The absence of ribonuclease H1 or H2 alters the sensitivity of *Saccharomyces cerevisiae* to hydroxyurea, caffeine and ethyl methanesulphonate: implications for roles of RNases H in DNA replication and repair

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Abstract

***Background*: RNA of RNA-DNA hybrids can be degraded by ribonucleases H present in all organisms including the eukaryote *Saccharomyces cerevisiae*. Determination of the number and roles of the RNases H in eukaryotes is quite feasible in *S. cerevisiae*.**

***Results*: Two *S. cerevisiae* RNases H, related to *Escherichia coli* RNase HI and HII, are not required for growth under normal conditions, yet, compared with wild-type cells, a double-deletion strain has an increased sensitivity to hydroxyurea (HU) and is hypersensitive to caffeine and ethyl methanesulphonate (EMS). In the absence of RNase H1, RNase H2 activity increases, and cells are sensitive to EMS but not HU and are more tolerant of caffeine; the latter requires RNase H2 activity. Cells missing only RNase H2 exhibit increased sensitive to HU and EMS but not caffeine**

***Conclusions*: Mutant phenotypes infer that some RNA-DNA hybrids are recognized by both RNases H1 and H2, while other hybrids appear to be recognized only by RNase H2. Undegraded RNA-DNA hybrids have an effect when DNA synthesis is impaired, DNA damage occurs or the cell cycle is perturbed by exposure to caffeine suggesting a role in DNA replication/repair that can be either beneficial or detrimental to cell viability.**

Introduction

Ribonucleases H are present in most species in one or more forms (Crouch & Dirksen 1982; Crouch 1990; Ohtani *et al*. 1999). These enzymes have been shown to be involved in retroviral replication and have been implicated in a number of different processes in bacterial cells (Champoux 1993; Hostomsky *et al*. 1993; Hughes *et al*. 1998; Kogoma & Foster 1998). Examination of these enzymes in eukaryotes has been a long-standing effort extending back to their original description in calf thymus cells (Hausen & Stein 1970). We have been using *Saccharomyces cerevisiae* as a source for study of these interesting enzymes that are capable of recognizing a duplex of RNA-DNA and degrading only the RNA strand. With the genomic DNA sequence known for *S. cerevisiae* as well as other organisms, we are provided with an opportunity to use computer searches for genes whose sequences are similar to those of known RNase H encoding genes. Moreover, the ability to manipulate the genes of *S. cerevisiae* provides us with the opportunity to ask questions as to the necessity of these genes for viability and the abundance or timing of appearance of the various RNases H as different cellular changes take place.

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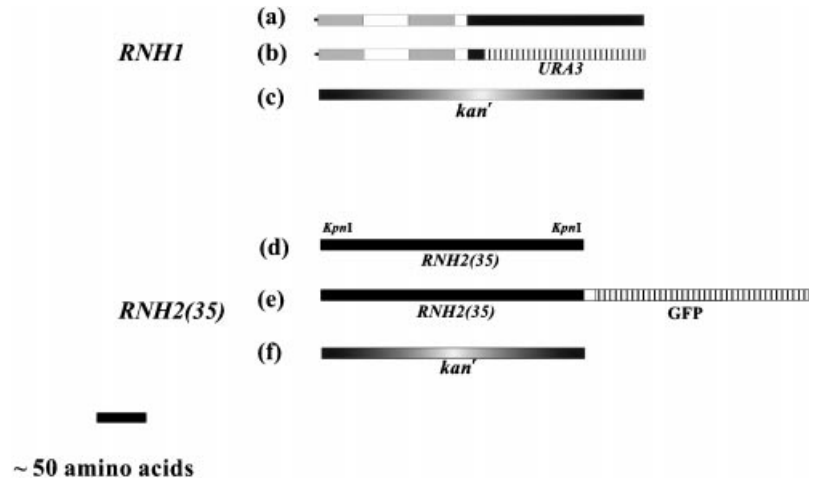

Figure 1 RNase H genes: wild-type, deletion-insertion mutants and protein fusion constructs. All drawings represent the DNA sequence of the gene described. Regions of the proteins corresponding to various domains are indicated by different shading. *S. cerevisiae RNH1*: (a) Wild-type gene/protein: Grey boxes represent the double-stranded RNA binding domains, the black region is the RNase H domain, and the white regions are connecting regions. (b) Mutant generated by insertion of the *S. cerevisiae URA3* gene between the first *Hin*dIII site and the *Xba*I site in the RNase H domain. (c) Deletion of the entire gene and replacement with the *kan*^{r} gene. This deletion was generated as described, removing the entire coding region. *S. cerevisiae RNH2(35)*: (d) Wild-type gene/protein: The black area represents the *RNH2(35)* gene/protein. (e) *RNH2(35)-GFP* fusion. The *GFP* gene was connected to the C-terminus of the *RNH2(35)* gene by eliminating the TGA stop codon and adding a linker DNA sequence (GCGTCGACTCTAGAGGAT CCCCGGTACCGGTCGCCACC) with the GFP coding sequence starting with the ATG codon of the E-GFP gene (Clontech, Palo Alto, CA). (f) Deletion-insertion of the *RNH2(35)* gene replaced the *Kpn*I fragment with a 1.4-kb the *kan*^{r} gene-cassette generated from the pFA6-kanMX6 plasmid using the two primers (AACCCGGGGGTACCCGTACGCTG CAGGTCGACGGATCC and AACCCG GGGGTACCATCGATGAATTCGAGC TCGTTTAAAC) for PCR amplification. Following amplification, the DNA was digested with *Kpn*I and cloned into the plasmid carrying the 3 kbp *Eco*RI fragment containing the *RNH2(35)* gene.

The RNases H1 and H2 of eukaryotes are similar in sequence to RNases HI and HII, respectively, of bacteria (Cerritelli & Crouch 1998a; Ohtani *et al.* 1999). In addition to the RNase H domain, eukaryotic RNases H1 have a domain(s) capable of binding dsRNA and RNA-DNA hybrids (Fig. 1 and Crouch & Cerritelli 1998; Cerritelli & Crouch 1995, 1998, Cerritelli *et al.* 1998).

The RNase HII-encoding gene *rnhB* of *Escherichia coli* was the first of the RNase HII genes to be identified, and was obtained by complementation of the RNase HI-defective *E. coli* strains (Itaya 1990). Other genes with similar sequences were also obtained following either complementation selection or by computer assisted searches for DNA/protein sequences related to *E. coli* RNases H. In one instance, partial protein sequence data obtained from highly purified calf thymus RNase HII was used to query the databases and the cDNAs for human RNase HI were identified (Frank *et al.* 1998a). Homologues of the RNase HII of *E. coli* were identified in *S. cerevisiae*, human, mouse, several bacteria and several Archae (Crouch & Cerritelli 1998; Itaya *et al.* 1999; Ohtani *et al.* 1999). Further studies on some bacterial and Archael RNase HII-like proteins showed there are two classes now called RNase HII and RNase HIII that differ in divalent metal ion preference, sites of cleavage within sequences of RNA of RNA-DNA hybrids, and specific activities.

The results obtained for the RNase H-like genes from *B. subtilis* (Itaya *et al.* 1999; Ohtani *et al.* 1999) point out that identification of a protein as being an active RNase H simply by sequence similarities is insufficient. A protein with strong sequence similarity to *E. coli* RNase HI has no demonstrable RNase H activity. A similar limitation of computer searches is apparent in the absence of TY element RNases H in BLAST searches using numerous RNase HI/1 proteins as query sequences even though these RNases H have the conserved catalytic residues (data not shown).

