Yeast RNase H(35) is the counterpart of the mammalian RNase HI, and is evolutionarily related to prokaryotic RNase HII

Peter Frank, Christa Braunshofer-Reiter, Ulrike Wintersberger*

Department of Molecular Genetics, Institute of Tumor Biology and Cancer Research, University of Vienna, Borschkegasse 8a, A-1090 Vienna, Austria

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Abstract We cloned the *Saccharomyces cerevisiae* homologue of mammalian RNase HI, which itself is related to the prokaryotic RNase HII, an enzyme of unknown function and previously described as having minor activity in *Escherichia coli*. Expression of the corresponding yeast 35 kDa protein (named by us RNase H(35)) in *E. coli* and immunological analysis proves a close evolutionary relationship to mammalian RNase HI. Deletion of the gene (called *RNH35*) from the yeast genome leads to an about 75% decrease of RNase H activity in preparations from the mutated, still viable cells. Sequence comparison discriminates this new yeast RNase H from earlier described yeast enzymes, RNase H(70) and RNase HI.

Key words: Ribonuclease H; RNA-DNA hybrid; *Saccharomyces cerevisiae*; Mammalia; *Escherichia coli*; Evolution

1. Introduction

Since the availability of the complete sequence of the *Saccharomyces cerevisiae* genome [1] it has been convenient to search for genes related to those known from other organisms. Using the well developed genetics of the budding yeast, *S. cerevisiae*, functional information can in many cases be obtained more easily than from studies with a complex organism from which a certain gene was originally cloned.

We have been studying ribonucleases H (RNases H), enzymes which specifically hydrolyze the RNA moiety of RNA-DNA hybrids, from different organisms [2–6], and recently cloned the large, enzymatically active subunit of human RNase HI (Frank et al., in preparation). In mammalia, RNase HI is the major RNase H activity and probably plays a role during DNA replication [7,8]. Earlier we had characterized a RNase H from budding yeast (RNase H(70), see [2]), and now asked ourselves whether this enzyme might be the homologue of the large subunit of human RNase HI. Although this was not the case, a new RNase H from yeast which we have named RNase H(35) was found; this enzyme does indeed represent the homologue of human RNase HI, and in addition, is related to the prokaryotic RNase HII. Here we will describe the cloning of the gene, *RNH35*, the partial purification of the protein and the reduced RNase H activity of a deletion mutant.

*Corresponding author. Fax: (43) (1) 406 07 90.
E-mail: Ulrike.Wintersberger@univie.ac.at

Abbreviations: RNase H, ribonuclease H; ORF, open reading frame; PCR, polymerase chain reaction; IPTG, isopropyl-1-thio-β-D-galactopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride

We dedicate this work to the memory of our colleague Robert Karwan (1959–1997).

2. Materials and methods

2.1. Yeast strains and growth media

Strain K699a (Mat**a** ura3 ade2-1 trp1-1 can1-100 leu2-3,112 his3-1,15 ssdl) was originally supplied by K. Nasmyth, strain BFRH35a (Mat**a** rnh35Δ::*HIS3* ura3 ade2-1 trp1-1 can1-100 leu2-3,112 his3-1,15 ssdl) was constructed in the course of this study. YPD growth medium (1% yeast extract, 2% peptone, 2% dextrose) was used as a standard medium, synthetic complete medium was prepared as described by Sherman [9].

2.2. PCR amplification and cloning procedures

Using the recently sequenced gene for the large subunit of human RNase HI (Frank et al., in preparation) for screening for a homologous gene in the yeast genome, ORF YNL072w on chromosome XIV turned out to be the best candidate. In order to amplify YNL072w for cloning into an expression vector, PCRs were performed with genomic yeast DNA (Promega) as a template. The following primers were used for PCR with the Expand PCR system (Boehringer Mannheim): cty1/*Bgl*II, which covers the 5′ end of the ORF, including the ATG start codon (underlined), and introduces a *Bgl*II site (bold): 5′-GATGTCG**AGATCT**ATGGTACCCCCCACGGTAGAAGCATC-3′, and cty3/*Sal*I, which covers the 3′ end of the ORF and introduces a *Sal*I site (bold): 5′-GATGTC**GTCGAC**ATATAGTATGTGCAAACTGGAGGTGATCACCGG-3′. A PCR product of 975 bp in size was obtained, digested with *Bgl*II and *Sal*I, gel purified and ligated into a *Bgl*II/*Sal*I digested pXa1 expression vector (Merck). This vector system allows the expression of β-galactosidase fusion proteins after induction with IPTG. The ligation sample was transformed into competent XL1 Blue *Escherichia coli* cells. Bacterial cultures were grown in the presence of IPTG and harvested by centrifugation. Bacterial pellets were processed for SDS-PAGE and analyzed on 8.5% gels for the presence of a fusion protein of the correct size (160 kDa).

