Purification of *Saccharomyces cerevisiae* RNase H(70) and identification of the corresponding gene

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Abstract We purified *Saccharomyces cerevisiae* RNase H(70) to homogeneity, using an optimized chromatographic purification procedure. Renaturation gel assay assigned RNase H activity to a 70 kDa polypeptide. Sequencing of tryptic peptides identified the open reading frame YGR276c on chromosome VII of the *S. cerevisiae* genome as the corresponding gene, which encodes a putative polypeptide of molecular mass of 62 849. We therefore renamed this gene *RNH70*. Immunofluorescence microscopy using a *RNH70*-EGFP fusion construct indicates nuclear localization of RNase H(70). Deletion of *RNH70* from the yeast genome did not result in any serious phenotype under the conditions tested. Homology searches revealed striking similarity with a number of eukaryotic proteins and open reading frames, among them the chimpanzee GOR protein, a homolog of a human autoimmune antigen, found to elicit autoimmune response in patients infected with hepatitis C virus.

Key words: Ribonuclease H; RNA-DNA hybrid; *Saccharomyces cerevisiae*; DNA replication; Nuclease; Evolution

1. Introduction

According to current knowledge RNA-DNA hybrids occur transiently in living cells, necessarily during DNA replication and transcription, and during the probably very rare events of retrotransposition. Whereas no special mechanism for dealing with the short hybrids arising during regular transcription is known, RNA primers of Okazaki fragments – which are intermediates of lagging strand DNA synthesis and are hybridized to the DNA template strand – have to be removed enzymatically [1]. Enzymes hydrolyzing specifically the RNA strand of RNA-DNA hybrids, called ribonucleases H (RNases H), were found in all organisms yet examined [2]. Nevertheless, the most intensively studied and best understood proteins exhibiting RNase H activity are retroviral reverse transcriptases [3]. Their RNase H activity catalyzes an essential step in the life cycle of retroviruses, i.e. the destruction of the RNA genomes after their retrotranscription into DNA. Another well studied RNase H is RNase HI of *Escherichia coli*, which is evolutionarily related to the RNase H domain of reverse transcriptases [4]. Interestingly, this enzyme was found to play a regulatory role during DNA replication: in wild type cells it seems to be on the watch for superfluous RNA-DNA hybrids which might have arisen from discontinued transcripts (for review see [5]). In *E. coli*, RNA primers of Okazaki fragments are removed by the RNase H activity of DNA polymerase I [1]. Nevertheless, still another enzyme with RNase H activity exists in *E. coli*, RNase HII [6], the primary structure of which is not related to the RNase H domain of reverse transcriptases, and the physiological role of which is as yet unknown. Remarkably, open reading frames, characterized by their sequences them as orthologs of *E. coli* RNase HII, were found in all bacterial genomes examined. Genes encoding orthologs of the two bacterial proteins were recently cloned from eukaryotic organisms, e.g. from the budding yeast *Saccharomyces cerevisiae* [7,8], from the fruit fly *Drosophila melanogaster* [9] and from human cells [10–13].

S. cerevisiae RNase H1 is structurally related to *E. coli* RNase HI [7]; the biological role of that enzyme, however, is as yet unknown. The yeast protein representing the evolutionary relative of bacterial RNase HII was, on the basis of its molecular weight, called RNase H(35) by us [8]. It is probably the main RNase H of that organism because deletion of the respective gene causes a drastic reduction of RNase H activity in yeast cell extracts [8]. Surprisingly, when RNase H activity originally was enriched by then classical purification steps from yeast cell extracts, an enzyme of molecular weight around 70 kDa was found and called RNase H(70) [14]. Following studies intended to isolate the gene encoding RNase H(70) revealed that the original preparation contained copurified proteins, the most prominent of which was poly(A) binding protein (PABP [15]) possessing a very similar molecular weight.

In this communication we report an improved purification procedure for RNase H(70), which removed PABP as well as a having reverse transcriptase activity which earlier was considered a property of RNase H(70) [16]. The new procedure resulted in a small amount of homogeneous protein which could be used for sequencing of several tryptic peptides which finally paved the way for the identification of the gene, *RNH70*.