We have studied two *S. cerevisiae* RNases H. These genes have been called *RNH1* (Itaya *et al.* 1991; Crouch & Cerritelli 1998), producing a protein similar in sequence to *E. coli* RNase HI and *RNH(35)* (Frank *et al.*

1998b), yielding a protein similar in sequence to *E. coli* RNase HII. For a uniform nomenclature, we will use the name RNase H2(35) for the 35 kDa protein from *S. cerevisiae* whose sequence is similar to RNase HII of *E. coli*. *S. cerevisiae* RNase H2(35) has been studied by Frank *et al.* (Frank *et al.* 1998b) who showed that deletion of the *RNH2(35)* gene is nonessential for viability but the level of RNase H activity decreased to 30–40% of wild-type strain extracts. We have examined several properties of yeast strains with deletions in one or both of these genes that suggest RNase H1 is a housekeeping gene and RNase H2 is involved in DNA replication and repair which is possibly mediated by RNA.

Results

Viability of strains carrying deletions of *RNH1* and *RNH2(35)*

We examined more than one type of deletion of the *RNH1* gene of *S. cerevisiae*; one obtained through the *S. cerevisiae* deletion project and one we generated that deletes the RNase H region, leaving the dsRNA-binding region intact (Fig. 1b, c). Strains carrying either of these mutations are viable in several different genetic backgrounds. Strains with deletions of the *RNH2(35)* gene (Fig. 1f) of *S. cerevisiae* are also viable. We have found that double deletion strains are also viable, indicating that cells have other means of processing RNA-DNA hybrids. Frank *et al.* have reported the presence of a third gene (*RNH70*) (Frank *et al.* 1999) that could possibly supply RNase H activity for any cellular process requiring such an activity, or there may be additional nucleases or helicases capable of removing RNA-DNA hybrids.

Changes in RNase H activity resulting from mutations in *RNH1* and *RNH2(35)* of *S. cerevisiae*

We have employed several assays for measuring the RNase H activity of various proteins; the most straightforward of which is to make a lysate and assay the supernatant for the ability of the protein(s) to degrade the RNA of RNA-DNA hybrids to acid soluble products (liquid assay). A second assay is to lyse the cells by boiling in the presence of SDS and performing SDS-PAGE in a gel containing labelled RNA-DNA substrate. This latter we called the gel-renaturation assay because of its dependence upon the protein renaturing, degrading the substrate, and products exiting from the gel. We did not detect an decrease in total RNase H activity using the liquid assay from extracts obtained from *rnh1*Δ strains (Fig. 2A), indicating that RNase H activity employing the liquid assay is due almost entirely to enzymes other than RNase H1. Because RNase H1 associates tightly with nucleic acids, we thought that RNase H1 might be present mainly in the pellet fraction of the crude lysate used for the liquid assay. Therefore, we checked for the presence of RNase H1 in the pellet and supernatant fractions using the gel-renaturation assays and were able to detect the enzyme in both fractions (Fig. 2C). Thus, RNase H1 is present in the extracts but does not contribute in any significant way to the RNase activity detected using the liquid assay, even when we employed various concentrations of Mg^{2+} or used Mn^{2+} as the divalent metal ion (data not shown). It is not clear why this activity is masked (Fig. 2A), but Cazenave (Cazenave *et al.* 1994; Cazenave 1998) has noted a similar problem in detecting the RNase H activity of this type in *Xenopus laevis* oocyte extracts using a liquid assay when the renaturation gel assay shows the enzyme to be present. Two other aspects of the gel assay should be noted: (i) there are two bands of activity present, one corresponding to the full-length protein (39 kDa) and the other to a proteolytic product that is missing the first copy of the dsRNA-binding motif (30 kDa) and (ii) no other activity is detected by this assay as indicated in assays employing *rnh1*Δ strains (Fig. 2C) even when replacing Mg^{2+} by Mn^{2+} as the divalent metal ion (data not shown).

In contrast to *rnh1*Δ strains, RNase H activity is significantly reduced in *rnh2(35)*Δ-strains when using the liquid assay (Fig. 2A). The polyrA-polydT substrate is still degraded to some extent using extracts derived from *rnh1*Δ *rnh2(35)*Δ mutant strains. RNase H70 does not contribute to the RNase H activity that is detected in crude extracts (Frank *et al.* 1999). Thus, the source of this activity remains unknown but could be due to other enzymes that degrade the substrate but are not specific for the RNA of RNA-DNA hybrids.

Interestingly, RNase H activity increases when a *rnh1*Δ mutation is present in the strain. This increase is most likely due to RNase H2, since we see a corresponding increase (1.4–1.7-fold) in the level of an HA-tagged RNase H2(35) polypeptide in the *rnh1*Δ-strain (Fig. 2B) compared to the wild-type *RNH1* strain. Additionally, a strain carrying a deletion of the *RNH2(35)* gene has the same low level of RNase H activity, regardless of the status of the *RNH1* gene (Fig. 2A).

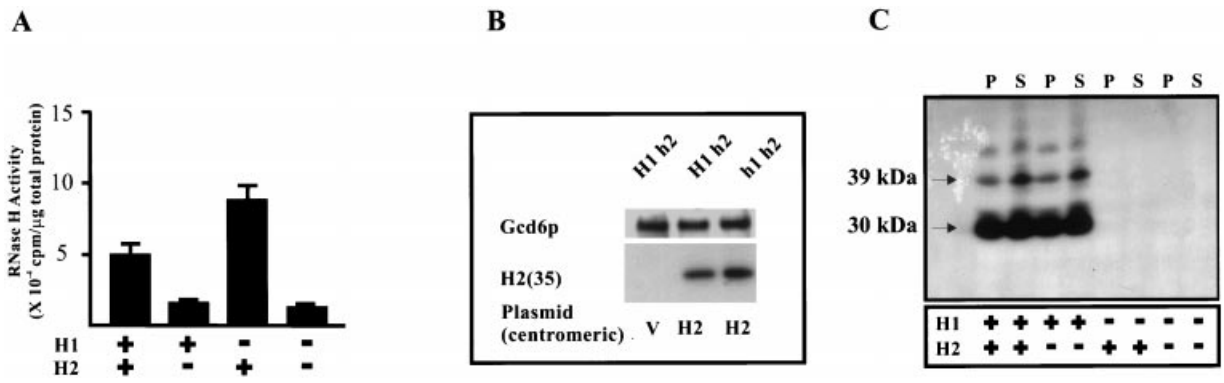

Figure 2 RNase H activity in wild-type and deletion strains. (A) Liquid assay for RNase H activity in crude lysates of *S. cerevisiae*. RNase H activity of total cell extracts of *S. cerevisiae* is shown. H1 and H2 indicate the presence (+) or deletion (−) of the *RNH1* and *RNH2(35)* gene is presents, respectively. Activity is expressed in relation to the total protein of the crude lysates. The data shown are the average of results from four different experiments. (B) Increase in RNase H2(35) in *rnh1Δ* strain as detected by western blot. Total protein extracted from cells that are H1 h2 [HIRO16D *RNH1 rnh2(35)Δ*], and h1h2[HIRO27AF *rnh1Δ rnh2(35)*Δ] containing a centromeric plasmid carrying the *RNH2(35)* gene tagged with two HA epitope encoding sequences were electrophoresed on a 10–20% gradient SDS-polyacrylamide gel, transferred to a PDF membrane by electroblotting and probed using anti-HA antibodies. Gcd6p was used as an internal control. (C) RNase H activity determined by the gel-renaturation assay. An autoradiogram of a gel following SDS-PAGE of extracts prepared as described in experimental procedure and electrophoresis in a 12% SDS-PAGE containing ^{32}P-labelled substrate. The data are shown as a negative image for clarity. H1 and H2 represent *RNH1* and *RNH2(35)* genes, respectively. P marks the lanes loaded with the pellet fractions following extraction and S lanes are those in which the supernatant fractions were loaded. Approximately equal amounts of protein were loaded on to each lane. Molecular masses of the two major forms of RNase H1 are indicated on the right of the figure. The slower migrating activity is most likely due to a dimeric form of the enzyme.

