional Homolog—A cDNA clone (number 843301) harboring a putative human exonuclease 1 was obtained from ATCC. Sequence of cloned cDNA was determined in our laboratory and deposited in GenBank (accession number AF060479) and translated into the protein sequence. Sequence of *S. cerevisiae* exonuclease 1 was obtained from the NCBI protein data base. They were then aligned using Optimal Global Alignment of Two Sequences at EERIE (Nimes, France) (Fig. 1). Overall sequence identity and similarity between human and *S. cerevisiae EXO1*s are 27 and 55%, respectively. The identity and similarity between the putative nuclease domains of the yeast Exo1 and Rad27 nuclease in are 28 and 57%. High sequence conservation between the human and *S. cerevisiae* predicts that they are functional homologs and leads us to test in yeast if the human enzyme can complement the characterized functions of the yeast enzyme.

Expression of HEX1 Restores the Recombination Rate in an exo1Δ Strain—ScEXO1 is involved in spontaneous mitotic and

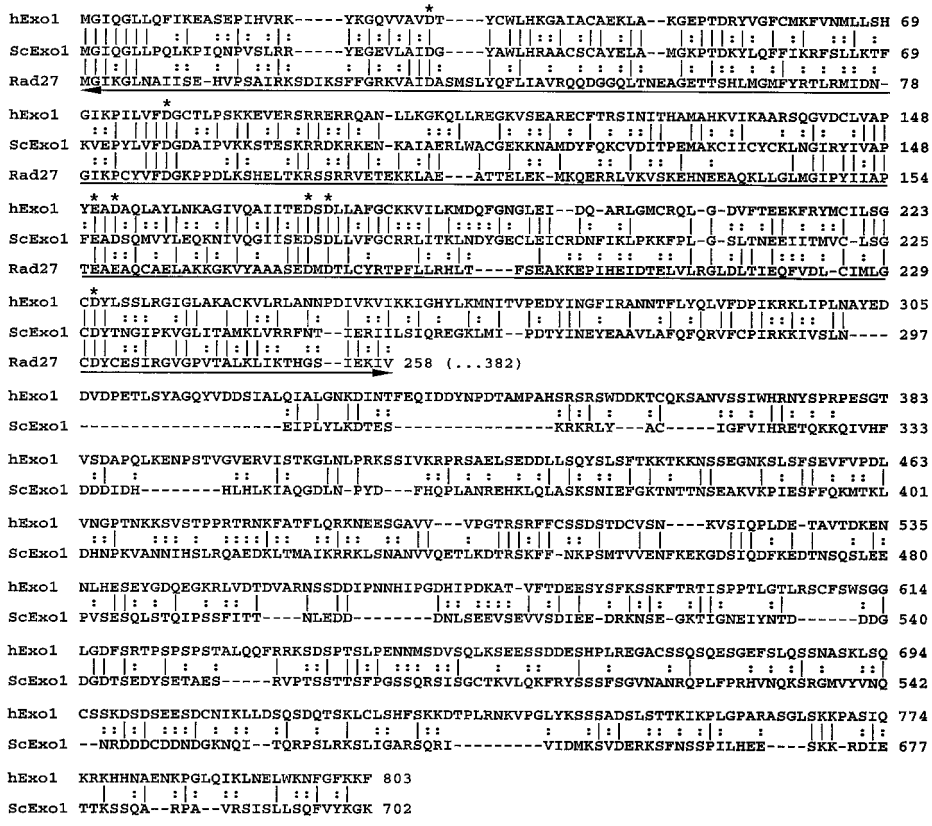

FIG. 1. **Sequence alignment of h*Exo1*, *ScExo1*, and the putative nuclease domain of *ScRad27*.** | indicates identical amino acids; : indicates similar amino acids; and * the conserved amino acids composing a nuclease active site as demonstrated in crystallography and site-directed mutagenic experiments of homologous enzymes of FEN-1 (61, 62). Overall sequence identity and similarity between human and *S. cerevisiae EXO1*s are 27 and 55%, respectively. *Underlined* are the conserved regions between exonuclease 1 and Rad27 nuclease.