For the production of a deletion allele of the YNL072w gene, the following primers were used for generating a 1650 bp fragment to be cloned into pUC18: cty3d/*Eco*RI: 5′-TGTGACT**GAATTC**GGCTGTGTGGATGATGTAAACAGGCAG-3′, and cty4d/*Hin*dIII: 5′-CGGATGT**AAGCTT**CCGGGAGACAATTGGTCACCTTCCTTC-3′. A PCR product of the expected size was obtained, digested with *Eco*RI and *Hin*dIII, gel purified and ligated into *Eco*RI/*Hin*dIII of the pUC18 vector. A fragment of 910 bp including nearly the complete ORF was replaced by a 1890 bp fragment carrying the *HIS3* gene. The isolated and gel purified deletion allele was used for integrative transformation [10], and His^+ transformants were isolated. They were checked by specific PCR for successful gene replacement and analyzed for RNase H activity. One of the mutants, BFRH35a, was used for further experiments.

2.3. Expression, purification and factor Xa digestion of the YNL072w-β galactosidase fusion protein

Recombinant fusion protein from the pXa1 expression plasmid was purified as follows: 100 ml of a bacterial overnight culture was grown in Luria broth in the presence of 1 mM IPTG and 25 μg/ml ampicillin. The cells were harvested by centrifugation and opened by ultrasonic treatment in ice-cold buffer A (PBS+5 mM EDTA). Then the extract was treated with benzonase (Merck) to destroy RNA and DNA and 8 M urea to solubilize inclusion bodies. The preparation was loaded onto a BioRad 691 prepcell (preparative gel electropho-

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resis) and separated on a cylindrical 4% gel. Elution fractions were analyzed for the presence of purified fusion protein by analytical 8.5% SDS-PAGE. Fractions containing the fusion protein were pooled and dialyzed three times against buffer A. Then they were concentrated in the dialysis bag by external treatment with aquacide II (Calbiochem). The concentrated fraction was digested with factor Xa protease at room temperature for different time periods. The efficiency of the digestion was analyzed by 12% SDS-PAGE.

2.4. Enzyme activity assay and standard techniques for protein analysis

RNase H assays were performed as described earlier [2]. Protein concentration was determined by the method of Bradford [11]. Discontinuous SDS-PAGE was performed according to Laemmli [12], and protein bands were visualized with Coomassie brilliant blue G250 or by silver staining [13]. Western blotting was performed as described earlier [14] using an antiserum raised against purified calf thymus RNase HI [15].

2.5. Enrichment of total RNase H activity from yeast cell extract

To minimize proteolysis, all purification steps were carried out at 4°C, and protease inhibitors (0.2 mM PMSF and 1 mM sodium sulfite, pH 8.0; 0.1 mM sodium tetrathionate; 1 μM each of *Nα-p*-tosyl-L-lysine-chloromethylketone, *N*-tosyl-L-phenylalanine-chloromethylketone, pepstatin A, and antipain) and 0.1% β-mercaptoethanol were included in all buffers. The strain in question was grown to mid-log phase, harvested by centrifugation, washed once with buffer B (20 mM Tris-HCl pH 7.9, 1 mM EDTA, 10% glycerol) containing 2 M NaCl, and frozen in liquid nitrogen. The cell pellet (10 g wet weight) was resuspended in 17 ml of buffer B containing 2 M NaCl, and after addition of an equal volume of acid washed glass beads (diameter 0.45 mm), the suspension was homogenized and separated from the glass beads, undisrupted cells and cell fragments as described [2]. The cell extract was subsequently centrifuged for 100 min at 50 000 rpm in a Beckman 55.2 Ti rotor. To remove nucleic acids, the supernatant was treated with polyethylene glycol 6000 (10% w/v final concentration) for 30 min on ice followed by centrifugation for 35 min at 22 000 rpm (Beckman 55.2 Ti rotor). After dialysis of the supernatant against buffer B containing 0.1 M NaCl and centrifugation of the dialysate for 25 min at 24 000 rpm (Beckman 55.2 Ti rotor), the clear supernatant was chromatographed on a 40 ml DNA-cellulose column as described earlier [2]. The bound protein was completely eluted with buffer B containing 2 M NaCl. Protein containing fractions were pooled and analyzed for RNase H activity.

