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# Excision of misincorporated ribonucleotides in DNA by RNase H (type 2) and FEN-1 in cell-free extracts.

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ABSTRACT

Misincorporated ribonucleotides in DNA will cause DNA backbone distortion and may be targeted by DNA repair enzymes. Using double-stranded oligonucleotide probes containing a single ribose, we demonstrate a robust activity in human, yeast and *E. coli* cell-free extracts that nicks 5' of the ribose. The human and yeast extracts also make a subsequent cut 3' of the ribonucleotide releasing a ribonucleotide monophosphate. The one-nucleotide gap produced is an ideal substrate for polymerase and ligase to complete a proposed repair sequence that effectively replaces the ribose with deoxyribose. Screening of yeast deletion mutant cells reveals that the initial nick is made by RNase H(35), a RNase H type 2 enzyme, and the second cut is made by Rad27p, the yeast homologue of human FEN-1 protein. RNase H type 2 enzymes are present in all kingdoms of life and are evolutionarily well conserved. We knocked out the corresponding *rnhb* gene in *E. coli* and show that extracts from this strain lack the nicking activity. Conversely, a highly purified archaeal RNase HII type 2 protein has a pronounced activity. To study substrate specificity, extracts were made from a yeast double mutant lacking the other main RNase H enzymes (RNase H1 and RNase H(70)), while maintaining RNase H(35). It was found that a single ribose is preferred as substrate over a stretch of riboses, further strengthening a proposed role of this enzyme in repair of misincorporated ribonucleotides rather than (or in addition to) processing RNA/DNA hybrid molecules.

## Introduction

There is presently a nearly total lack of information about repair of deoxyribose modifications in DNA. Such modifications can be caused by external agents such as oxidizing agents and ionizing radiation (1-3), and can also occur naturally by misincorporation of ribonucleotides into DNA during DNA replication (4). The presence of ribose in DNA is a hindrance to formation of normal B-form DNA as evidenced by the structure of RNA/DNA hybrid molecules (5) and consequently a single ribose in DNA will result in a local DNA backbone distortion (6). Other bulky modified sugars are also likely to cause backbone distortions and it can be hypothesized that they pose a hindrance for DNA polymerases and that they can be mutagenic.

Progressive DNA and RNA polymerases are similar in structure and belong to the same class of proteins (4), probably with a common evolutionary origin (7). The specificity towards deoxyribonucleotides (dNTPs) or ribonucleotides (rNTPs) has been found to be determined by subtle differences at the active site (4). Gao *et al.* (8) could largely eliminate the discrimination between the rNTPs and dNTPs by introducing a single amino acid change in a reverse transcriptase, and similar observations in mutant polymerases have recently been made by several investigators (7, 9-13). Based on such observations, it has been suggested that the discrimination against ribonucleotides by DNA polymerases is largely accomplished by a "steric gate" that will not give enough space for the 2' hydroxyl group present in rNTPs (4). However, the discrimination against rNTPs is not 100%, and detectable incorporation of rNTPs has been found *in vitro* using purified DNA polymerases with a wide variety of discrimination factors ranging from a few thousandfold (7, 11) to several millionfold (13). However, it is presently not known to what extent ribonucleotides are misincorporated into DNA during normal *in vivo* DNA replication. The intranuclear milieu contains both ribonucleotides and deoxyribonucleotides with the ribonucleotide concentration generally higher than the deoxyribonucleotide concentration (14). The deoxyribonucleotides are produced from the ribonucleotide pool by the enzyme ribonucleotide diphosphate reductase. This enzyme can be inhibited in eukaryotic cells by hydroxyurea (HU), which blocks DNA replication when given to cell cultures in sufficient concentration.

Gao and Hoff (15) mutagenized a viral polymerase to increase ribose misincorporation during viral DNA replication *in vivo*. Although some minus strand DNA was synthesized with significant ribose incorporation in virally infected cells, the synthesis was not completed and the mutation was lethal. A possible explanation is that ribose in the template DNA strand acts as a replication block. Here we demonstrate in cell-free extracts an activity that excises a single ribonucleotide from DNA and identify the enzymes involved.

## Materials and Methods

**Probes with misincorporated ribose.** Two partly complementary 100-mer oligonucleotides (**Figure 1**, probe 1) were extended with a modified T7 DNA polymerase (Sequenase Version 2, USB) in a 50 µl reaction mixture containing 20 mM Tris-HCl pH 7.5, 2 mM $MnCl_2$, 5 mM DL-isocitrate, 5 mM DTT, 100 µM dGTP, dATP and dTTP, 3 µM dCTP, 3 µM [$\alpha$-$^{32}$P]rCTP (800 Ci/mmole) (Amersham Pharmacia Biotech), 1 pmole annealed oligonucleotide and 13 units of Sequenase. Reaction was for 30 min at 37°C followed by addition of 100 µM dCTP and continued incubation for 20 min. Non-incorporated $^{32}$P was then completely removed by three consecutive Sephadex G50 spin columns. The discrimination factor against rCTP compared to dCTP under these conditions was about 300-fold. Incorporation of $^{32}$P-labeled rGTP, rATP or rUTP was performed with the same strategy as for rCTP.