meiotic recombination between direct repeats (45, 47). Complementation of this function during *S. cerevisiae* mitotic growth by *HEX1* was determined by measuring the rate of recombination between nontandem direct repeats of the *HIS3* genes (Fig. 2). The two *HIS3* alleles are truncated at their 3′ and 5′ ends, respectively, and are separated by plasmid vector sequences including the *URA3* gene. The end repeats of the *HIS3* gene in this system are 413 bp in length. Strains containing this duplication form can grow on the SD medium without uracil. Recombination events that excise the DNA between the repeats and restore the *HIS3* gene are visualized as the recombinant cells can grow on the medium without histidine. Recombination rate in a strain containing this direct repeat and a disruption of the *EXO1* gene were 58% lower than that of the wild type strain hosting the *HIS3* gene construct as a control. Expression of either *ScEXO1* or *HEX1* restored the recombination rate to the wild type level as shown in Table II.

Expression of HEX1 Ablates the UV Sensitivity of an exo1Δ Strain—Deletion of the *EXO1* gene in *S. cerevisiae* causes a minor UV sensitivity (51). Epistatic analysis indicated that the observed phenotype was not due to the defective *RAD2*-dependent nucleotide excision repair or loss of its known function in *RAD51*-dependent double strand break-induced recombination pathway or mismatch repair pathway. It is most likely due to its involvement in a DNA replication by-pass pathway. Double deletion of *EXO1* and *RAD51* made the strain very sensitive to UV treatment. Introduction of the human and yeast *EXO1* expression plasmids into this double deletion mutant strain recovered the UV sensitivity close to the level of the *rad51*Δ mutant (Fig. 3).

Purified hExo1 Efficiently Removes RNA Primers from Okazaki Fragment Model Substrates—Full-length human exonucleases-1 cDNA was cloned into the pET28a vector and expressed in *E. coli* using sorbitol medium as described previously (54, 58). PCR primers were designed to add both N- and C-terminal His tags onto the recombinant protein. The

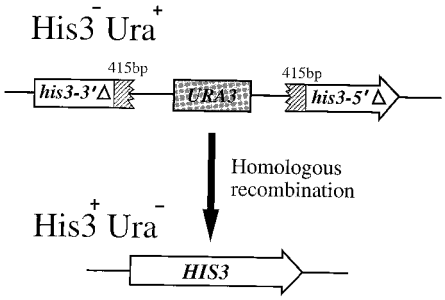

FIG. 2. **A recombination assay used in this study.** The yeast strain, HUWT (wild type) and HUWT1 (*exo1*Δ), containing a recombination substrate on the chromosome that consists of two nontandem direct repeats of truncated alleles (415 bp) of the *his3* gene separated by the *URA3* sequence were used in this experiment. They were transformed with three plasmids: PRS314, PRS-ScE, and PRS-hE to measure the complementation ability of *HEX1* in recombination (see the results in Table II).

TABLE II
His⁺ recombination frequency in strains containing the his2–3′Δ::URA3::his3–5′Δ

Strains	Genotype	Recombinants per 10^4 viable cells	Relative frequency
			%
HUWTa	Wild type/pRS314	2.65 ± 0.44	100
HUWT1a	*exo1*/pRS314	1.12 ± 0.23	42
HUWT1b	*exo1*/PRS-ScE	2.55 ± 0.29	96
HUWT1c	*exo1*/PRS-hE	2.16 ± 0.31	82

final products of fast protein liquid chromatography purification using a nickel affinity column contained two protein bands (approximately 93 and 45 kDa). Microsequencing of both polypeptides revealed the expected N-terminal sequence of the human exonuclease 1 deduced from its cDNA sequence (MGIQGLL). Therefore, the smaller polypeptide is a C-termi-