Expression of mRNA and RNase H activity during cell-cycle progression

Much of the speculation and data on the function of RNases H centres on their involvement in DNA synthesis. Early work indicated the presence of two types of RNase H in eukaryotic cells (Büsen & Hausen 1975). One type seemed to be correlated with the onset of DNA synthesis, while the second type increased when RNA synthesis was most abundant (Büsen *et al.* 1977). To make some inference as to the cellular roles performed by RNase H1 and H2(35), we examined the changes in mRNA levels for these genes as a function of the cell cycle. The amount of mRNA from the *RNH1* gene was relatively constant throughout the cell cycle (Fig. 3A), whereas transcripts of the *RNH2(35)* gene exhibited two periods of maximal expression, one in S-phase (DNA replication) and the second in the G2/M (repair) transition period (Fig. 3A). Transcript levels of *CDC28* (two peaks at G1 and G2) and *CLB1* (G2) were used for marking the stages of the cell cycle and *G3PDH* was used for normalization to a constant transcript. These results are consistent with those of Cho *et al.* (Cho *et al.* 1998) and Spellman *et al.* (Spellman *et al.* 1998) but the second peak observed for *RNH2(35)* mRNA, has not been reported.

The liquid assay carried out with ^{32}P-polyrA-polydT substrate to measure total RNase H activity throughout the cell cycle showed two peaks of activity at 15 and 90 min post α-factor release, corresponding to the two periods of maximal mRNA expression (Fig. 3B). Attribution of the RNase H activity detected at the 15 and 90 min time points to RNase H2 is based on the absence of these peaks in studies using an isogenic *rnh2(35)Δ*-strain (Fig. 3B). RNase H activity detected by the gel assay (RNase H1) indicated a fairly uniform enzymatic activity throughout the cell cycle for this protein as observed for the mRNA expression (Fig. 3C).

Cell localization of *S. cerevisiae* RNase H2(35)

For the RNases H to be involved in transcription, DNA replication or DNA repair, nuclear localization would be expected. Therefore, we determined the cellular location of RNase H2(35) by expressing a fusion protein containing RNase H2(35) at its N-terminus and the Green Fluorescent Protein (GFP) at the C-terminus. As can be seen in Fig. 4, the majority of the fusion protein is concentrated in the nucleus, whereas the GFP protein that is expressed without being fused to RNase H2(35) is distributed uniformly throughout the cell. Cell localization of the RNase H1 protein has been

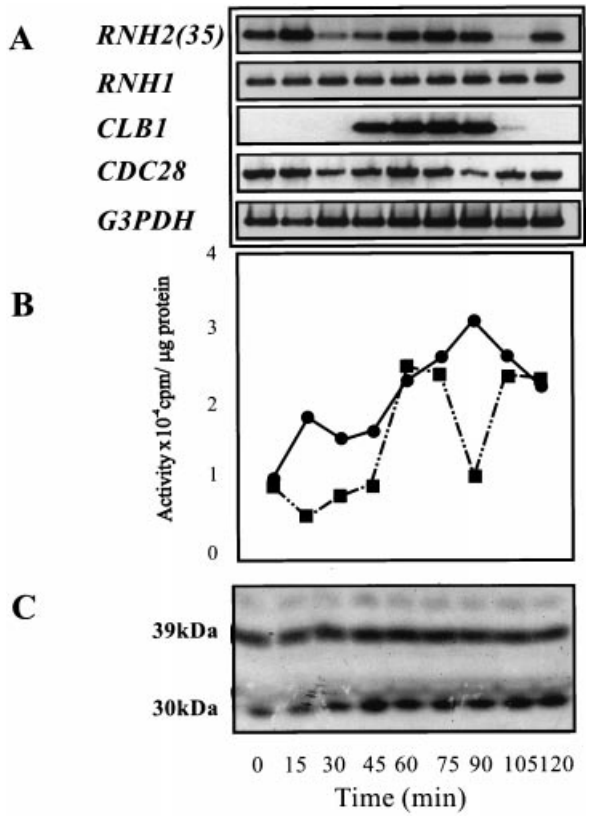

Figure 3 Regulation of RNH1 and RNH2(35) during cell cycle. (A) Expression of *RNH1* and *RNH2(35)* mRNAs *S. cerevisiae* strain X2180-1A was synchronized by the addition of α-factor, as described in Experimental procedures. Samples were taken at the times indicated, RNA extracted, and quantitative RT-PCR reactions were performed as described in Experimental procedures. The cell cycle was followed by observing the morphology of cells in a light microscope and counting the number of cells in various stages of budding. Under the conditions employed in our experiments, the cell cycle for X2180-1A was about 100–105 min in length. RNAs for control genes are *G3PDH* (a noncycling mRNA), *CLB1* (a gene encoding a cyclin expressed in the G2-phase of the cell cycle (Amon *et al.* 1993), and *CDC28* [a gene whose mRNA peaks at two different times during the cell cycle (Cho *et al.* 1998)]. (B) RNase H activity for RNH2(35)p as a function of cell cycle. Total cell extracts obtained at various time periods of the cell cycle were used to measure the activity by liquid assay using the ^{32}P-labelled polyrA-polydT substrate. Activity obtained for the wild-type strain (●), and for the *rnh2(35)* deletion strain (■) is indicated throughout various time periods of the cell cycle. (C) RNase H activity for RNase H1 as a function of cell cycle. Total cell extracts obtained at various time periods of the cell cycle were subjected to 12% SDS-PAGE. The gel containing ^{32}P-polyrA-polydT substrate was renatured as described in Experimental procedures following electrophoresis and an autoradiogram was obtained. The result is shown as a negative image for clarity. 39 kDa is the full-length protein and 30 kDa is a proteolytic fragment comprising the second copy of the dsRNA-binding domain and the RNase H domain (Fig. 1a).

studied in *Schizosaccharomyces pombe* (H. Yamada & R.J. Crouch, unpublished), in *X. laevis* oocytes (Cazenave *et al.* 1994; Cazenave 1998), and in calf thymus (Büsen 1980) and has been also found in the nucleus.

Phenotypes of RNH-mutant strains

Cell localization, appearance at various stages of the cell cycle and their specificity for the RNA of RNA-DNA hybrids support the notion that RNases H1 and/or H2 participate in replication and repair of DNA and possibly in transcription and recombination. Therefore, we examined *rnh1Δ*, *rnh2(35)Δ* and *rnh1Δ rnh2(35)Δ* strains for sensitivity to UV-irradiation and chromosome loss, and we observed no significant difference between the mutant and wild-type parental strains (data not shown). We also tested *rnh1Δ*-strains for alterations in mitotic and meiotic recombination and the stability of mitochondrial DNA. We noticed very little effect of the *rnh1Δ* mutation in either recombination or stability of mitochondria, although there was a slight increase in loss of mitochondrial function (ability to grow on glycerol) in the presence of ethidium bromide (data not shown). Others have reported an increase in mutation frequency for strains deleted for *RNH1* and *RNH2(35)* (Qiu *et al.* 1999). We continued our search by examining the susceptibilities of the *rnhΔ*-mutant strains to other chemicals that target processes in which RNase H might be involved, three of which showed differential effects on growth of the wild-type and mutant strains.