A 16-mer primer annealed to a 32-mer template (**Figure 1**, probe 2) was extended in the reaction mixture specified above modified with the addition of 50 mM NaCl and excluding dCTP. Incubation was for 30 min at 37°C followed by two consecutive Sephadex G25 spin columns.

Primers 3 and 4 (18-mers) containing either a single ribose modification at the 3' end (**Figure 1**, probe 3) or consisting exclusively of ribonucleotides (**Figure 1**, probe 4) were obtained from Integrated DNA Technologies (IDT). They were annealed to a 5' phosphorylated 40-mer template and extended using Klenow polymerase in a 100 µl reaction mixture containing 10 mM Tris-HCl pH 7.9, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, 200 µM dATP, dTTP, dCTP and dGTP, 37 pmole annealed template and 5

units of Klenow polymerase. Incubation was for 30 min at 37°C. The extended oligonucleotides were then purified with a Quickstep2 purification kit (Edge Biosystems) to remove protein, ssDNA and low molecular weight products. Small aliquots of the purified double-stranded probe were subsequently 5' end-labeled with $^{32}$P using T4 polynucleotide kinase. Non-incorporated $^{32}$P was removed by two consecutive Sephadex G25 spin columns.

**_E. coli_ strains.** An *E. coli rnhB* knockout strain was constructed using the recombination system of Yu *et al.* (16). The host strain DY330 (W3110 Δ*lacU169 gal490 λc1857 Δ(cro-bioA)*) was obtained from Dr. Donald Court, NIH. The method employs an inducible λ bacteriophage recombination system (RED) by which a targeted genomic sequence can be replaced by a drug resistance cassette. We first used PCR to construct an ampicillin-resistance cassette with flanking sequences that targeted upstream and downstream regions of the *E. coli rnhB* gene. The primers were 5'ATT TGT TTA TCC GCA CAC GCA GCT GGT TGC GGG TGT GGA TCA TTC AAA TAT GTA TCC GCT C and 5' AAG TCC CAG TGC GCG TTT GAC AGG CCC AAA GCT GCG CCG AAG AGT GGT AGT CTC TTG ATC using a previously amplified ampicillin-resistant cassette *amp* from pBluescript (Stratagene) as template. The underlined sequences are complementary to the *amp* template, while the non-underlined sequences target the *rnhB* gene The gel-purified cassette was subsequently electroporated into DY330 cells with heat-induced RED system followed by selection of ampicillin-resistant colonies as described by Yu *et al.* (16). Three ampicillin-resistant colonies were picked and tested for Rnase HII function.

An *E. coli rnhA1* mutant strain FB2 (CGSC# 6585) and parental rnhA+ strain KS351 (CGSC# 6586) was obtained from *E. coli* Genetic Stock Center, Yale University. Additional markers in these strains were *lacZ482*(Am), *relA1, spoT1, rha-9* and *thi-1*.

**Yeast strains and procedures.** Construction of a complete set of yeast mutants deleted for each known non-essential gene or open reading frame has been described in detail elsewhere (17). In these strains, each deleted gene is replaced by a functional gene, *KANMX4,* that confers resistance to the antibiotic geneticin (G418). A haploid set of these deletions was obtained from Research Genetics, Huntsville, AL (now Invitrogen

Corporation, Carlsbad, CA). Appropriate deletion strains were used for initial biochemical assays. Standard yeast genetic techniques (18) were used to demonstrate co-segregation of the *rnh35* deletion with the absence of ribonucleotide excision activity in meiotic tetrads. The *rnh35Δ* and other deletion segregations were followed by scoring geneticin resistance, using 100 μg/ml of geneticin (obtained from Sigma) added in solution to cooled YEPD medium (18) prior to pouring plates. Genetic crosses with tetrad analysis were used to construct the *rnh1Δ rnh70Δ* double deletion mutant strain, and the *rnh1Δ rnh35Δ rnh70Δ* triple deletion mutant. To construct the triple mutant, we replaced by transformation the geneticin-resistance gene in the *MATα rnh35Δ* mutant with a functional *LEU2* gene, (provided by Jim Brown (Department of Radiation Oncology, Stanford University School of Medicine) so that we could use leucine prototrophy to monitor the segregation of the *rnh35* deletion independently of the *rnh1* and *rnh70* deletions. We confirmed the genotypes of double and triple mutant strains by appropriate back-crosses to the single mutants.

**Cell-free extracts.** A 10 liter HeLa cell pellet (obtained from NIH National Cell Culture Center) was washed 3 times in PBS, treated with a hypotonic buffer (10 mM Hepes-KOH pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT and protease inhibitors) and the cells broken with a Dounce homogenator (20 strokes with tight pestle). The homogenate was spun 2K for 15 min followed by 12.5K for 20 min and the supernatants collected and pooled as a low salt extract. The pellet was resuspended in a high salt buffer (20 mM Hepes-KOH pH 7.9, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 25% glycerol and protease inhibitors), stirred on ice for 30 min and spun at 12.5K for 30 min. The supernatant was recovered as a high salt extract and stored aliquoted at −70°C. A small scale cell-free extract from human Jurkat cells was also prepared with the same protocol.