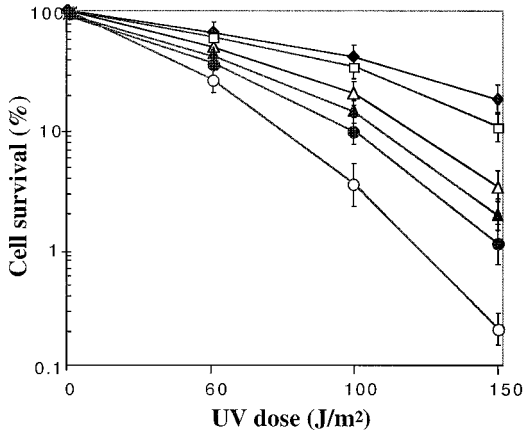

FIG. 3. **UV sensitivity of *S. cerevisiae exo1* null mutatnt is reversed by the expression of *Sc*- or h-*EXO1*.** As revealed previously (51), the single deletion of *S. cerevisiae EXO1* only exhibits a minor UV sensitivity whereas the double deletion of *EXO1* and *RAD51* has a synergistic effect. For this reason, the double deletion mutant was used to test if the *HEX1* could complement the function of *S. cerevisiae EXO1* in the UV DNA damage repair. ♦, AB15Be (wt/pRS 314); □, AB1Ba (exo1Δ/pRS314); Δ, U687a (rad51Δ/pRS314); ○, FDERa (exo1Δrad51Δ/pRS314); ▲, FDERb (exo1Δrad51Δ/PRS-ScE); ●, FDERc (exo1Δrad51Δ/PRS-hE). The data was averaged from six independent experiments.

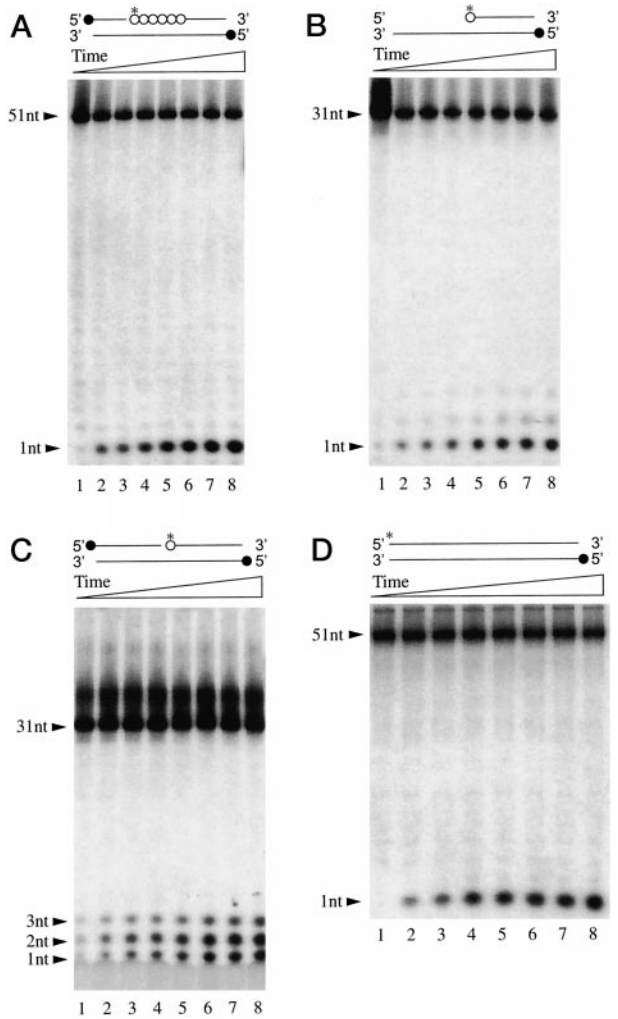

FIG. 4. **The purified Exo1 protein shows the 5′-3′ double strand exonuclease activity and 5′-3′ riboexonuclease activity.** Exonuclease activity of human recombinant Exo1 was tested with a blunt ended DNA duplex substrate and illustrated in *panel D*. The 5′-3′ riboexonuclease activities were tested with Okazaki fragment-like substrates and illustrated in *panels A-C*. Sequence of oligoes used to make the substrates is given in the text. The assays were performed as described under "Experimental Procedures." Time points were 0, 1, 1.5, 2.5, 5, 7.5, 10, and 20 min. *Lanes 1–8, triangles* denote increasing reaction time. *Dots* in the substrate scheme represent RNA portion. The *filled dots* indicate the 5′ single strand blockage to the 5′ Exo1 activity. *Arrows* with *nt* (nucleotide) show the length of the substrates and products; * indicates the radioactively labeling position.