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Abbreviations: PCR, polymerase chain reaction; RNase H, ribonuclease H; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; EST, expressed sequence tag; ORF, open reading frame; PMSF, phenylmethylsulfonyl fluoride; PEG, polyethylene glycol; EGFP, enhanced green fluorescent protein; DAPI, 4′,6-diamino-2-phenylindole; TPCK, *N*-tosyl-L-phenylalanine chloromethylketone; TLCK, *Nα-p*-tosyl-L-lysine chloromethylketone; p*I*, isoelectric point

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2. Materials and methods

2.1. Yeast strains and growth media

Strains MZ3 (*Mat*α; *pep4-3 trp1 leu2-Δ1 ura3-Δ1*) and MZ3ΔPABP (derived from MZ3 by deleting a 405 bp *Spe*I/*Sph*I fragment from the C-terminus of the PABP gene and introducing the *URA3* marker [17]) were used for enzyme purification. Strain K699 (*Mata ura3 ade 2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ssdl* [8]) was used for constructing strain *BC70 (Mata ura3 ade 2-1 trp1-1 can1-100 leu2-3,112 his3-11,15 ssdl rnh70*Δ::*URA3*); strain AK606 (*Mata ura3 leu2 trp1 his3*), an adenine-prototrophic meiotic segregant from a cross between strains K699 and MZ3, was employed for transformation with plasmid pUG36-RNH70. YPD growth medium (1% yeast extract, 2% peptone, 2% dextrose) and synthetic complete medium (SC) were as described [18].

2.2. Enzyme activity assays, standard techniques for protein analysis, materials and enzymes

The standard RNase H assay in solution was performed as described [14], and the reverse transcriptase assay was performed as reported earlier [16]. The renaturation gel assay for the in situ detection of RNase H activity was performed according to Frank et al. [19] using Mn^{2+} as a divalent cation. Protein concentration was determined by the method of Bradford [20]. Discontinuous SDS-PAGE was performed according to Laemmli [21]; protein bands were visualized with Coomassie brilliant blue G250 or by silver staining [22]. [α-^{32}P]ATP, 3000 Ci/mmol, 10 mCi/ml and [5-^{3}H]uridine 5′-triphosphate, 15.6 Ci/mmol, 1 mCi/ml, were from American Radiolabeled Chemicals (ARC). DNA cellulose was prepared according to [23]. Oligo(dT), RNA polymerase (*E. coli*), phenyl-Sepharose, and Mono P for chromatofocusing were purchased from Pharmacia-LKB. Hydroxyapatite and Affi-Gel Blue were from Bio-Rad. Restriction enzymes, antipain, pepstatin A and PCR-related products were from Boehringer Mannheim. PMSF, TPCK and TLCK were purchased from Fluka.