Strains of *Saccharomyces cerevisiae* were grown at 30°C in 40 ml of liquid YEPD growth medium (18) and collected in late log phase. After 3 washes in $H_2O$, the pellets were resuspended in one volume 20 mM Tris-HCl pH 7.5, 100 mM NaCl and protease inhibitors. To the slurry was added an equal volume glass beads (size 425-600 μm, acid-washed, Sigma) and the mixtures were vortexed in 1.5 ml Eppendorf tubes at maximum speed with intermittent cooling on ice for a total of 2 minutes to break the cells. The tubes

were then spun at 14K for 10 min at 4°C. The supernatants were collected, supplemented with glycerol to a final concentration of 20% v/v, and stored at -70°C.

*E. coli* cells were grown in 100 ml Luria Broth until late log phase, washed twice in 10 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA and the pellets frozen to $-70$°C. Thawed pellets were suspended in 1 ml 20 mM Tris-HCl pH 7.5, 100 mM NaCl, 2 mM EDTA, 10 mM DTT, 0.5% NP-40, 10% glycerol, 5 mg/ml lysozyme and protease inhibitors and incubated on ice for 15 min. The cells were then disrupted by sonication on ice for 1 min, spun at 14K for 10 min and the supernatants collected and stored at $-70$°C.

**In vitro assay.** In a standard 10 μl reaction, 1-10 fmole $^{32}$P-labeled probe was mixed with 1 μl of variously diluted cell-free extracts in a buffer containing 10 mM Tris-HCl pH 7.5, 35 mM KCl, 1 mM DTT, 1mM EDTA, 4 mM MgCl$_2$, 5 % glycerol. The mixture was incubated for 15 min at 37°C and the reaction terminated by the addition of an equal volume of loading buffer (80% formamide, 10 mM EDTA pH 8, 1mg/ml xylene cyanol FF, 1 mg/ml bromophenol blue). Separation of the products was achieved on denaturing polyacrylamide gels using standard techniques, followed by exposure on phosphor screens and evaluation on a Phosphor Imager (Molecular Dynamics).

# Results

**Probes.** We constructed a set of oligonucleotide probes that contain a single ribonucleotide (or in one case a stretch of ribonucleotides) (**Figure 1**). Probes 1 and 2 are obtained by *in vitro* misincorporation of $^{32}$P-labeled ribonucleotides during primer extension, using a modified T7 DNA polymerase in a Mn-containing buffer. Probe 1 is a 172 bp double-stranded oligomer obtained by extending two partly complementary 100-mers. Random misincorporation of any $^{32}$P-labeled rNTP present in the reaction results in labeled probes containing a ribose, while products formed without ribose misincorporation will be unlabeled. Since misincorporation is a rare event, most labeled molecules will have a single ribose. Probe 2 is obtained by extending a 16-mer primer on a 32-mer template containing a single location of G for misincorporation with $^{32}$P-rCTP. This probe will thus have a site-specific location for a single ribose with a $^{32}$P present immediately 5' of the ribose sugar. Probe 3 is a 40-mer obtained by extending a

commercially obtained primer (Integrated DNA Technologies) that is modified with a single ribose at the 3'end, using Klenow polymerase. This probe is $^{32}$P-labeled at the 5' end with T4 polynucleotide kinase before use. Probe 4, which is used for comparative purposes, is obtained by extending an 18-mer RNA primer with deoxyribonucleotides on a 40-mer template. Probes 3 and 4 are identical in sequence, with probe 3 containing a single ribose while probe 4 contains a stretch of 18 ribonucleotides on the 5' end annealed to the complementary normal DNA strand. We verified that ribose was present in the probes by specific cleavage 3' of the ribose by potassium hydroxide or piperidine (an example is seen in Figure 3 below).

**Nicking activity in cell-free extracts.** Cell-free extracts were prepared from human cells (Jurkat cells and HeLa cells), yeast cells (*Saccharomyces cerevisiae*), and *E. coli* using standard techniques (19), employing Dounce homogenization (human cells), disruption with glass beads (yeast cells), or sonication (*E. coli*). Double-stranded probes 1 and 2 containing a single ribose with adjoining $^{32}$P were then incubated with the cell-free extracts and analyzed on denaturing PAGE gels. A robust nicking activity was detected in all cell-free extracts, with a nicked molecule seen as product using lower extract concentrations and with release of free cytosine monophosphate (CMP) at higher extract concentrations (**Figure 2**). The activity is dependent on magnesium and is completely abolished by EDTA (data not shown). The release of CMP was not caused by general degradation of the oligomers as could be determined by the use of end-labeled probes (results shown below). The initial nick occurred 5' of the $^{32}$P label as evidenced by the use of probe 1 (**Figure 3**). As can be seen, KOH hydrolysis generated as expected a nick at the 3' side of the ribose sugar, generating labeled products of 100-172 bp. On the other hand, the nicking activity in the extracts generated $^{32}$P-labeled products of 1-72 bp as is expected from nicking on the 5' side of the $^{32}$P in this substrate. The $^{32}$P-CMP seen for higher extract concentrations co-migrated with authentic CMP in several gel systems (data not shown). These results strongly suggest an activity that initiates the repair of misincorporated ribose in DNA. The one-nucleotide gap that results from excision of the ribonucleotide could probably be filled in by one of several pathways, for example by polymerase β and ligase III in association with XRCC1 in human cells (20). **Figure 4** shows the indicated locations of the nicks in relation to the ribose sugar. The position of

the initial nick will classify the responsible enzyme as a class II endonuclease, belonging to the same family as for example the human AP endonuclease and most RNase H enzymes.