nal degradation product of the 93-kDa protein. All of the Exo1 homologs (*S. cerevisiae*, *S. pombe*, and human) possess a 5′-3′ double-stranded DNA exonuclease activity. To explore the possibility that the enzyme might participate in RNA primer removal during eukaryotic DNA replication lagging strand DNA synthesis, three Okazaki fragment model substrates were prepared as ribo-substrates-1, -2, and -3 (Fig. 4) that mimic dynamic lagging strand DNA replication intermediates. Indeed, the enzyme possesses a 5′ riboexonuclease activity on all of the substrates. A blunt ended double-stranded DNA duplex was included as a control (Fig. 4, *A-D*). The time course of the enzyme reaction indicates that the reactive efficiency of the enzyme with ribo-substrate-1, -2, and double-stranded DNA substrate are similar (Fig. 5, *A-D*). hExo1 digests both RNA and DNA with a duplex substrate with or without an upstream primer. However, the enzyme releases mononucleotides with the substrate-1, -2, and DNA duplex while it cleaves mono-, di-, and trinucleotides simultaneously from the 5′ end with substrate-3 (an upstream primer was added to the substrate-2, Fig. 4*C*).

Expression of Both ScEXO1 and HEX1 Suppressed the Lethality of rad27Δ Mutant at 37 °C—Yeast Rad27 nuclease and its mammalian homolog FEN-1 functions in RNA primer removal during lagging strand DNA synthesis (29, 38, 43). *rad27* null mutants displayed a conditional lethality phenotype: the cells grow slowly at 30 °C but were arrested in S-phase at 37 °C. The mutants also have a hyper-recombination phenotype. This is indicative of long-lived regions of single-stranded DNA in the chromosome and is symptomatic of a defect in Okazaki fragment processing. A partial defect in this process indicates that an inefficient nuclease backs up the function of the Rad27 nuclease. Exonuclease-1 shares a conserved nuclease domain with the Rad27 nuclease (Fig. 1). Deletion of both *EXO1* and *RAD27* in *S. cerevisiae* leads to complete lethality of cells (data not shown). Expression of *ScEXO1* and *HEX1* in a *rad27* null mutant promotes the colony formation at 37 °C even though the human gene has less capacity to remove RNA primers in yeast as the transformants formed smaller colonies (Fig. 6). All of these strains were grown in the liquid YPD medium at 37 °C to determine the growth curve. The results indicated that the expression of both *Sc*- and human *EXO1* gene made the rad27 null mutant grow more closely to the wild type even though the strain harboring the human *EXO1* gene grew more slowly than the rad27Δ mutant with the *ScEXO1* (Fig. 7).

Expression of Both ScEXO1 and HEX1 Reversed the Mutator Phenotypes—Due to the functions of the *EXO1* gene in recombinational mismatch correction and probably in RNA primer removal, deletion of the gene increased yeast mutation rates 29-fold in the cycloheximide resistance assay (Table III). The rate increased about 6-fold in the CAN^r and HOM assays (59). Expression of both *ScEXO1* and *HEX1* reversed the mutator phenotypes of an *exo1* null mutant and the mutation rate was reduced to the wild type level. These data indicate that the human gene is a functional homolog of the yeast gene and can play a role in mutation avoidance in heterogeneous cellular environment.