Sensitivity of *rnhΔ*-mutant strains to hydroxyurea, caffeine and ethyl methanesulphonate

Hydroxyurea

In the presence of HU, DNA replication is altered by depletion of dNTP precursors, causing cells to undergo slow process of DNA replication during the S-phase arrest (Weinert *et al.* 1994; Lydall & Weinert 1997). RNA-DNA hybrids may accumulate in the presence of HU, creating substrates for RNases H and strains deleted for *RNH1*, *RNH2(35)* or both might exhibit a noticeable phenotype. The cytotoxic effect of HU was tested by plating cells from different serial dilutions on YPD plates containing 100 mM HU. After two days, all cells exhibited similar growth on YPD plates but no colonies were visible for any strain on plates containing HU (Fig. 5A). On day 4, wild-type and the *rnh1Δ*-strains formed large colonies on plates containing HU,

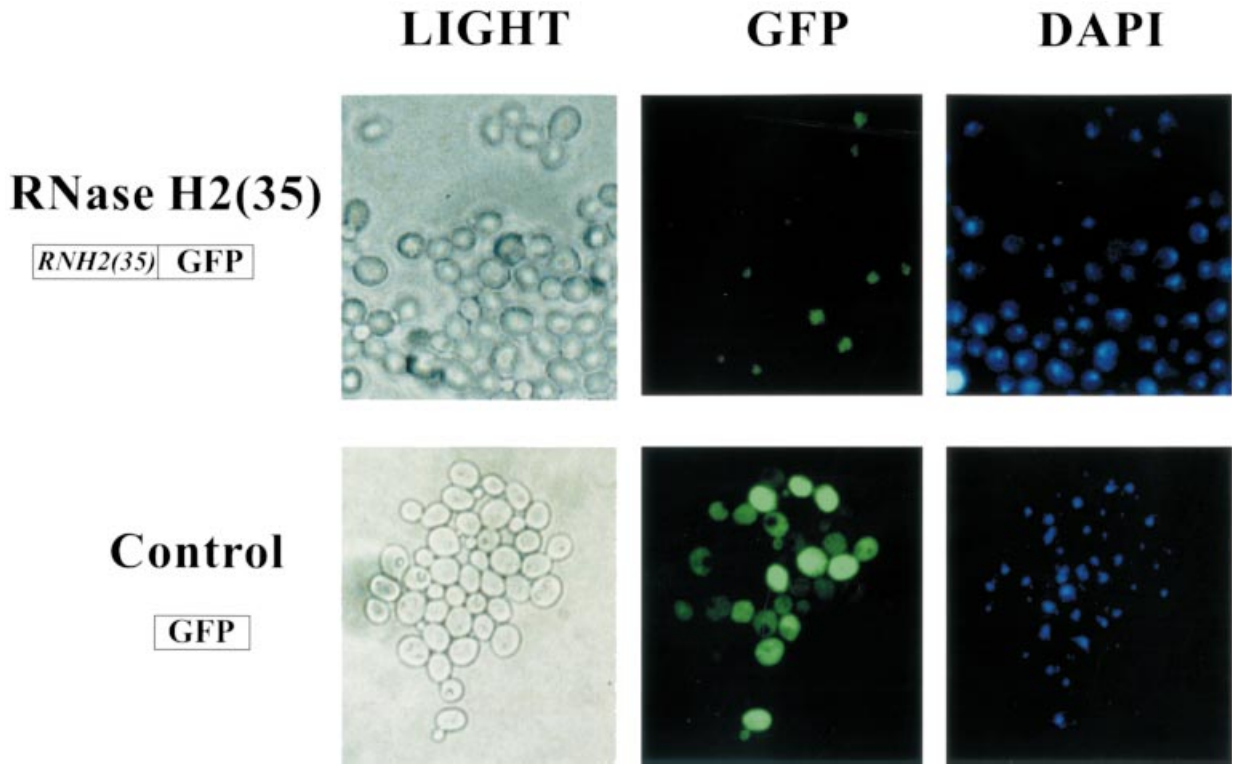

Figure 4 Cell localization of *S. cerevisiae* RNase H2(35). RNase H2(35) was expressed in cells as a fusion protein Fig. 1e. A plasmid bearing the *RNH2(35)* gene fused to the *GFP* gene was expressed as well as the *GFP* gene in the same vector not fused to the *RNH2(35)* gene. Light microscopy was used for visualization of cells.

in contrast to the two strains carrying the *rnh2(35)Δ* (Fig. 5A: h1 h2; H1 h2) that formed smaller colonies. The prolonged HU-mediated arrest occurring in *rnh2(35)Δ*-mutant strains implies that RNase H2 is important for overcoming the defect(s) created as a result of HU treatment. Eventually, the *rnh2(35)Δ*-mutant strains surmount the effects of HU treatment (see for example day 6 Fig. 5A), suggesting that an alternative, less-efficient mechanism can function in the absence of RNase H2.

Caffeine

Caffeine is an analogue of xanthine and has been known to interfere with several cellular processes, including delaying cell cycle progression, inhibition of cAMP phosphodiesterase, and interference with DNA repair and recombination pathways (Benko *et al.* 1997). Furthermore, caffeine treatment induces cells to enter mitosis before DNA replication is completed, thereby enabling cells to bypass the G2/M checkpoint.

To examine the effects of caffeine on strains carrying deletions of *RNH1*, *RNH2(35)* or both genes, we plated various numbers of cells on YPD plates containing caffeine in concentrations from zero to 20 mM and examined their growth after one to three days. No colonies appeared on plates containing 20 mM caffeine, even after five days [data not shown—see also Costigan *et al.* (1992)].

Strains that were wild-type for *RNH1* and *RNH2(35)* or *RNH1 rnh2(35)::kan* exhibited similar sensitivities to caffeine (Figs 5B, 10 mM). The double deletion-strain was significantly more sensitive to caffeine than any of the other strains. In contrast, the strain deleted for *RNH1* and wild-type for *RNH2(35)* showed greater tolerance for caffeine than the wild-type strain. In Fig. 5B (YS33, h1 H2), the *rnh1Δ RNH2(35)* strain exhibited much better growth at a 1 : 100 dilution on the plates containing 10 mM caffeine than did the other strains. Even on plates containing 5 mM caffeine, this strain formed colonies that were larger and more uniform in size than those of the other strains (data not shown). These results suggest that the absence of RNase H1 in a strain that has active RNase H2 diminishes the deleterious effect(s) of caffeine.

Ethyl methanesulphonate

Alkylating agents produce DNA adducts that are removed mainly by base excision repair or nucleotide excision repair. To test the sensitivity of the *rnhΔ*-strains,