**Screen of yeast deletion mutants.** To identify the enzyme that causes nicking we screened selected mutants from an annotated library of yeast deletion strains recently constructed by an international consortium (17). This strain collection contains more than 4,600 separate mutants involving almost every open reading frame in the genome that can be deleted without causing loss of viability. There are fewer than 100 known or putative endonucleases in *S. cerevisiae* and only some of them are known as class II endonucleases. In the first test of 10 nuclease knockout strains we included the deletion mutants for genes encoding each of the three yeast RNase H enzymes previously known to degrade the RNA strand in a DNA/RNA hybrid molecule, i.e., *rnh1Δ, rnh35Δ* and *rnh70Δ*. As shown in **Figure 5**, the *rnh35Δ* strain completely lacked the nicking activity at a single ribose, while all the other strains in the panel had normal levels. We later confirmed this finding by back-crossing the *rnh35Δ* knockout strain with wild-type cells and finding that the lack-of nicking phenotype cosegregated as expected with the drug resistance marker used to replace the *rnh35* gene (data not shown). Based on primary sequence analysis (21, 22), RNase H(35) is a type 2 RNase H enzyme, while RNase H1 and RNase H(70) belong to type 1.

In previous studies related to removal of Okazaki primers (22, 23), the human enzyme FEN-1 was found to be able to remove a single ribonucleotide at the 5' end of DNA. This makes FEN-1 a candidate enzyme for the second cut that we observe in substrates with a single ribose. For this reason we also studied yeast *rad27* deletion mutant cells, since yeast Rad27p is the homologue of human FEN-1 (24). As seen in **Figure 6A**, the second cut was indeed severely compromised in these knockout cells, suggesting that FEN-1 is the major enzyme involved in removal of the ribonucleotide after the initial nick has been made by the RNase H(35). Since the activities of the two enzymes depend on salt concentration, the activity was also measured in a wide range of salt concentrations in the assay. As seen in **Figure 6B**, the initial nicking activity is similar in wild type and *rad27* knockout strains, while the release of free CMP is virtually absent in extracts of the *rad27* mutant. The results support a novel DNA repair pathway

acting on misincorporated ribonucleotides in DNA that consists of an initial nick by an RNase H type 2 enzyme followed by removal of the ribonucleotide by FEN-1 (Rad27p), as depicted in **Figure 4**.

**Substrate specificity.** Using probe 1 we tested nicking activity in probes containing misincorporated rC, rG, or rA and found that crude extracts from HeLa cells, yeast, and *E. coli* could nick the probes, with slight preference for rG and rA over rC. Interestingly, misincorporated rU was also found to be a good substrate in human and yeast extracts, including yeast extracts lacking Apn1p, the main AP endonuclease in yeast cells, while extracts from RNase H(35) knockout cells lacked the nicking activity (data not shown). This suggests that the ribose repair activity described here, rather than uracil DNA glycosylase and AP endonuclease, predominantly acts at such sites.

Using probes 3 and 4, we also compared the efficiencies of nicking by RNase H(35) at a single ribose with nicking of a RNA/DNA hybrid molecule as has been mainly studied previously (6, 22, 23). Using extracts from a *rnh1Δ rnh70Δ* double knockout strain, lacking the other two main RNase H enzymes present in yeast cells, we find that the single ribose is cut 3-fold more efficiently than all cuts together within the stretch of 18 ribonucleotides present in probe 4 (**Figure 7**). This further supports the notion that a main function for this enzyme is in repair of single misincorporated ribonucleotides. We also crossed the *rnh1Δ rnh70Δ* double mutant strain with a *rnh35Δ* strain, and observed viability and normal colony size in all 84 spore clones isolated from 21 meiotic tetrads from the triple heterozygote. This demonstrates viability for each of the double mutant genotypes and the triple mutant involving these three genes. We were readily able to identify a triple mutant spore-clone, and observed normal colony formation on plates in this strain. Hence, viability of the three single mutant *rnh* strains in yeast cannot be attributed to compensatory functions of either of the others in any essential process.

**Nicking by bacterial and archaeal Rnase HII.** Based on sequence homology (23), the *E. coli rnhB* gene product RNase HII corresponds to the yeast RNase H(35) and human RNase H1, all type 2 enzymes. We therefore constructed an *E. coli rnhB* gene knockout strain using the RED recombination system of Yu *et al.* (16). As we expected, extracts made from *rnhB* knockout strains lack the nicking activity at a single ribose present in wild type cells as well as in *rnhA-* cells lacking the RNase HI activity in *E. coli*

(**Figure 8**). The *E. coli* RNase HII enzyme is very weak in degrading RNA/DNA hybrid molecules, with less than 1% of the activity of the well-known *E. coli* RNase HI enzyme (25). However, the nicking activity at a single ribose is very robust, pointing to a strong preference for a single ribose or deoxyribose/ribose link for this enzyme.