3. Results

3.1. The open reading frame YNL072w of the S. cerevisiae genome codes for a RNase H

When searching the budding yeast genome for homologies with the sequence of the enzymatically active subunit of human RNase HI (Frank et al., in preparation) we found ORF YNL072w on chromosome XIV, which encodes a hypothetical protein of 34.9 kDa. The overall identity between the 307 aa yeast sequence and the 299 aa human sequence is 36.6%, and therefore we considered it a reasonable assumption that we had discovered a yeast gene for a RNase H. Interestingly, this gene is also related to gene *rnhb* from *E. coli* (Fig. 1) which was earlier found to encode a so-called minor RNase H activity, named RNase HII [16]. Further searching revealed the presence of related, but biochemically and genetically uncharacterized sequences in many of the eubacterial, archaebacterial and eukaryotic organisms as we report elsewhere (Frank et al., in preparation), and as others have noticed [17,18] while this manuscript was in preparation. Fig. 1 shows that, if the three sequences with experimentally proven RNase H identity, the yeast, human, and *E. coli* RNase H, are compared, the overall identities are limited, nevertheless there exist

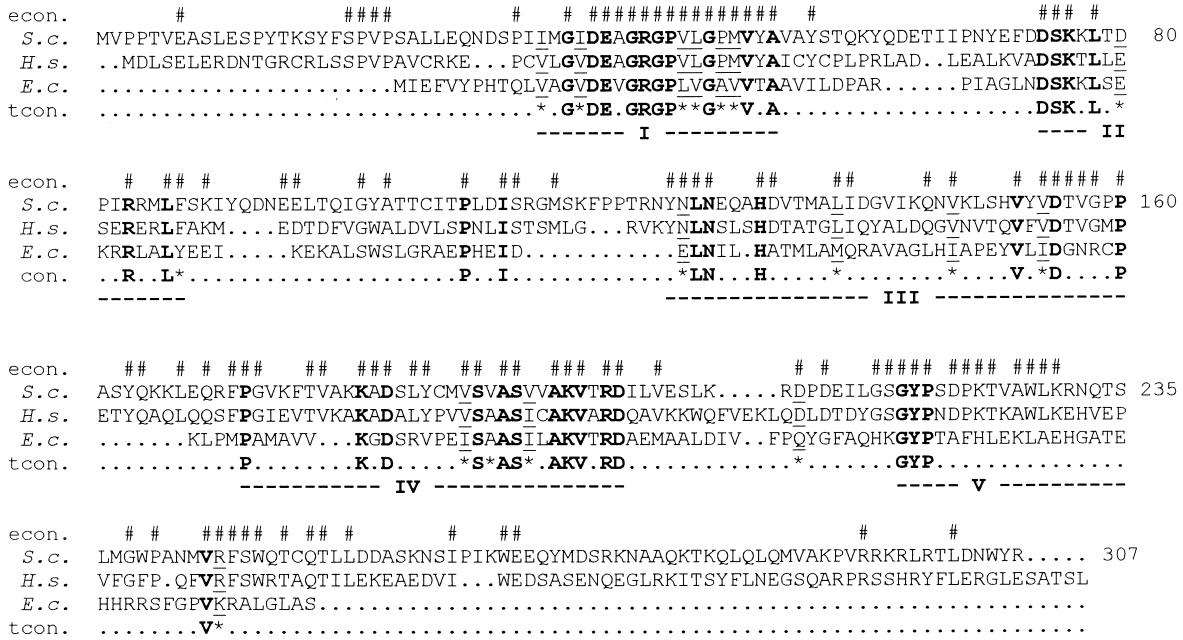

Fig. 1. Overall sequence alignment of the yeast ORF YNL072w (marked S.c., EMBL accession number Z71348), the large subunit of human RNase HI (marked H.s., EMBL accession number Z97029) and the *E. coli* RNase HII (marked E.c., SwissProt accession number P10442) [16]. The alignment was generated using the 'Multiple sequence alignment with hierarchical clustering method' of Corpet [28], and manually modified as follows: amino acids identical in all three sequences are marked in bold letters, similar amino acids are underlined. In the consensus sequence (tcon.), bold letters indicate identical amino acids, and asterisks similar amino acids. Parameters used for the alignment: symbol comparison table: blosum62; gap weight: 12; gap length weight: 2. In addition, identities between the two eukaryotic sequences are marked as # in the line (econ.) above the alignment.

five regions of pronounced homology, indicated by numbers I–V in Fig. 1.