2.3. Purification of RNase H(70)

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 TLCK, TPCK, pepstatin A, and antipain) and 0.1% β-mercaptoethanol were included in all buffers. After having used strain MZ3ΔPABP for working out a purification procedure by which RNase H activity could unambiguously be separated from PABP the following large-scale preparation was carried out (see Fig. 1A). Cells (strain MZ3) were grown in a 4 l fermenter as a continuous culture at pH 6.2 and 30°C using YPD as a medium, and starting with a cell density of 1.6×10^{6}/ml. The cells were harvested by centrifugation, washed once with buffer A (20 mM Tris-HCl pH 7.9, 1 mM EDTA, 10% glycerol) containing 2 M NaCl, and frozen in liquid nitrogen. The cell pellet (120 g wet weight per frozen aliquot) was resuspended in 180 ml of buffer A 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 glass beads, undisrupted cells and cell fragments as described [14]. The cell extract was subsequently centrifuged for 120 min at 43 000 rpm in a Beckman 45 Ti rotor and filtered through a paper filter. To remove nucleic acids, the supernatant was treated with polyethylene glycol 6000 (PEG, 10% w/v final concentration) for 30 min on ice followed by centrifugation for 35 min at 22 000 rpm (Beckman 45 Ti rotor). After dialysis of the supernatant against buffer A containing 0.1 M NaCl (2×2 h) and centrifugation for 25 min at 24 000 rpm (Beckman 45 Ti rotor), the clear supernatant was loaded on a 360 ml DNA cellulose column (flow rate 60 ml/h). After extensive washing (until no protein was detectable in the flow-through), the bound protein was completely eluted with buffer A containing 2 M NaCl. Protein containing fractions (10 ml each) were analyzed for RNase H activity and pooled. The pool was adjusted with ammonium sulfate up to 60% saturation and, after 20 h of storage, centrifuged (35 000 rpm; 45 min; 45 Ti rotor). The precipitated proteins were dissolved in buffer A containing 1 M ammonium sulfate and loaded onto a 100 ml phenyl-Sepharose column (flow rate 30 ml/h). After washing, protein was eluted with 500 ml of a decreasing gradient (1–0 M ammonium sulfate in buffer A). In the fractions PABP was detected with the aid of a rabbit antiserum raised against the protein expressed in and purified from *E. coli* [17], and reverse transcriptase by determination of enzyme activity. Both proteins eluted in fractions well separated from those containing RNase H activity. The latter (6 ml each) were pooled, precipitated with ammonium sulfate (60% saturation) and centrifuged (35 000 rpm; 45 min; 45 Ti rotor). The precipitated protein was dissolved in 40 ml buffer K1 (25 mM K_2HPO_4/KH_2PO_4, pH 6.8, 10% glycerol), dialyzed against buffer K1 (2×2 h) and loaded onto a 40 ml hydroxyapatite column (flow rate 24 ml/h). After washing, the bound protein was eluted with a linear increasing phosphate gradient of 300 ml (25–500 mM K_2HPO_4/KH_2PO_4, pH 6.8, 10% glycerol). RNase H containing fractions (6 ml each) were pooled. The pool was dialyzed two times against buffer K2 (10 mM K_2HPO_4/KH_2PO_4, pH 6.8, 5 mM $MgCl_2$, 10% glycerol) and loaded onto a 40 ml Affi-Gel Blue column (flow rate 30 ml/h). After washing, the elution took place using a 300 ml linear increasing gradient (0–2 M NaCl in buffer K2). RNase H containing fractions (6 ml each) were pooled. The pool was desalted with a PD10 column (Pharmacia) and applied to a Mono P column (5×200 mm), which was equilibrated with 25 mM BisTris-HCl, pH 7.1, 10% glycerol, PMSF and β-mercaptoethanol. Elution was done with Polybuffer 74 (Pharmacia), pH 4.0, 10% glycerol, PMSF and β-mercaptoethanol. Fractions (1 ml each) were collected and analyzed for the presence of RNase H using the renaturation gel assay [19].

2.4. Peptide sequencing

The RNase H(70) preparation was separated on a preparative 12% SDS-PAGE, stained with Coomassie brilliant blue R-250 (Serva) and the band corresponding to the RNase H(70) protein was cut out and concentrated on a specially designed concentration gel according to Vandekerckhove et al. [24]. Approximately 5 μg of the purified and concentrated RNase H(70) polypeptide was blotted from the Vandekerckhove gel onto a poly(vinylidene difluoride) (PVDF) membrane (Millipore, Bedford, MA, USA) using a standard blotting tank system. The transfer buffer contained 13 mM sodium carbonate pH 9.9 and 20% methanol. The protein spot was detected by staining with Coomassie brilliant blue R-250 and afterwards excised from the membrane. The blotted protein sample was digested with sequencing grade trypsin (Promega, Madison, WI, USA) as described by Fernandez et al. [25], except that the detergent Triton X-100 was replaced by octyl-β-D-glucopyranoside (Boehringer Mannheim, Germany). Peptide mapping was carried out on a 1×250 mm Vydac C18 column at 40°C with a flow rate of 50 μl/min using the HP 1100 HPLC system (Hewlett-Packard, Waldbronn, Germany). Selected peptide fractions were analyzed by automated Edman degradation using the HP G1005A protein sequencing system (Hewlett-Packard, Palo Alto, CA, USA). Three peptide sequences, TSVQEDDHT, NTFNEISD, and CVEDDET, were detected in ORF YGR276c on chromosome VII of the *S. cerevisiae* genome. YGR276c was renamed *RNH70* and cloned.

2.5. PCR amplification and cloning procedures

In order to amplify the RNase H(70) coding sequence for the production of a deletion allele of the *RNH70* gene, PCRs were performed with yeast genomic DNA (Promega) as template. The following primers were used for PCR with the Expand PCR system (Boehringer Mannheim) for generating a 2273 bp fragment to be cloned into pUC18: forward primer rh70d3/*Eco*RI: 5′-TGTGACTGAATTC-TAATGTACTTGGAGGAAGGGAGGAAGACG-3′ and reverse primer rh70d4/*Hin*dIII: 5′-CGGATGTAAGCTTTCTACAGAGG-GAGAGCTGTGAGGATATATT-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 digested pUC18 vector. A *Ssp*BI/*Sma*I fragment of 1549 bp, including nearly the complete ORF, was replaced by a 1125 bp fragment carrying the *URA3* gene. The isolated and gel purified deletion allele was used for integrative transformation [26], and Ura^{+} transformants were isolated. They were checked by specific PCR for successful gene replacement and analyzed for phenotypes. One of the mutants, BC70, was used for further experiments.