To further test the substrate specificity of this type of highly conserved RNase H type 2 enzymes, we used a cloned and purified RNase HII enzyme from the archaea *Pyrococcus furiosus* (a generous gift from Dr. John Tainer at Scripps). This enzyme is very similar to the *Archaeoglobus fulgidus* RNase HII, studied by Chapados *et al.* (22). On SDS-PAGE we found the purity of the enzyme to be about 90%. We tested the activity of this preparation on our ribose-containing substrates, and found a robust nicking activity down to a $10^{-5}$ dilution (original concentration 50 mg/ml) (**Figure 9**).

## Discussion

**A repair pathway for removal of misincorporated ribose in DNA**.

Our results show biochemical evidence for a DNA repair pathway in bacteria, yeast and human cells for removal of ribose residues in DNA. Such residues may be misincorporated during normal DNA replication, and it may be essential for cells to remove such residues. Based on the *in vitro* results, the pathway looks intriguingly simple. An endonuclease (RNase H, type 2) cuts 5' to the lesion followed by FEN-1 (or homologous enzyme) cutting 3' to the lesion leaving a one-nucleotide gap. The gap has the proper end-groups to be a direct substrate for a DNA polymerase and DNA ligase without any further modifications. In human cells DNA polymerase β and DNA ligase III in association with XRCC1 can complete repair at such sites (24). We believe the suggested simplicity of this repair pathway may hold true in prokaryotic systems. However, as has been generally demonstrated by other DNA repair pathways, a new level of complexity typically comes into play in higher eukaryotic systems. An indication of this is the presence of a second smaller subunit of the mammalian RNase H1 type 2 enzyme (26) with hitherto unknown function. It is the large subunit of the mammalian protein that has substantial sequence homology to yeast and prokaryotic RNase H type 2 enzymes (26) and the cloned large subunit functions as an RNase H enzyme on its own *in vitro*.

11

The suggested pathway for repair of sugar lesions has similarities to repair of AP sites, where an AP-endonuclease makes a first cut at the same position relative to the damaged nucleotide as the RNase H type 2 enzyme. However in this case DNA polymerase β is generally believed to remove the abasic sugar fragment present on the 5' end and repair can be completed without participation of FEN-1 (27).

**RNase H enzymes and their proposed functions.**

The functional definition of RNase H enzymes is the ability to endonucleolytically degrade the RNA strand of a DNA/RNA hybrid molecule. RNase H enzymes are present in all kingdoms of life, often in multiple forms, but their cellular functions are still not well understood. Based on the primary amino acid sequence, two major unrelated families of RNase H enzymes are known, proposed to be called type 1 and type 2 (21, 22). Type 1 enzymes have been proposed to be involved mainly in functions related to transcription, while the predominantly suggested function of type 2 enzymes is in removal of Okazaki primers during lagging strand DNA replication (22, 23). The main support for this function is given by *in vitro* studies of hybrid molecules that simulate the situation of an RNA primer extended as DNA on a DNA template. This substrate is preferred by the enzyme over a simple RNA/DNA hybrid molecule, with the strongest nicking occurring one base 5' of the RNA-DNA link (6, 22, 23). The last remaining ribonucleotide still attached to the DNA can then be removed by FEN-1 (23). However, evidence for this function for RNase H type 2 enzymes *in vivo* is lacking and, as an alternative pathway, removal of Okazaki primers is believed to be efficiently accomplished by strand displacement followed by cutting by FEN-1, thus not requiring the activity of the RNase H enzyme in the process (23). An alternative view that these enzymes are involved in removal of misincorporated ribose in DNA has been expressed peviously (6, 28, 29). In particular the early work by Eder *et al.* (28) shows that purified human RNase H1 (a type 2 enzyme) is able to incise at the site of a single ribose, as opposed to *E. coli* RNase H1 (a type 1 enzyme) that requires a stretch of at least 4 ribose nucleotides for activity. This ability, which is studied in more detail in the present paper, suggests a possible repair function of misincorporated ribose for type 2 enzymes. However this possible function has not been emphasized before either in literature about

RNase H enzymes, or in literature about DNA repair. In recent years it has become evident that type 2 enzymes are highly conserved and universally present in organisms of all kingdoms with no known exceptions (26). A search of current databases identifies over 100 RNase H type 2 enzymes with significant sequence homology from Rickettsia to Homo sapiens, indicating an important biological function.

The phenotypic properties of RNase H(35) type 2 knockout cells of the yeast *Saccharomyces cerevisiae* have recently been studied (23, 29, 30). Such knockout cells have normal growth characteristics, arguing against an essential role for the enzyme in DNA replication. However, in a double mutant combination *rnh35Δ* has been shown to further slow the reduced growth rate of *rad27Δ* mutants at $30^0$, and this has been interpreted as implying an accessory role in replication (23). Also, *rnh35Δ* mutants are sensitive to hydroxyurea (HU) (29), which is an inhibitor of ribonucleotide reductase, the enzyme that produces deoxyribonucleotides from ribonucleotides. High levels of HU may result in the collapse of replication forks and the sensitivity to HU can be argued to imply a function in replication, such as Okazaki primer processing. However, equally possible is the notion that depletion of deoxyribonucleotides promotes an increase in misincorporation of ribonucleotides into DNA, and lacking the ability to repair such misincorporation will result in sensitivity to HU. Knockout cells also have a moderate increase in spontaneous mutation rate (23) which also is consistent with a repair function. The spontaneous mutations are mainly frameshifts in the form of small deletions, reportedly with a preference for 4-basepair deletions (30). Knockout cells are also moderately sensitive to EMS, an alkylating agent, suggesting a repair deficiency of lesions other than ribose (29).