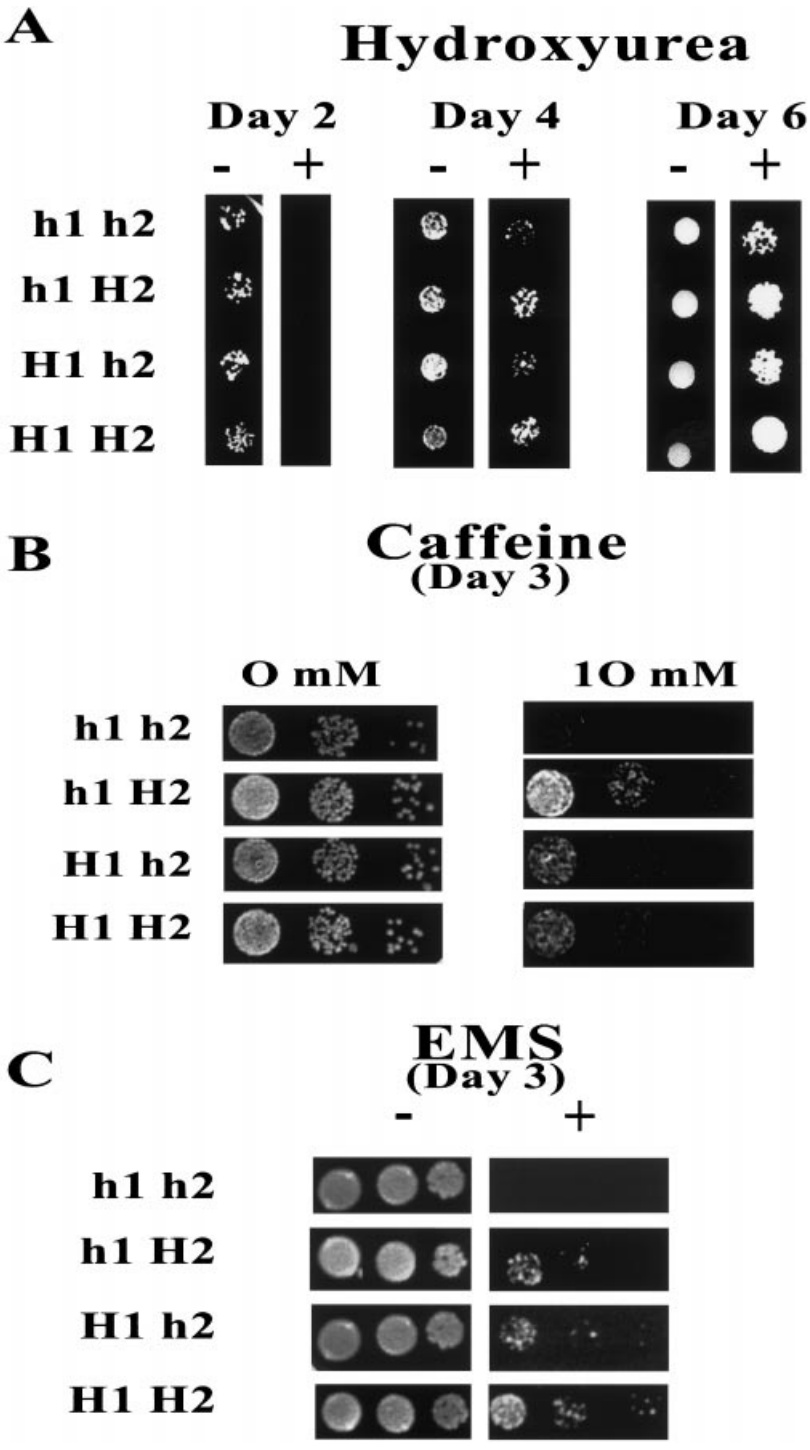

Figure 5 Effects of caffeine, hydroxyurea, and ethyl methanesulphonate on wild-type and RNH mutant strains of *S. cerevisiae*. (A) Hydroyurea treatment. Three μL of 1 : 1000 dilutions of log-phase cells were plated on YPD containing either no hydroxyurea (−) or 100 mM hydoxyurea (+). Growth was followed and cells photographed on days 2, 4 and 6. H1 indicates the wild-type *RNH1* gene, H2 the *RNH2(35)* gene. The corresponding lower case letters indicate the mutant form of the gene. (B) Caffeine. Three μL of dilutions (1 : 10, 1 : 100, and 1 : 1000—shown left to right for each strain) of log-phase cells grown at 32 °C in YPD medium were spotted on plates containing 0 or 10 mM caffeine. Photographs were taken at day 3. (C) EMS Log-phase cells grown at 32 °C in YPD medium were treated with EMS (3% V/V) for 1 h, centrifuged and resuspended in YPD. Three μL of dilutions (1 : 10, 1 : 100, and 1 : 1000—shown left to right for each strain) were spotted on YPD plates and the plates were photographed after three days growth at 32 °C.

we treated exponential phase cell cultures with ethyl methanesulphonate (EMS) and examined viability by plating on YPD plates. Each of the strains with a single *rnh* deletion grew less well than the wild-type strain while the double *rnh1Δ rnh2(35)Δ* strain grew very poorly (Fig. 5C). The synergistic effect observed with the double-mutant strain is sometimes an indication that each protein recognizes the same modification and resolves the damage using different pathways.

Discussion

RNases H of *S. cerevisiae* related in sequence to RNases HI and HII of *E. coli* have been examined at the protein and gene level. We have found that deletion of either the *RNH1* or *RNH2(35)* gene or both does not lead to inviability under a variety of normal growth conditions. Both proteins are nuclear in location, as would be expected for enzymes that recognize RNA-DNA hybrids, but each is expressed in a different temporal manner. RNase H1 is expressed throughout the cell cycle at a relatively constant level, while RNase H2(35) is most abundant during S-phase with a second peak of activity being present during the G2/M-phase. The appearance in S-phase is consistent with an interaction of this protein with Okazaki fragments whose removal may involve Rad27p (Bambara *et al.* 1997; Qiu *et al.* 1999). Interestingly, there is an increase in RNase H2 activity when the *RNH1* gene is deleted, suggesting that RNase H2 may replace RNase H1 in the degradation of some RNA-DNA hybrids. Even though RNase H1 activity (gel assay Fig. 3C) and mRNA (Fig. 3A) are relatively constant as the cells progress through the cell cycle, there remains the possibility that RNase H1 is regulated post-translationally, since we see no RNase H attributable to RNase H1 using a liquid assay, possibly due to the presence of an inhibitor. The *in situ* gel assay detects RNase H1 activity but not RNase H2 activity (Fig. 2C). RNase H70, a third RNase H of *S. cerevisiae,* was not detected by either assay [Frank *et al.* (1999) and Fig. 2A,C]. The gel assay relies on the ability of the protein to renature to form an active protein. Low abundance and poor refolding can limit the detection of activity in this assay but most important for recovering activity is the subunit structure of the enzyme. Multi-subunit proteins are unlikely to be able to form active enzymes due to a dissimilar migration of the two (or more) polypeptides in the SDS-gel. Likewise, the co-migration of an inhibitor and the protein would be unlikely, freeing the enzyme to be active in the gel assay.

rnh2(35)Δ strains exhibit increased sensitivity to HU

Deletion of the *RNH2(35)* gene but not the *RNH1* gene increases the sensitivity of *S. cerevisiae* to HU. Treatment of cells with HU leads to a depletion of dNTPs and results in an extension to the S-phase, with late firing origins being delayed to a much greater extent than early activated origins (Santocanale & Diffley 1998). Genes in which mutations result in an increase in sensitivity to HU fall into three categories: (i) genes whose products are required to arrest cell division prior to entry into M-phase (*MEC1* and *RAD53*), (ii) the *RAD24* group whose proteins are required for checkpoint control at G1/S and G2/M, and (iii) recombination and repair genes *RAD51* and *RAD52* (Allen *et al.* 1994; Desany *et al.* 1998). Thus, HU exposure not only causes delays in DNA replication but also results in DNA damage. The biphasic nature of expression of RNase H2(35) suggests the possibility that this protein participates in DNA replication during S-phase and DNA repair during G2/M-phase. Considerable evidence has been reported that RNase H2 is involved in removal of the majority of RNA primers of lagging strand DNA, and some of the sensitivity to HU could be related to this function.

Why is it that RNase H1 does not substitute for RNase H2 in the *rnh2(35)Δ* strain and maintain an HU-sensitivity that is similar to that of the wild-type strain? A requirement for specific recruitment of RNase H2 to its site of action could be one explanation. Yet another possibility is a difference in specificity of the two enzymes. Either of these possibilities could explain the lack of effect of deleting the *RNH1* gene on sensitivity to HU treatment (Fig. 5A). Various types of RNA-DNA hybrids that may be *in vivo* targets for these RNases H are shown in Fig. 6. The 'hybrid' containing a single ribonucleotide in an otherwise duplex DNA molecule has been shown to be a target for RNase H2 derived from human tissue culture cells (Eder & Walder 1991; Eder *et al.* 1993) as well as from calf thymus (Rumbaugh *et al.* 1997; Murante *et al.* 1998). It has been proposed that recognition of such substrates may be useful when a ribonucleotide is misincorporated during DNA synthesis. Cleavage to the 5′-side of the ribonucleotide would permit polymerization from the adjacent 3′-OH, displacing the ribonucleotide-containing strand with an enzyme such as Fen1/Rad27p, eliminating the single stranded tail. During normal growth, in the absence of RNase H2 activity, the frequency of misincorporating ribonucleotides is not a significant problem and could be eliminated by a second pathway. However, when dNTPs are limiting, as when the cells are exposed to HU, there may be a greater tendency for replication errors of this type, and RNase H2 could play a significant role in their removal. RNase H2 levels present in wild-type strains seems to be sufficient to have maximal response to HU-induced effects since the excess RNase H2 activity seen in the *rnh1Δ* strain (Fig. 2A) does not increase its resistance to HU exposure (Fig. 5A).