2.6. Cellular localization of yeast RNase H(70) enhanced green fluorescent protein fusion

Primers RH70/*Eco*RI and RH70/*Sal*Irev2 were used to introduce *Eco*RI and *Sal*I restriction sites at the beginning and end of the RNase H(70) coding sequence, respectively; RH70/*Eco*RI: 5′-G-ATGTCGGAATTCATGCAAGTAGAAGGGCCTGACACTAA-3′; RH70/*Sal*Irev2: 5′-GATGTCGTCGACTATAGTCCTTTGAATTG-

GCACCTTCAT-3′. The amplified fragment of *RNH70* was inserted into the pUG36 vector (U. Güldener and J. Hegemann, manuscript in preparation). This system allowed the expression of *RNH70* fused to the carboxy-terminus of the enhanced green fluorescent protein (EGFP) under the control of the yeast *MET25* promoter. The strain AK606 was transformed with the resulting pUG36-RNH70 vector, grown in SC medium lacking uracil and induced by addition of methionine for 4 h. Formaldehyde fixed and DAPI stained cells were viewed with a Nikon FXA microscope (EX450–490 nm), and photographed on Fujichrome 400 film.

3. Results

3.1. Purification of RNase H(70)

When purification of RNase H activity from yeast cells was originally attempted in our laboratory we ended up with a protein fraction which showed in SDS-PAGE one prominent band around 70 kDa, and by glycerol gradient centrifugation an activity peak at a sedimentation value corresponding to the same molecular weight [14]. Further studies, however, revealed the presence of PABP (molecular mass 64 344) and of a reverse transcriptase activity. As PABP at that time was just known to be somehow involved in transcription termination it would have been reasonable that it possessed the RNase H activity. To find out whether this was the case or whether PABP represented a separate but copurified protein, a strain, MZ3ΔPABP, containing a PABP gene with a shortened C-terminus and thus a PAB protein of molecular mass different from that of RNase H(70), namely 55 429, was constructed, and an antiserum was raised against *E. coli* expressed PABP. As described in detail elsewhere [17] these experimental means finally enabled us to unambiguously identify PABP as an impurity of the RNase H preparation. By initially using strain MZ3ΔPABP for purification we confirmed the existence of a separate RNase H molecule exhibiting in SDS-PAGE a molecular weight around 70 kDa, for which therefore the name RNase H(70) was retained.

The newly worked out purification procedure, indicated in Fig. 1A, and described in detail in Section 2, allows separation of PABP as well as of the reverse transcriptase activity from RNase H(70) by using a hydrophobic interaction chromatography (HIC) on phenyl-Sepharose as second chromatographic step. A further improvement compared to the original procedure is achieved by the employment of Affi-Gel Blue chromatography and chromatofocusing on a Mono P column (Table 1). Even when starting with 120 g of yeast cells (wet weight) the amount of protein after this last chromatography was very low and an overall purification factor could not be determined. Analysis of the fractions from the Mono P column by separation of proteins on SDS-PAGE and silver staining (Fig. 1B) revealed a prominent band around 70 kDa in the same fractions which exhibited RNase H activity in the in situ renaturation gel assay (see Fig. 1B). Fraction 6 from the Mono P column was further processed for isolation of RNase H(70), peptide sequencing and identification of the corresponding gene.