The structure of the archaeal *Archaeoglobus fulgidus* Rnase HII protein, which belongs to the family of type 2 enzymes, has recently been resolved at 1.95 Å resolution (22). Among the most interesting features is a DNA binding cleft with evolutionarily conserved charged amino acids proposed to form a phosphate ruler motif that interacts in particular with the distorted backbone resulting from the inability of a RNA/DNA hybrid to form B-form DNA. From such a mode of substrate discrimination based on backbone distortion, it can be speculated that the substrate specificity would be quite wide and could include other sugar modifications in addition to ribose.

**Acknowledgements**

**Reference list.**

1. von Sonntag, C. & Schulte-Frohlinde, D. (1978) *Mol. Biol. Biochem. Biophys.* **27,** 204-226.

2. Isildar, M., Schuchmann, M. N., Schulte-Frohlinde, D. & von Sonntag, C. (1981) *Int. J. Radiat. Biol.* **40,** 347-354.

3. von Sonntag, C. (1984) *Int. J. Radiat. Biol.* **46,** 507-519.

4. Joyce, C. M. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 1619-1622.

5. Horton, N. C. & Finzel, B. C. (1996) *J. Mol. Biol.* **264,** 521-533.

6. Eder, P. S., Walder, R. Y. & Walder, J. A. (1993) *Biochimie* **75,** 123-126.

7. Patel, P. H. & Loeb, L. A. (2000) *J. Biol. Chem.* **275,** 40266-40272.

8. Gao, G., Orlova, M., Georgiadis, M. M., Hendrickson, W. A. & Goff, S. P. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 407-411.

9. Sousa, R. & Padilla, R. (1995) *Embo J.* **14,** 4609-4621.

10. Tabor, S. & Richardson, C. C. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6339-6343.

11. Astatke, M., Ng, K., Grindley, N. D. & Joyce, C. M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 3402-3407.

12. Gardner, A. F. & Jack, W. E. (1999) *Nucleic Acids Res.* **27,** 2545-2553.

13. Bonnin, A., Lazaro, J. M., Blanco, L. & Salas, M. (1999) *J. Mol. Biol.* **290,** 241-251.

14. Kornberg, A. & Baker, T. A. (1992) *DNA replication* (Freeman, New York).

15. Gao, G. & Goff, S. P. (1998) *J. Virol.* **72,** 5905-5911.

16. Yu, D., Ellis, H. M., Lee, E. C., Jenkins, N. A., Copeland, N. G. & Court, D. L. (2000) *Proc. Natl. Acad. Sci. USA* **97,** 5978-5983.

17. Winzeler, E. A. *et al*. (1999) *Science* **285,** 901-906.

18. Kaiser, C., Michaelis, S. & Mitchell, A. (1994) *Methods in yeast genetics* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY).

19. Deutscher, M. P. (1990) *Guide to Protein purification* (Academic Press).

20. Kubota, Y., Nash, R. A., Klungland, A., Schar, P., Barnes, D. E. & Lindahl, T. (1996) *Embo J.* **15,** 6662-6670.

21. Ohtani, N., Haruki, M., Morikawa, M., Crouch, R. J., Itaya, M. & Kanaya, S. (1999) *Biochemistry* **38,** 605-618.

22. Chapados, B. R., Chai, Q., Hosfield, D. J., Qiu, J., Shen, B. & Tainer, J. A. (2001) *J. Mol. Biol.* **307,** 541-556.

23. Qiu, J., Qian, Y., Frank, P., Wintersberger, U. & Shen, B. (1999) *Mol. Cell Biol.* **19,** 8361-8371.

24. Lieber, M. R. (1997) *Bioessays* **19,** 233-240.

25. Itaya, M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 8587-8591.

26. Frank, P., Braunshofer-Reiter, C., Wintersberger, U., Grimm, R. & Busen, W. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 12872-12877.

27. Bennett, R. A., Wilson, D. M., 3rd, Wong, D. & Demple, B. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 7166-7169.

28. Eder, P. S. & Walder, J. A. (1991) *J. Biol. Chem.* **266,** 6472-6479.

29. Arudchandran, A., Cerritelli, S., Narimatsu, S., Itaya, M., Shin, D. Y., Shimada, Y. & Crouch, R. J. (2000) *Genes Cells* **5,** 789-802.

30. Chen, J. Z., Qiu, J., Shen, B. & Holmquist, G. P. (2000) *Nucleic Acids Res.* **28,** 3649-3656.

**Figure legends**

**Figure 1**. Primers used for construction of probes containing a single ribose residue (probe 1 –3) or a stretch of ribose residues (probe 4). Capital letters are used for deoxyribonucleotides and lower case letters for ribonucleotides. A triangle (Δ) indicates position of ribose misincorporation for probe 2 and location of ribose residue in probe 3.

**Figure 2**. Nicking of probe 2 by crude cell-free extracts. Lanes 1 and 8: no extract. Lanes 2-7: increasing amounts of HeLa cell extract (.001 - .1 µl/assay). Lanes 9 – 15: increasing amounts of *S. cerevisiae* extract (.004 - .5 µl/assay). CMP = cytosine monophosphate.