3.2. Expressing the S. cerevisiae 34.9 kDa ORF in E. coli yields a protein immunologically closely related to mammalian RNase HI

In order to examine the immunological relationship of the yeast ORF YNL072w with mammalian RNase HI, we expressed it in *E. coli*. Using PCR primers with appropriate cloning sites at both ends, we amplified the corresponding DNA fragment from genomic yeast DNA (Promega) and cloned it into the *E. coli* expression vector pXa1, as described in Section 2. There it was expressed as a fusion protein with β-galactosidase. Purification of the fusion protein and cleavage with the protease factor Xa led to the expected 34.9 kDa protein. As shown in Fig. 2, this protein is specifically recognized by the antibody against calf thymus RNase HI in Western blot analysis. Therefore we named the ORF YNL072w *RNH35*, and its protein product RNase H(35).

3.3. Deletion of the gene RNH35 from the genome of S. cerevisiae results in a decrease of total RNase H activity

Using integrative transformation [10] with the deletion construct, described in Section 2, we obtained the viable haploid strain BFRH35a. A cell extract of this strain exhibited a 50% decrease in overall RNase H activity compared to an extract from the corresponding RNase H(35) proficient strain (data not shown). RNase H activity was enriched from cell extracts of both strains by removal of nucleic acids and DNA cellulose chromatography (see Section 2), and the resulting fractions were tested for RNase H activity using Mg^{2+} as divalent cation. As shown in Fig. 3, RNase H activity of the fraction derived from the deletion mutant is about 75% lower than that derived from the strain with the intact *RNH35* gene. Thus, RNase H(35) obviously is the main RNase H activity of the unicellular eukaryote *S. cerevisiae*, a finding in agreement with its homology to RNase HI, which in mammals also represents the major RNase H active enzyme.

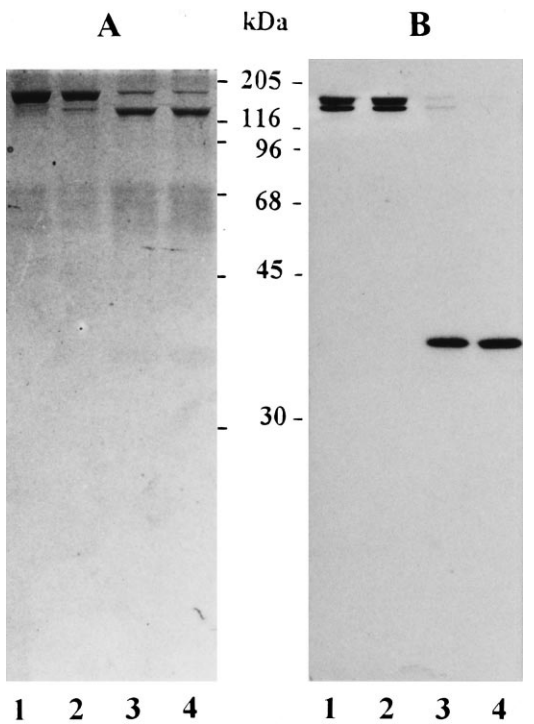

Fig. 2. Expression of the of yeast RNase H(35) in *E. coli*. Purified yeast RNase H(35)-β-galactosidase fusion protein (lane 1) was digested with factor Xa for 0 min (lane 2), 90 min (lane 3) and 120 min (lane 4) and analyzed on a 12% gel. A: Silver staining. B: Western blotting with anti-calf thymus RNase HI antibody. Positions of protein M_r markers are indicated.