3.2. Identification of the corresponding yeast gene

To obtain the 70 kDa polypeptide for protein sequencing,

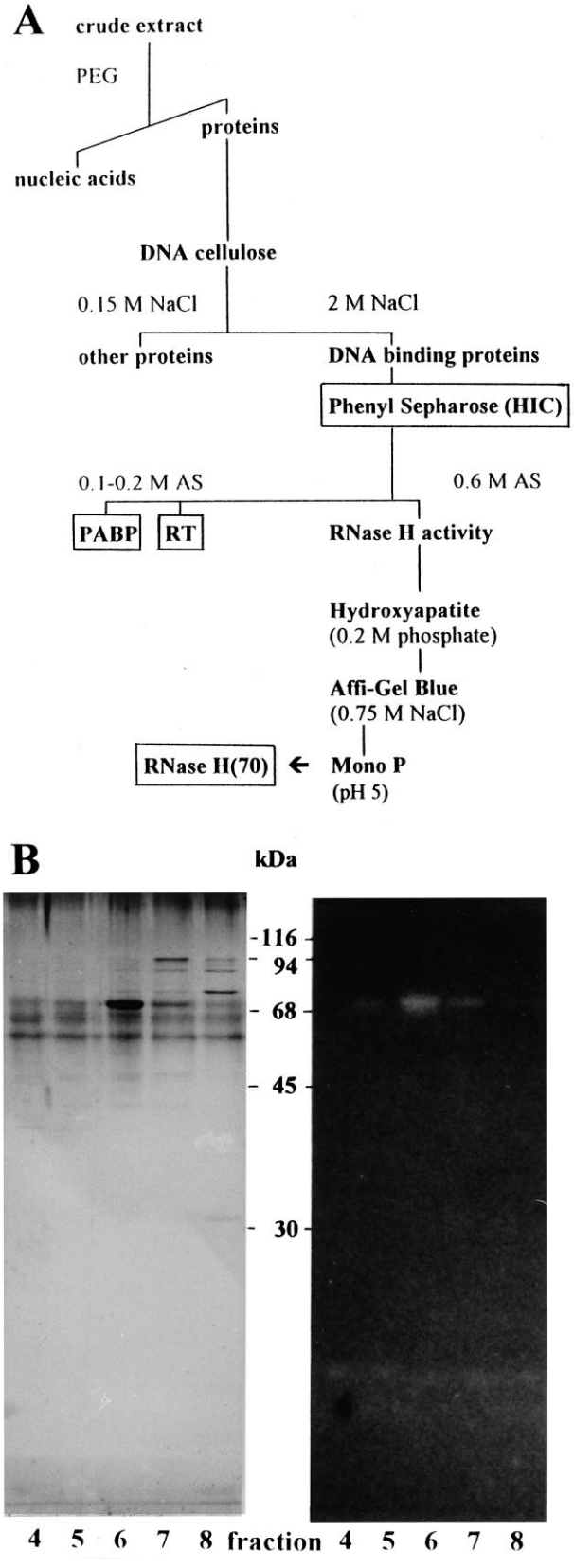

Fig. 1. Purification of RNase H(70). A: Purification scheme; AS, ammonium sulfate. B: Protein and activity profile of the final step, chromatofocusing on Mono P. Proteins from fractions 4–8 from the Mono P column were separated by 12% SDS-PAGE and silver stained (left panel, the bands identical in all fractions result from a staining artifact), as well as analyzed by in situ renaturation gel assay (right panel). Protein size markers are indicated between the two panels.