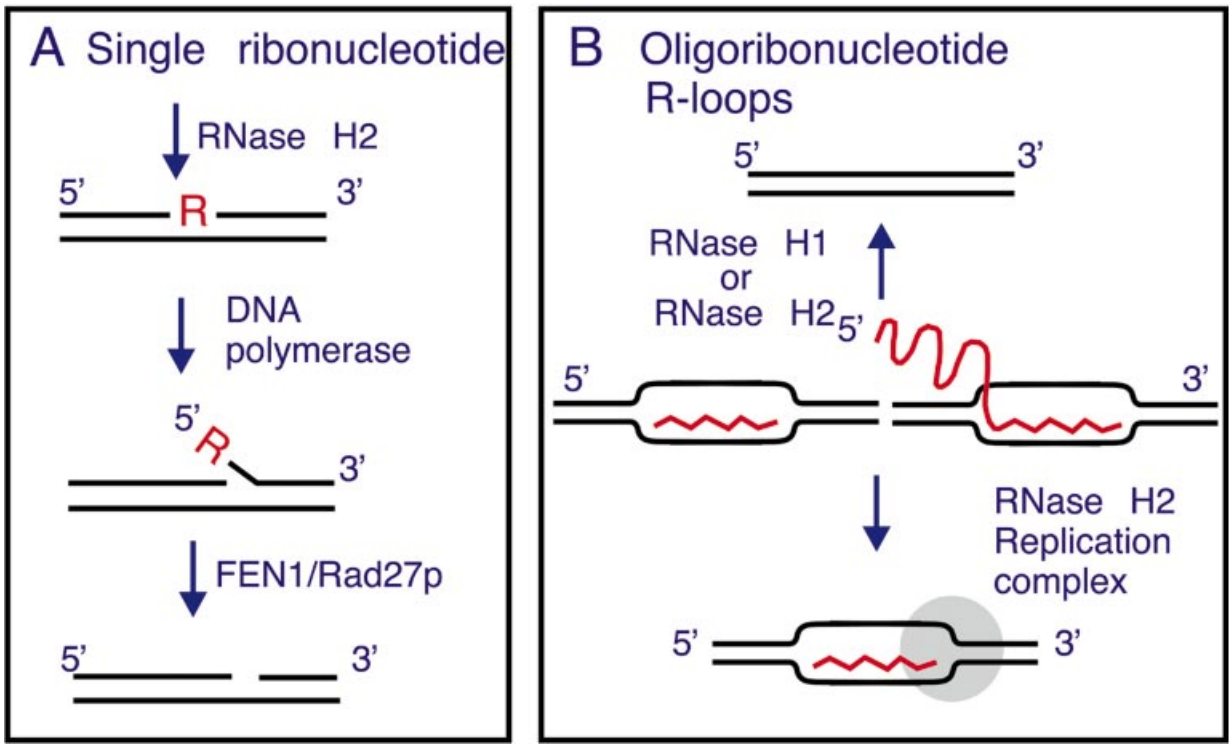

Figure 6 Substrates for RNases H. Two types of RNA-DNA hybrids are shown. (A) A single ribonucleotide in a DNA duplex possibly arising via mis-incorporation of a ribonucleotide during DNA synthesis or by ligating the single ribonucleotide left after RNase H2 cleavage of Okazaki fragment to the upstream DNA (Rumbaugh *et al*. 1997). Removal of the ribonucleotide could involve cleavage by RNase H2, polymerization of DNA and removal of the displaced ribonucleotide-containing DNA by FEN1/Rad27p. (B) R-loops formed either by transcription (right R-loop) or some other mechanism (left R-loop). RNases H could remove the RNA of either R-loop, returning the DNA to its normal duplex structure. RNase H1 would be expected to resolve R-loops exclusively to duplex DNA whereas RNase H2 might degrade the RNA or be involved in replication starting from either R-loop. The frequency of degradation vs. DNA replication could change depending on the condition of the cell at the time of R-loop formation.

Tolerance of cells to caffeine is influenced by RNases H1 and H2(35)

Caffeine treatment induces cells to enter premature mitosis, bypassing the G2/M checkpoint. Failure to repair defects in the DNA can lead to a loss of viability. If DNA synthesis were completed more quickly, or if DNA repair were more efficient, cells might be more tolerant of caffeine. In an *RNH1* wild-type strain, the presence or absence of RNase H2 activity has no discernable effect on growth in the presence of different caffeine concentrations. However, in a *rnh1Δ* background, the state of the *RNH2(35)* gene becomes important. When the wild-type *RNH2(35)* gene is present in a *rnh1Δ* strain, cells exhibit a greater tolerance to caffeine whereas in the *rnh1 rnh2(35)* double-deletion strain, growth of cells is very sensitive to caffeine (Fig. 5B). To explain these results, we suggest that in a *rnh1Δ* strain other enzymes recognize RNA-DNA hybrids normally degraded by RNase H1. Resolution of these hybrids by a non-RNase H1 pathway seems to involve RNase H2 since the *rnh1 rnh2(35)* double-deletion strain is much more sensitive than either the wild-type or *rnh2(35)Δ* strain. One possibility is that the increase in RNase H2 activity seen in the *rnh1Δ* strain is even better than RNase H1 in removing RNA-DNA hybrids. However, unless RNA-DNA hybrids remain in wild-type cells and contribute to caffeine-sensitivity, it is not obvious why caffeine-sensitivity would be reduced by a faster or better removal of RNA-DNA hybrids. A second possibility is that RNase H2, acting on these RNA-DNA hybrids, results in activation of a caffeine-insensitive checkpoint stopping cells prior to entering mitosis. A third possibility, that we favour, is suggested by studies in *E. coli*, in which R-loops are formed in the absence of RNase HI, and these R-loops (Fig. 6) provide a substrate for initiation of DNA synthesis.

Early activation of origins of replication or formation of entirely new sites of replication initiation could result in the completion of DNA synthesis more quickly or provide more sites for DNA repair. In either circumstance, RNase H2(35) is required for whatever mechanism is employed, since the *rnh1 rnh2(35)* double-deletion strain grows so poorly on caffeine-containing plates (Fig. 5B). Whether it is the only protein involved remains to be determined.

Deletion of the *RNH1* and *RNH2(35)* genes leads to greater sensitivity to the alkylating agent EMS

Sensitivity to the alkylating agent EMS for both *Δrnh1* and *rnh2(35)Δ* strains and the greater sensitivity of the double-deletion strain (Fig. 5C) is yet another indication that RNases H respond to DNA damage. Similar to *rad27* mutant strains (Johnson *et al.* 1998) sensitivity of *rnh1Δ* and *rnh2(35)Δ* strains to alkylating agents, these strains are not more sensitive to UV-irradiation. Exposure of *S. cerevisiae* to the alkylating agent methyl methanesulphonate (MMS) results in a slower progression through S-phase, mediated by *MEC1* and *RAD53* (Shirahige *et al.* 1998). In addition, alkylating agents can form DNA adducts that arrest transcription complexes inducing transcription-coupled repair (Tornaletti & Hanawalt 1999). Any R-loops (Fig. 6) that may form in cells would normally be eliminated by the cellular RNases H, but in the absence of these enzymes the R-loops could affect either or both of the responses to treatment of cells with alkylating agents. For example, R-loops could mimic a stalled transcription complex and divert some or all of the transcription coupled repair enzymes away from the damaged DNA sites, thereby increasing lethality. A slower progression through S-phase following exposure of the cells to MMS is due in large part to a delay in the firing of late origins of replication. A *rad53* mutation permits replication of these late-firing origins, greatly increasing the sensitivity of such strains to MMS (Shirahige *et al.* 1998). If the RNase H1 is absent, normally late-firing origins may initiate at inappropriate times, resulting in a lethality similar to that observed in MMS-treated cells bearing a *rad53* mutation. In the absence of both RNase H1 and RNase H2 activities, the cells would then be supersensitive to alkylating agents, as is the case for the *rnh1Δ rnh2(35)Δ* strain (Fig. 5C).