**Figure 3**. Nicking of probe 1. Lanes 1-4: control probe with no ribose incorporation. Lanes 5-12: with ribo C incorporation. Lanes 1 and 5: no extract. Lanes 2 and 6: probe treated with KOH. Lanes 3 and 7-11: incubation with various amount of Jurkat crude extract. Lanes 4 and 12: incubation with heat-inactivated extract.

**Figure 4**. Excision of ribonucleotide from oligomer probes by crude cell-free extracts.

**Figure 5**. Crude extracts from ten *S. cerevisiae* knockout strains were assayed for nicking activity using probe 2. Each strain was assayed with 2 extract concentrations (1 µl and 0.1 µl/assay). *RNH1*, *RNH35* and *RNH70* are the genes for the three known RNase H type enzymes present in *S. cerevisiae. NGL1*, *NGL2*, *NGL3* and *YEN1* are putative endonuclease genes as determined by sequence homology. *APN1* and *APN2* are AP-endonuclease genes and *RAD1* is a gene for a 5'-endonuclease that functions in nucleotide excision repair. Note absence of nicking in the extract generated from the *rnh35Δ* knockout strain. The amount of protein in the extracts was in the range of 1.4 – 2.1 mg/ml.

**Figure 6   A.** Increasing amount of extract from a *rad27Δ* knockout cell line was incubated with probe 2. Compared to extracts with wild-type *RAD27* (Fig 5), only very

limited amount of CMP is seen, with the majority of product being the 16-mer even at

high concentration of extract/assay. **B.** Products generated as a function of salt
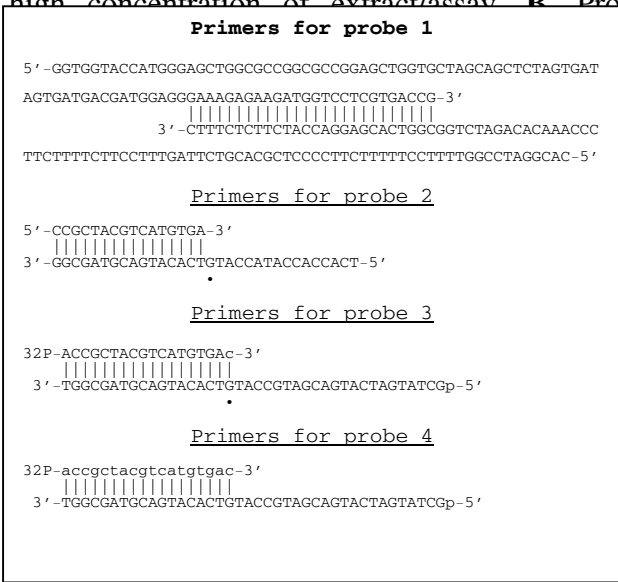
...er panel) and *rad27Δ* knockout cell line

...e reaction using extract from the *rad27Δ*

...ncreasing amounts of crude extract from a

...aseH(70). The single ribose in probe 3 is

...st lane) by increasing amounts of *E. coli*

...endent *rnhB* knockout strains and a *rnhA-*

strain. Laddering is due to non-specific nucleases in the extracts. Note the lack of specific

nicking for the *rnhB* knockouts.

```
                    Primers for probe 1

5'-GGTGGTACCATGGGAGCTGGCGCCGGCGCCGGAGCTGGTGCTAGCAGCTCTAGTGAT
AGTGATGACGATGGAGGGAAAGAGAAGATGGTCCTCGTGACCG-3'
                    ||||||||||||||||||||||||
               3'-CTTTCTCTTCTACCAGGAGCACTGGCGGTCTAGACACAAACCC
TTCTTTTCTTCCTTTGATTCTGCACGCTCCCCTTCTTTTTCCTTTTGGCCTAGGCAC-5'

                    Primers for probe 2

5'-CCGCTACGTCATGTGA-3'
   ||||||||||||||||
3'-GGCGATGCAGTACACTGTACCATACCACCACT-5'
               .

                    Primers for probe 3

32P-ACCGCTACGTCATGTGAc-3'
    ||||||||||||||||||
 3'-TGGCGATGCAGTACACTGTACCGTAGCAGTACTAGTATCGp-5'
                   .

                    Primers for probe 4

32P-accgctacgtcatgtgac-3'
    ||||||||||||||||||
 3'-TGGCGATGCAGTACACTGTACCGTAGCAGTACTAGTATCGp-5'
```

**Figure 9**. Nicking of probe 2 by purified *P. furiosus* RNase HII.  Leftmost lane: no

enzyme. Next three lanes: 1 μl enzyme added after dilution of the original stock solution