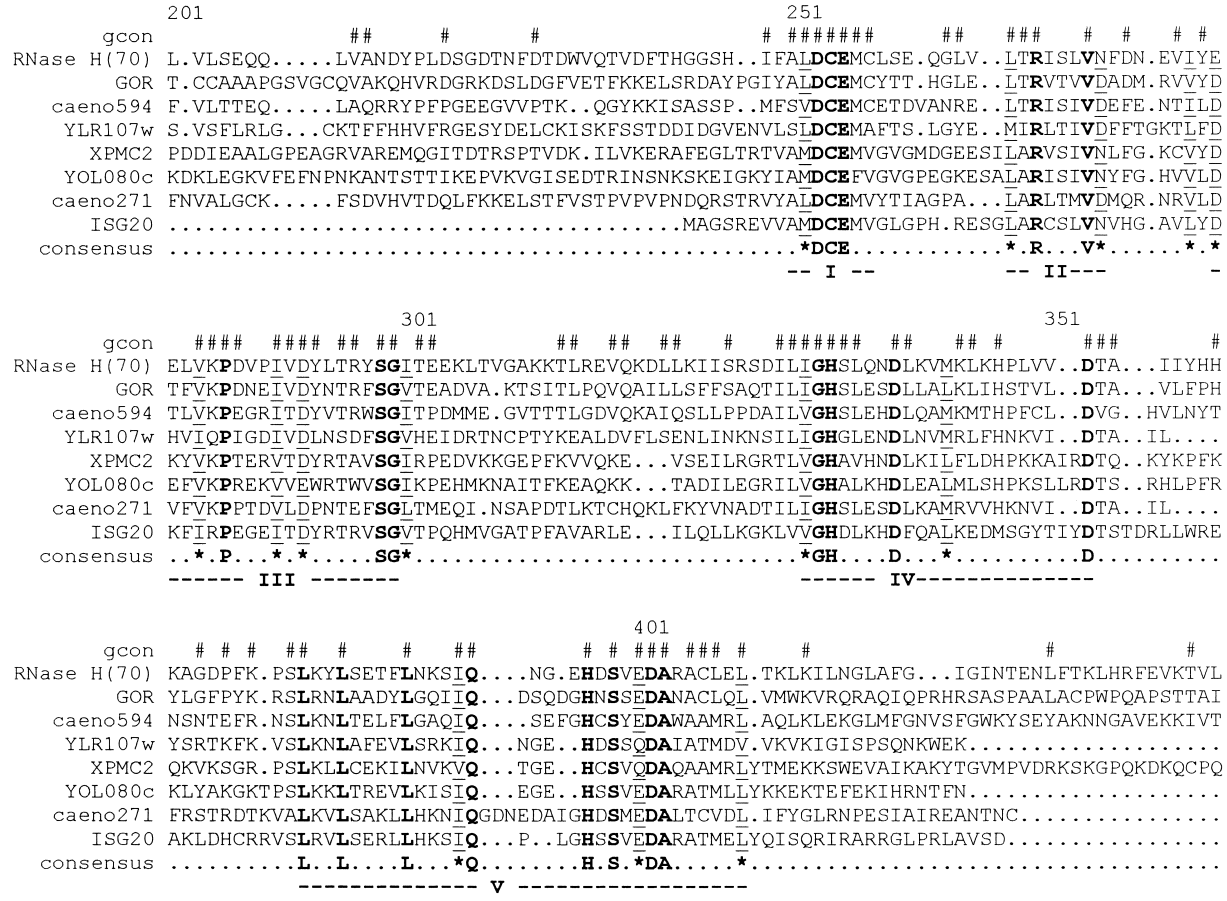

Fig. 2. Multiple sequence alignment of homologous parts from yeast RNase H(70), and corresponding eukaryotic open reading frames. The alignment was generated, using the 'multiple sequence alignment with hierarchical clustering' method of Corpet [27], and sequences from the *S. cerevisiae* ORF YGR276c (553 aa, marked RNase H(70), Swiss-Prot accession number P53331), the GOR antigen of the chimpanzee *P. troglodytes* (525 aa, partial sequence; EMBL accession number D10017), a *C. elegans* ORF (594 aa; marked caeno594; GenBank accession number AF016430), the *S. cerevisiae* ORF YNL107w (130 aa; EMBL accession number Z73280), the *Xenopus laevis* XPMC2 protein (421 aa; GenBank accession number U10185), the *S. cerevisiae* ORF YO080c (289 aa; GenBank accession number Z74822), a *C. elegans* ORF (271 aa; marked caeno271; Swiss-Prot accession number Q101024), and the human ISG20 protein (179 aa; GenBank accession number X89773). Numbering of amino acids above the alignment corresponds to the consecutive sequence of yeast RNase H(70) (only regions with existing consensus are shown). Amino acids identical in all sequences are marked in bold letters, and similar amino acids are underlined. In the consensus sequence (marked consensus), bold letters indicate identical and asterisks similar amino acids. In addition, identities between RNase H(70) and GOR are marked # in the line 'gcon' above the alignment. Parameters used for the alignment: Symbol comparison table: blosum62; gap weight: 12; gap length weight: 2. Five regions of clustered identical or similar amino acids are indicated as I, II, III, IV, and V below the consensus.

the RNase H(70) preparation was processed as described in Section 2, including preparative SDS-PAGE, concentration on Vandekerckhove gel, blotting onto nitrocellulose, and Coomassie brilliant blue R-250 staining. The stained spot was cut out and digested overnight with trypsin. HPLC purification of the tryptic peptides and Edman sequencing yielded the following three peptide sequences: TSVQEDDHT, NTFNEISD, and CVEDDET. Using those sequences, we unambiguously identified ORF YGR276c as the gene for RNase H(70) in the *S. cerevisiae* genome. We therefore renamed this