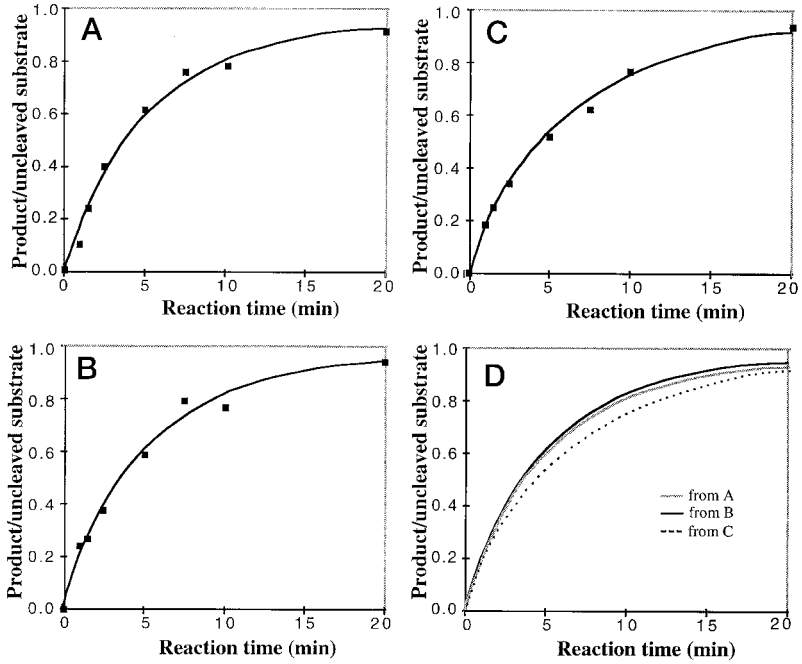

FIG. 5. **Human exonuclease 1-driven reactions with three different RNA-DNA/DNA and DNA/DNA duplex substrates.** The reactions have been conducted under the conditions described in the legend to Fig. 4 and under "Experimental Procedures" with excessive substrate to the enzyme. 10^3-10^6-fold more of unlabeled oligoes than the labeled oligo were used to construct the substrates. Densitometry was used to quantify the conversion of substrate to product. *A,* reaction with blunt ended double-stranded DNA substrate; *B,* reaction with RNA-DNA/DNA substrate (ribo-substrate-1); *C,* reaction with monoribonucleotide-DNA/DNA substrate (ribo-substrate-2); *D,* superimposition of *A, B,* and *C*. The data is from the average of three independent experiments.

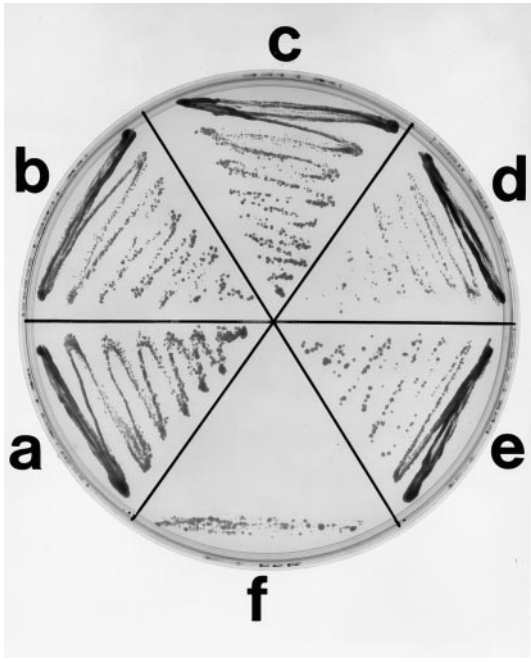

FIG. 6. **Overexpression of *Sc*- or h-Exols suppresses the lethality of RAD27/FEN-1 mutant at 37 °C.** Wild type (wt) and rad27 mutant (IC2) strains were grown on the SD-URA plate with and without the introduction of *Sc*- and h-*EXO1*s. *a*, AB15Ba (wt/pDB20); *b*, AB15Bb (wt/PDB-*ScE*); *c*, AB15Bc (wt/PDB-*hE*); *d*, IC2-1c (rad27/PDB-hE); *e,* IC2-1b (rad27Δ/PDB-ScE); *f*, IC2-1a (rad27Δ/PDB20).