Our results indicate that RNA-DNA hybrids are sometimes accessible to both RNase H1 and RNase H2(35), and at other times are recognized preferentially by one of these enzymes, leading to different resolutions of the hybrids. Expression of RNase H2(35) at two distinct stages of the cell cycle may also contribute to the disposition of RNA-DNA hybrids. RNA-DNA hybrids present during phases of the cell cycle in which RNase H2(35) is at its lowest level may be substrates for RNase H1 or some other pathway for resolution. It is also likely that the reverse is true; that during times of its maximal expression RNase H2(35) is the enzyme most often involved in recognition and processing of RNA-DNA hybrids. RNase H2(35) and RNase H1 may have special roles during periods of DNA damage or periods of impairment of DNA replication. To understand the interplay of these two RNases H, it will be important to have more insight into the regulation of synthesis of RNase H2(35) and what other genes might be affected when one or both of the two RNases H is missing.

Experimental procedures

Strains

S. cerevisiae X2180-1A (*MAT*α *SUC2 mal mel gal2 CUP1*) (Moore 1984) was obtained from the Genetic Stock Center, LH330B (*MAT*α *ade2 leu2 trp1 ura3 aro2 can1 hom3 sap3*) was from Lee Hartwell (Fred Hutchinson Cancer Research Center, Seattle), N484 (*MAT*α/a *ura*3-52/ura3-52 *leu*2-3, *leu2-112/LEU2 his1-24/HIS1 ino*1/INO1) was obtained from Alan Hinnebusch (NIH). YS33 (*MAT*α *ade2 leu2 trp1 ura3 aro2 can1 hom3 sap3 rnh1*::*URA3*) was generated by transformation of LH330B by a *Sph*I-*Bam*HI fragment (*Bam*HI site in adjacent *RNA1* gene) containing the *URA3* gene inserted between the first *Hin*dIII site and the *Xba*I site of pMY21 (Itaya *et al.* 1991). YS31V (*MAT*α *ade2 leu2 trp1 ura3 aro2 can1 hom3 sap3 rnh2(35)::kan*^{r}) was derived by transformation of LH330B with a DNA fragment starting with the *Xho*I restriction site, approximately 600 bp upstream of the *RNH2(35)* gene and extending to the *Eco*571 site about 600 bp downstream of the *RNH2(35)* gene. Confirmation of the disruptions was made by Southern analysis (Southern 1975) (data not shown). This DNA fragment had the *kan*^{r} gene from the kan-cassette of pFAMX6 (Wach *et al.* 1994) inserted between the *Kpn*I sites at either end of the *RNH2(35)* gene. YS33V [*MAT*α *ade2 leu2 trp1 ura3 aro2 can1 hom3 sap3 rnh1*::*URA3 rnh2(35)::kan*^{r}] was generated as for YS31V except the recipient strain was YS33. N484 was the parent of all of a second set of strains. N484 was transformed with the *Sph*I-*Bam*HI fragment used to generate YS33. The diploid strain was sporulated and tetrads analysed for growth and genotypes. All four spores proved to be viable. HIRO29 [*MAT*α *ura3-52 leu2-3, leu2-112 his1-24 INO1 rnh1*::*URA3 RNH2(35)*] was used as a *rnh1Δ* strain. HIRO15A [*MAT*α *ura3-52 leu*2-3, *leu2-112 his1-24 INO1 RNH1 RNH2(35)*] the *RNH1 RNH2(35)* and HIRO16A [*MAT*α *ura3-52 leu*2-3, *leu2-112 his1-24 ino1 RNH1*

rnh2(35)::kan^r] were derived by transformation of N484 with the kan-cassette DNA fragment used to produce YS31V, *rnh2(35)Δ* strain. After sporulation and tetrad dissection (all four spores were viable) HIRO15A [*RNH1 RNH2(35)*] and HIRO16A [*RNH1 rnh2(35)Δ*] were obtained. A second spore from the same sporulation event was obtained (HIROV5D [*MAT*α *ura3-52 LEU2 HIS1 ino1 RNH1 rnh2(35)::kan*^{r}]). HIROV5D was crossed with HIRO29, spores were generated and tetrads were dissected to obtain HIRO26A and HIRO27A [*MAT*α *ura3-52 leu2-3, leu2-112 his1-24 ino1 rnh1::URA3 rnh2(35)::kan*^{r}]. HIRO27AF was obtained by selecting HIRO27A on FOA plates.

Plasmids

Plasmids of pYX242 and pYX113 were obtained from Novagen. pCRII was from Invitrogen. The GFP-containing plasmid (pEGFP) was from Clontech. pMY21 has been previously described (Itaya *et al.* 1991). pFAMX6 was a gift from A. Hinnebusch (NIH). pBluescriptSK$^-$ was from Stratagene. pGRL1 is a derivative of YEp51 (Rose & Broach 1991), modified to carry the *lacZ* gene of *E. coli* downstream of the *GAL10* promoter with a synthetic multilinker site for cloning purposes. pGRL-03 is a derivative of pGRL1 containing the region of *RNH1* from pMY21 and contains the region from second Met residue through the *Bam*HI site of pMY21 placing the RNase H domain of the gene downstream of the *GAL10* promoter.

Media

S. cerevisiae cells were grown in liquid and solid yeast media as previously described (Kaiser *et al.* 1994). Synthetic dropout media (Bio101) contained sugars at 2% and all amino acids and supplements except those dropped-out.

Genetic methods

Standard yeast genetic methods (Kaiser *et al.* 1994) were employed except where noted. Sporulation was carried out on KAC plates for 2–3 days at 23 °C and random spore analysis was performed as described by Hollingsworth and Byers (Hollingsworth & Byers 1989). Transformations were performed by the LiCl method (Ito *et al.* 1983). Mitotic chromosome loss and recombination for haploid strains disomic for chromosome *VII* were performed as described by Meeks-Wagner and Hartwell (Meeks-Wagner *et al.* 1986) or loss of *ADE2* (Shero *et al.* 1991).

RNase H assays

RNase H activity assay of lysates

Mid-log phase yeast cells were harvested, washed with ice-cold water and suspended in 200 μL extraction buffer (50 mM Tris-HCl pH 7.9, 1 mM DTT, 1 mM PMSF, 5 μg/mL leupeptin, pepstatin, aprotenin). Acid-washed glass beads were added, and the cells were broken by mixing using a vortex mixer. The supernatant was used in liquid assay to detect the total RNase H activity with appropriate dilution in the dilution buffer (40 mM Tris-HCl, 10 mM β mercaptoethanol, 50% glycerol and 1 mg/mL BSA). ^{32}P-polyrA-polydT substrate was used in this assay (Hall & Crouch 1977). Protein content was measured by using the Coomassie Plus protein assay reagent (Pierce). Activity is expressed relative to the total protein content of lysates. Assays were performed as previously described (Carl *et al.* 1980).

Renaturation assay

S. cerevisiae cells were grown to early stationary phase in glucose (YPD or SD). For cells that were used with Gal-promoters, cells were grown in synthetic medium with raffinose until they were in log phase (10^6–10^7 cells/mL) then centrifuged and resuspended in synthetic medium plus galactose and grown for 12 h. Extracts were made by breaking the cells by vortexing with glass beads (0.4 mm, Sigma) in 0.1 M Tris pH 8.0, 20% glycerol and 1 mM 2-mercaptoethanol followed by addition of an equal volume of SDS-sample buffer and boiling prior to electrophoresis or by resuspension of cell pellets in 2 × SDS sample buffer and boiling for 5 min. Samples were loaded on to 15% polyacrylamide gels that were processed as previously described (Carl *et al.* 1980; Han *et al.* 1997). Pellets obtained upon centrifugation were boiled in 60 μL SDS-sample buffer for 3 min and 10 μL was used to detect the RNase H activity by gel assay. 12% SDS-PAGE incorporated with ^{32}P-labelled polyrA-polydT substrate (10^7 c.p.m./gel) was run and renatured in renaturation buffer (Han *et al.* 1997) to detect RNase H activity by gel assay.