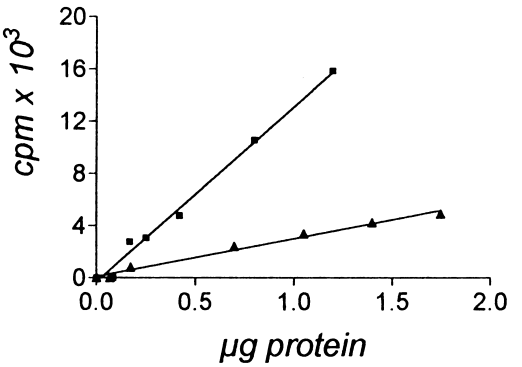

Fig. 3. Deletion of gene *RNH35* from the yeast genome causes a decrease in RNase H activity. A wild-type strain (■) and a strain harboring the deletion allele, rnh35Δ::*HIS3* (▲) were used for the preparation of DNA cellulose fractions enriched in RNase H activity (see Section 2), and assayed for RNase H activity (10^3 cpm corresponds to 22.2 nmol ribonucleotides released).

4. Discussion

Although ribonuclease H activity had been discovered in eukaryotic tissue [19] before it was detected in a retroviral reverse transcriptase [20], and before a RNase H was purified from the bacterium *E. coli* [21,22], the biological roles of eukaryotic RNases H are still elusive. Intuitively, removal of RNA primers from Okazaki fragments during lagging strand DNA synthesis is the function ascribed to cellular enzymes exhibiting RNase H activity (see however [23]). In *E. coli* this task is mainly carried out by DNA polymerase I [24] and the extensively studied RNase HI of this organism has a different function in the replication of the bacterial genome: it eliminates RNA transcripts, which may accidentally hybridize to the template, in order to prevent initiation of DNA replication at sites other than oriC, the canonical replication origin of the *E. coli* genome (for review see [25]). Unfortunately, the biological role of the second RNase H of *E. coli,* called RNase HII [16], which is the homologue of the large, enzymatically active subunit of the major human RNase H, called RNase HI (Frank et al., in preparation), and of the yeast enzyme described in this communication, is unknown.

The gene encoding a hitherto unknown budding yeast RNase H was found by screening the database of the total *S. cerevisiae* genome with the *complete* sequence of the cDNA of human RNase HI, large subunit. The ORF YNL072w, situated on the left arm of *S. cerevisiae* chromosome XIV (between genes *MSK1* and *LAT1*), was found to encode a protein very similar to the human enzyme (Fig. 1) in sequence, size (around 35 kDa) and recognition by an antiserum against the bovine RNase HI (Fig. 2). The following criteria convinced us that gene YNL072w does indeed encode a RNase H: (i) expression in and purification from *E. coli* resulted in a protein which is strongly recognized by an antibody, raised against purified calf thymus RNase HI, and (ii) a yeast strain

from whose genome gene YNL072w was deleted showed a significant decrease in total RNase H activity (Fig. 3). Therefore we renamed the gene *RNH35* and its product RNase H(35).

The fact that a haploid yeast strain completely missing gene *RNH35* is alive and does not show any serious phenotype under usual laboratory conditions may indicate that yeast possesses other enzymes which may substitute for RNase H(35), or that the newly discovered enzyme is essential under living conditions which may occur in nature and may be very different from the nutritionally rich environment of the laboratory. The behavior of the deletion mutant under various more specialized conditions, like starvation or other kinds of stress, remains to be investigated. Regarding other RNases H of yeast, the biological role of all of which is unknown, we refer to earlier studies of several laboratories (for review see [26]) but we have no information on whether any of these previously described enzymes may be identical to RNase H(35). As far as *S. cerevisiae* gene *RNH1* [27] is concerned, and yeast RNase H(70) which was discovered in our own laboratory [2], we know for certain that this is not the case (Frank et al., in preparation). The evolutionary conservation suggests that the RNase H family, of which RNase H(35) is a member, plays an important role in all living cells for managing the metabolism of RNA-DNA hybrids.

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