DISCUSSION

Sequence and in Vitro Functional Conservation of Eukaryotic 5′-Exonuclease 1—The enzyme has so far been identified in *S. pombe, S. cerevisiae*, *D. melanogaster*, and human (44–52). Sequence similarity of Exo1s from these eukaryotic organisms indicates evolutionary and functional conservation of this important group of 5′-exonucleases. The biochemical properties of hExo1 are similar to that of *Sc*Exo1. The enzyme is a non-processive 5′-3′ double-stranded and single-stranded DNA exonuclease. In *S. cerevisiae,* the 5′-3′ exonuclease activity is 2-fold more on double-stranded DNA than on single-stranded DNA. The purified protein from *S. pombe*, however, has very low 5′-3′ single-stranded DNA exonuclease activity. It is more specific for the double-stranded DNA. It is possible that the truncated protein purified from *S. pombe* lacks a structure responsible for 5′-3′ single strand DNA exonuclease activity. Resembling the *Sc*Exo1, human exonuclease 1 has both activities but prefers a double-stranded DNA substrate to single-stranded DNA. However, it does not discriminate between RNA and DNA (Fig. 4 and see below). Because the observed sequence conservation of the putative nuclease domains between the exonuclease-1 and flap endonuclease-1 (see Refs. 28, 29, 60, and 62 for additional information on FEN-1 nuclease), we tested the flap endonuclease activities of human exonuclease-1 with a typical flap DNA substrate with DNA or RNA as the single-stranded portion and human flap endonuclease-1 as a positive control. The result indicates that the enzyme does not possess a specific flap endonuclease activity. A detailed description of the human exonuclease 1 substrate specificity will be published elsewhere.

Human Exonuclease 1 Functionally Complements the Yeast DNA Recombination and UV Resistance and Mutation Avoidance—Expression of human exonuclease 1 in yeast complements several characterized phenotypes in the *exo1*Δ mutants. hExo1 complements the function of *Sc*Exo1 in recombination (Table II). The recombinational rate decreases 2-fold when the *EXO1* gene was deleted in our assay system, where the repeat fragments were about 400 bp. 7-fold decrease was reported when the repeats were extended to 900 bp (45). It was also demonstrated *in vitro* that the enzyme significantly promotes the recombination of the two DNA fragments with end overlapping sequence. As revealed in *S. pombe*, mRNA level and enzyme activity was induced during meiosis (44, 45) and in *D. melanogaster,* it is only expressed in the early embryogenesis of the female germline (46). We speculate that the functional 5′-exonuclease 1 may be involved in recombinational mismatch correction during meiosis in mammalian cells as no such eukaryotic enzyme has been identified to date.

In *S. cerevisiae*, it has been shown that Exo1 is involved in UV DNA damage repair, which is distinct from nucleotide excision repair pathway (49). In this study, we revealed that *hEXO1* complements the function of *Sc*Exo1 in UV DNA damage repair, indicating that hExo1 could be involved in UV DNA damage repair in human cells as well. Among the different UV