Localization of RNase H2(35) in *S. cerevisiae*

The *RNH2(35)* gene from *S. cerevisiae* was subcloned into the *Hin*dIII/*Sal*I sites of the pEGFP vector (Clontech). The *Hin*dIII/*Stu*I fragment containing the *RNH2(35)* gene, a 30-bp linker region (multiple cloning region) and the EGFP gene at the C-terminal position was cloned into the *Hin*dIII/*Eco*RV sites of the pYX242 (Ingenius) yeast expression vector. The *RNH2(35)-EGFP* construct was transformed into H16V (*INO leu ura his rnh2(35)::kan*^{r}) yeast strain and selected on SD-Leu plates. Individual colonies were isolated, streaked on SD-Leu plates, and grown in SD-Leu medium. Overnight cultures were diluted to 1 : 20 to fresh medium and were grown at 30 °C for 7 h. Cells were collected by centrifugation at 3000 r.p.m. for 5 min and washed with cold water and fixed with 30% paraformaldehyde in PBS buffer. After washing with PBS buffer, cells were mounted on slides in 50% glycerol containing 1 μg/mL DAPI and observed using a fluorescence microscope. The pEGFP plasmid vector was digested with *Nco*I and *Stu*I yielding the EGFP DNA fragment that was then subcloned into the pYX242 yeast vector at the *Nco*I and *Eco*RV sites and used as a control in this study. Expression of the RNase H2(35)-GFP fusion protein was confirmed by Western analysis using GFP monoclonal antibody (Clontech).

Expression of RNase H2(35)-double HA tag protein

The *GAL* promoter region and *Age*I restriction site were removed from the low copy pYX113 yeast vector (Ingenius,

Novagen Inc.) by a digestion with *Acc*65I/*Eco*RI and a filled in reaction with Klenow (New England Biolabs). *Xho*I/*NgoM*I fragment containing the ORF of *RNH2(35),* 605 bp upstream and 405 bp downstream regions was cloned (pVANPH2) into this vector. Expression of the message for *RNH2(35)* gene and the activity for the protein expressed from this native promoter were checked. Double HA tag was generated by two step PCR method in which the first step was carried out with two sets of primers BC786 (GGT TAT CCG TAT GAT GTG CCT GAC TAC GCA GGT TAT CCG TAT GAT GTG CCT GAC TAC GCA TGA TCA CCT CCA GTT TGC AC)/BC795 (CAG TGC CGG CAT ACC AAT AAT GGT GTG) and BC 637(TCT TGG AAC AGA ATG ACT CTC CA)/ BC787(ACC TGC GTA GTC AGG CAC ATC ATA CGG ATA ACC TAC GGG ACC GCT AGC TAG CCG GTA CCA ATT ATC TAG GGT TCT CAG C). Where BC786 has a part of the double HA tag, stop codon and the downstream region of the *RNH2(35)* gene and BC787 has a portion of the double HA tag and C-terminal region of the *RNH2(35)* gene. BC637 and BC795 code for the N-terminal region of the *RNH2(35)* gene and the down stream region of the *RNH2(35)* gene, respectively. First-step PCR products were gel purified and subjected to a second step using the BC637/BC795 primer set. The PCR amplified product was cloned into the TOPO vector, digested with *Xba*I/*NgoM*I and cloned into the yeast vector construct pVANPH2 to generate pVANPH2HA.

Western blot analysis

HIRO16A [*rnh2(35)Δ*] and HIRO27AF [*rnh1Δ rnh2(35)Δ*] yeast strains harbouring the plasmid pVANPH2HA were grown to log phase and extracts were obtained as described above (see Renaturation assay). Protein content was measured by using Coomassie blue reagent and equal amounts of protein samples were loaded on 10–20% gel. Blots were probed with anti- HA tag antibody (Covance) and as a control with antibody specific for GCD6 protein (a gift of Alan Hinnebusch, NIH). HIRO16A [*rnh2(35)Δ*] harbouring the vector was used as a control in the analysis. Bands were quantified using the IMAGEQUANT program following densitometry (Molecular Dynamics).

Synchronization of cells

Mid-log phase X2180-1A yeast cells (Moore 1984) were treated with three micromolar α factor for 2 h. Cells were released from α factor after 2 h (85–90% synchronization) by either filtration or centrifugation and were suspended in fresh YPD medium. A sample was withdrawn at zero time and cells were allowed to grow at 30 °C. Samples were harvested every 15 min, washed with water, and used to isolate total RNA. Microscopic observation of the cells after α-factor-release (% of budded cells) indicated a high degree of synchrony of the cells.

Computer analysis

Computer searches were performed on a VAX3500 using programs from the GCG Package (Genetics Computer Group, Program Manual for the GCG Package, Version 10).

Total RNA isolation

The cell pellet of a 10-mL culture of approximately 10^7 cells per mL was suspended in 200 μL cold extraction buffer A (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EDTA, 5% SDS) and in 200 μL of buffer B (83 μL phenol, 83 μL chloroform: isoamyl alcohol, 33 μL extraction buffer A without SDS) (Kaiser *et al.* 1994). The lysate was obtained by vortexing the cells with acid washed glass beads followed by a centrifugation at 2000 r.p.m. for 5 min. Extraction of RNA was carried out by adding 200 μL of buffer B and the aqueous solution obtained was mixed with 2.5 volume of ethanol and 0.2 M sodium acetate, pH 6.0. After a 30-min incubation on ice, RNA was centrifuged at 4 °C for 15 min at 10 000 r.p.m. The pellet obtained was washed with ice-cold ethanol (80%) and dissolved in buffer C (10 mM Tris-HCl pH 7.4, 10 mM $MgCl_2$, 10 mM NaCl). Three microlitres of RQ RNase free DNase (Promega) was added and phenol/chloroform extraction was performed. RNA was precipitated with 2.5 volumes of ethanol and 0.2 M sodium acetate, pH 6.0. The concentration of RNA was measured at 260 nm and the quality was analysed on 0.8% agarose gel.

Analysis of transcripts

Quantitative RT-PCR analysis was carried out with Super-script™ reverse transcriptase (Life Technologies). The cDNA obtained from different time periods of the cell cycle was run on 1.0% agarose gel, stained with Sypro Green II (Molecular Probes) and quantified in the Storm™ 860 system (Molecular Dynamics) using a blue laser. The data obtained were normalized to the G3PDH message. A control cDNA analysis was carried out with no RT in the cDNA synthesis reactions.

Hydroxyurea, caffeine and EMS treatment

Strains with either the HIRO or YS backgrounds were tested for sensitivity to all three drugs, with similar qualitative results. The major difference was a greater degree of sensitivity of the YS strains to HU. Figures reporting these results show the HIRO series for HU treatment and the YS series for both caffeine and EMS. Serial dilutions were made with log phase cells [wild-type (HIRO5A or YS31), *rnh1Δ* strain (HIRO29 or YS33), *rnh2(35)Δ* strain (HIRO6A or YS31V) and *rnh1Δ rnh2(35)Δ* double-deletion strain (HIRO26A or YS33V)] and three microlitres were plated on YPD plates incorporated with the above chemicals (100 mM HU, 10 mM caffeine). Dilutions were also plated on YPD plates as a control. For EMS treatment, log phase cells grown in YPD medium were treated with 3% V/V EMS for 60 min at 30 °C and three microlitres of 10-fold serial dilutions were spotted on YPD plates along with similar amounts of untreated cells. Plates were incubated at 30 °C and the colony formation was followed for several days.

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The *S. cerevisiae* deletion project can be found online at: http://www-sequence.stanford.edu/group/yeast_deletion_project/new_deletion_strategy.html.

Nomenclature

RNase H nomenclature is evolving so that it refers to the homologous enzyme in each organism having the same name (Cerritelli & Crouch 1998a; Wu *et al*. 1999). We have followed this process. There are references to mammalian (bovine and human) RNase HI (or sometimes RNase H1) and RNase HII. Because we now know their relationship, it is possible to create a uniform nomenclature for these enzymes.

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