$10^{-4}$ to $10^{-6}$ as indicated. Original stock solution had a concentration of 50 μg/μl.
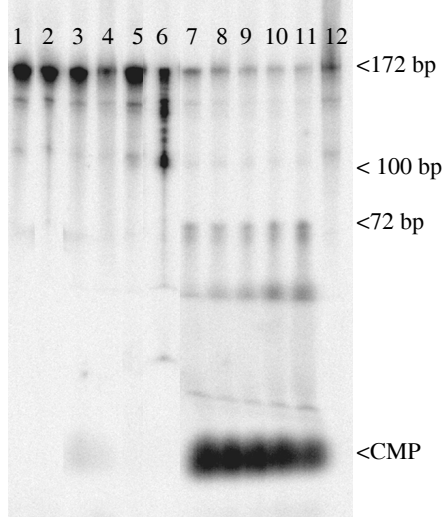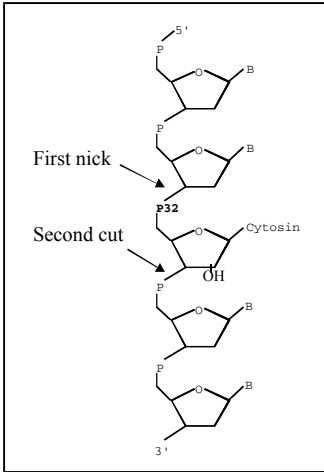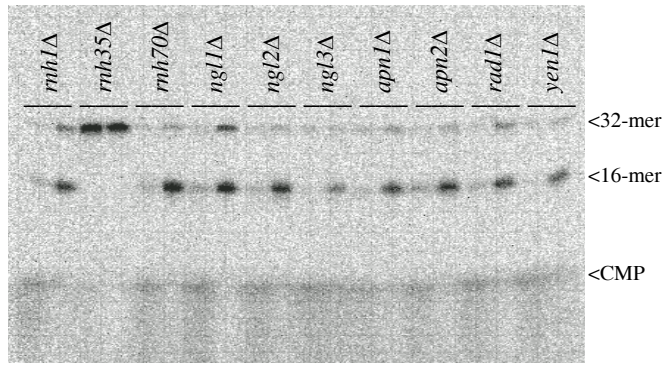
**Figure 1**

**Figure 2**

**Figure 3**

First nick

P32

Second cut

Cytosin

OH

Figure 4

**Figure 5**

A

< 32-mer

< 16-mer

< CMP

B

yeast WT

Percent

uncut
cut
CMP

Salt (mM)

yeast rad27

Percent

rad27Δ

uncut
cut
CMP

Salt (mM)

**Figure 6**

23
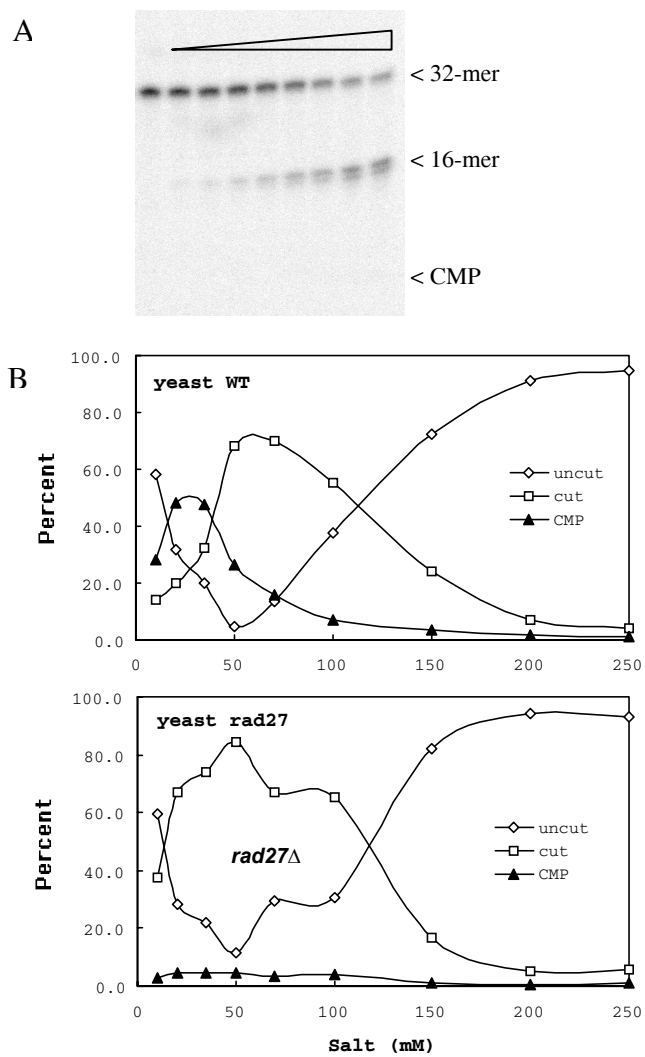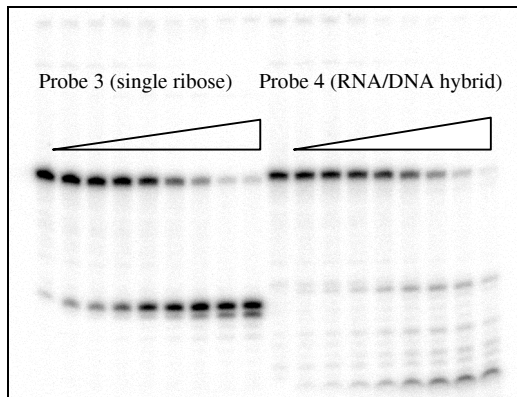
**Figure 7**

**Figure 8**

**Figure 9**