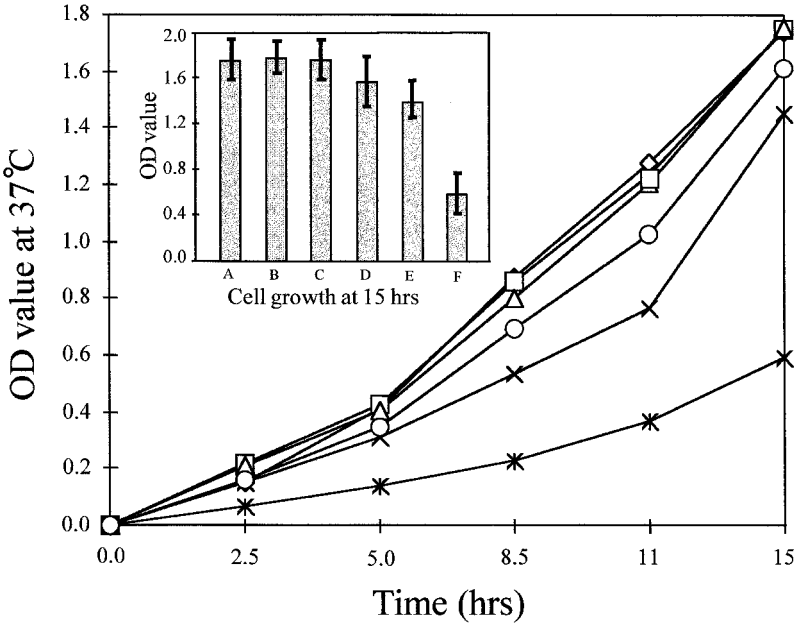

FIG. 7. **Cell growth curve of rad27 mutant strain with and without the introduction of *Sc*- and h*EXO*ls.** Cells were grown in SD-URA liquid medium at 37 °C starting with the same amount of initial culture. Samples were taken in every 2–3-h time intervals to measure the OD units. The data were averaged from three independent experiments. ◇, AB15Ba (wt/pDB20); □, AB15Bb (wt/PDB-*ScE*); Δ, AB15Bc (wt/PDB-*hE*); *, IC2-1a (rad27Δ/PDB20); ○, IC2-1b (rad27Δ/PDB-ScE); ×, IC2-1c (rad27/PDB-hE).

TABLE III
Expression of hEXO1 reverses cycloheximide resistance of the exo1 null Saccharomyces cerevisiae

Strains	Genotype	Mutation frequency ($\times 10^{-8}$)	Relative rate
AB15Be	Wild type/pRS314	0.22 ± 0.05	1
AB1Ba	*exo1*/pRS314	6.21 ± 0.81	29
AB1Bb	*exo1*/PRS-ScE	0.28 ± 0.09	1.3
AB1Bc	*exo1*/PRS-hE	1.12 ± 0.21	5.1

DNA damage repair pathways, the XPG/Rad2 nucleotide excision repair pathway is a major pathway. In humans, dysfunction of this repair pathway results in xeroderma pigmentosum syndrome. Epistatic analysis in yeast showed that the UV DNA damage repair involving Exo1 is independent of XPG/Rad2 nucleotide excision repair and other UV DNA damage repair pathways (51). The minor UV sensitivity is due to the failure of DNA replication-driven bypass compensation mechanism.

Failure of mismatch correction during DNA recombinational repair increases mutation rate. Deletion of *ScEXO1* caused a 29-fold increase in cycloheximide resistance in *S. cerevisiae*. The assay is based on the fact that mutation in the yeast gene *CYH2* can lead to resistance to cycloheximide, an inhibitor of eukaryotic protein synthesis. The gene product of *CYH2* is ribosomal protein L29, a component of the 60 S ribosomal subunit. In most cases, resistance to cycloheximide is due to a transversion mutation resulting in replacement of a glutamine by a glutamic acid in position 37 of Leu-29 (57). Mutation rates in a *ScEXO1* deletion mutants assayed with another system such as CAN^r and HOM3 are only increased 6-fold compared with wild type (59). The different mutation rates determined may be due to the following reasons: 1) the CAN^r assay is a forward assay but not particularly sensitive to the point mutations such as transversions; 2) the HOM3 assay is sensitive to single base deletions and additions, which happens frequently in mismatch repair defective cells. This is a reversion assay. The mutant strain causes at a high rate the deletion of a single T in a run of 7 Ts. Otherwise, the HOM3 protein does not tolerate much sequence variation elsewhere. Table III also shows that *HEX1* complements the function of *Sc*Exo1 and reverses the mutator phenotype in *exo1*Δ mutants. This result implies that hExo1 could play a role in the mutation avoidance mechanism in human cells. The result is also consistent with the role of *exo1* in mismatch correction in *S. pombe* (44). The protein interacts with Msh2 (48, 49) and double knockout of *EXO1* and *MSH2* showed the similar mutation rate as the single knockout of *MSH2*. Altogether, the evidence available indicates that these two proteins may work together in a mutation avoidance mechanism.

Human Exonuclease 1 Backs Up the Function of Flap Endonuclease 1 in DNA Replication RNA Primer Removal—Fig. 4 shows a time-dependent ribonuclease activity of hExo1 on RNA/DNA hybrid duplexed to a DNA template regardless of multiple or monoribonucleotide proceeding the DNA portion. This activity may indicate the involvement of Exo1 in RNA primer removal during lagging strand DNA synthesis. In prokaryotes, the role of RNA primer removal during DNA lagging strand synthesis is played by the 5′-3′ exonuclease activity of DNA polymerase I (24–27). In eukaryotes, the function of removing RNA primers of the lagging DNA strand is performed by the FEN-1/Rad27 nuclease in two alternative pathways. One pathway is that RNase H removes all ribonucleotides except for the last one adjunct to DNA fragments; then, the last ribonucleotide is removed by FEN-1/Rad27 nuclease. The second pathway is performed by FEN-1/Rad27 nuclease via its flap endonuclease activity independent of RNase H. If there are only these two pathways involved in the removal of the RNA primers, disruption of the *FEN-1/RAD27* gene in yeast should completely block the RNA primer removal pathways and the null mutant may be lethal. However, disruption of *ScRAD27* caused a conditional lethality phenotype (39–42): the null mutant cells could still grow at the normal temperature (30 °C) but become lethal at 37 °C. For this reason, a third pathway for removing RNA primers has been proposed (29) and Exo1 is one of the best candidates for this pathway due to its 5′-3′ exonuclease activity. The hExo1 was as efficient in removal of ribonucleotides as deoxynucleotides, resembling the function of Rad27 in RNA primer removal pathway in our experiments. As we have also shown, the overexpression of *HEX1* as well as *ScEXO1* in yeast *S. cerevisiae* cells suppressed the temperature-sensitive phenotype of rad27/fen-1 null mutant at 37 °C. This result indicates that the Exo1 enzyme is involved in removal of RNA primers during lagging strand DNA synthesis. Bambara's hypothesis (29) on the candidate nucleases for ini-

tiator RNA removal is supported by the fact that overexpression of *EXO1* suppresses the temperature-sensitive phenotype of rad27/fen-1 null mutant at 37 °C and the lethality of the double knockout of *RAD27* and *EXO1* (48).

Recently, however, it has also been proposed that the unligated Okazaki fragments of the *rad27* null mutant can also be repaired by the recombinational double strand break repair pathway (43). This hypothesis is based on the fact the disruption of *FEN-1/RAD27* can elevate recombination rate by 20–30-fold (43).[2] In addition, the double knockout of *RAD27* and *RAD51* or *RAD52* (the key protein components of double strand break repair) is lethal. The double strand break repair pathway requires 5′-3′ exonuclease to generate the single-stranded 3′-overhangs for strand invasion. *EXO1* is a good candidate for this process as well. Meanwhile, if *EXO1* is the only enzyme so far available, which possibly generates the single-stranded 3′-overhangs in a eukaryotic cell, deletion of *EXO1* would block the double strand DNA break repair pathway. The double knockout of *RAD27* and *EXO1* would result in the cell lethality as that of *RAD27* and *RAD51* or *RAD52*. This hypothesis is supported by the fact that the knockout of *EXO1* reduced recombination rate and the double knockout of *RAD27* and *EXO1* is indeed lethal. However, this hypothesis does not explain the result that the overexpression of *EXO1* suppressed the temperature-sensitive phenotype of rad27 null mutant unless the overexpression of Exo1 facilitates an alternative process for the RNA primer removal. With the above information in mind, we propose that Exo1 might be involved in two pathways relevant to DNA replication. On one hand, it could back up the function of FEN-1 to remove RNA primers; on the other hand, it is involved in the double strand DNA break repair pathway to generate 3′-overhang, correct the mismatches, and remove unligated Okazaki fragments in the *rad27* mutant.

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[2] J. Qiu and B. Shen, unpublished data.