Table 1
Purification of yeast RNase H(70)

	Total protein (mg)	Protein (mg/ml)	Specific activity (U/μg)	Total activity (U)	Yield (%)	Purification factor (fold)
Extract[a]	6396	31.2	137	8.8×10^8	100	1
DNA cellulose	280	1.6	1052	2.9×10^8	34	8
Phenyl-Sepharose	29	0.28	2260	6.5×10^7	7.6	16
Hydroxyapatite	2.88	0.16	20270	5.8×10^7	6.8	148
Affi-Gel Blue	0.13	0.003b	309910	4.0×10^7	4.7	2260
Mono P	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.: not determined by activity test in solution.
[a]Cell extract after PEG precipitation.
[b]Estimation by silver staining.

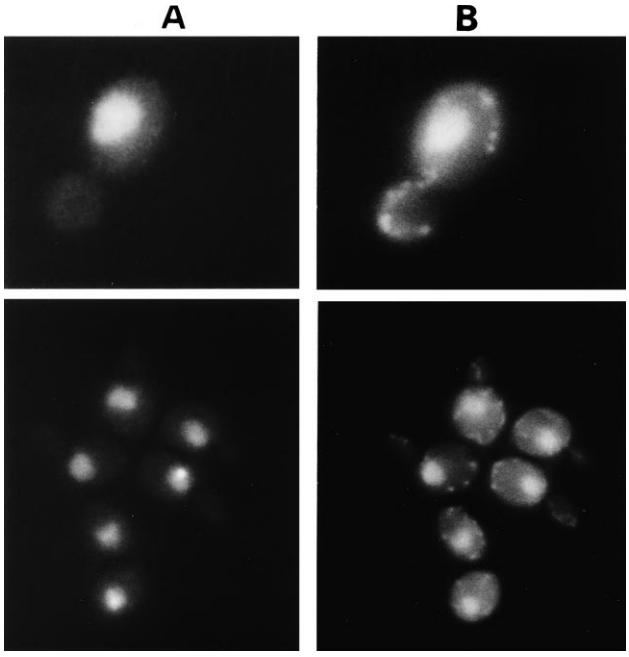

Fig. 3. Fluorescence microscopy of yeast cells expressing the RNase H(70)-EGFP fusion protein. Cells of strain AK606 were transfected with plasmid pUG36-RNH70 carrying the EGFP fusion construct, fixed and stained with DAPI. A: Localization of the expressed EGFP fusion protein. B: Costaining of DNA with DAPI.

ORF, which encodes a potential protein of molecular mass 62 849 and which is located on the right arm of chromosome VII, *RNH70*. The obvious molecular mass difference between the putative protein encoded by ORF *RNH70* and the isolated protein might be due to post-translational modification.

3.3. Sequence comparison uncovers a relationship between yeast RNase H(70) and other eukaryotic proteins

When searching the non-redundant protein database at NCBI using the BLAST program, we found highly significant homologies of the yeast RNase H(70) with the GOR antigen of the chimpanzee, *Pan troglodytes*, with a *Caenorhabditis elegans* ORF of 594 aa, with the *S. cerevisiae* ORFs YLR107w and YO080c, the *Xenopus laevis* XPMC2 protein, a *C. elegans* ORF of 271 aa, and the human ISG20 protein. An alignment of these sequences is shown in Fig. 2. Using the 'multiple sequence alignment with hierarchical clustering' method of Corpet [27], we detected five highly conserved motifs in all of the eight analyzed sequences. Another feature of the sequence becomes evident using the newly established position-specific iterated BLAST (PSI-Blast) program [28]. It indicates that RNase H(70) is additionally related to prokaryotic DNA polymerases I and III and prokaryotic RNases T (data not shown).

3.4. Deletion of the gene RNH70 from the genome does not result in a phenotype easily discernible under laboratory conditions

Using integrative transformation [26] with the deletion construct described in Section 2, we obtained the viable haploid strain BC70. A cell extract of this strain exhibited no significant decrease in overall RNase H activity compared to an extract from the corresponding RNase H(70) 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. Again no significant change in RNase H activity between the fraction derived from the deletion mutant and that derived from the strain with the intact *RNH70* gene was found. Additional experiments were performed to look for a mutant phenotype of the deletion strain: there was no difference in the proliferation rates of the RNase H(70) proficient or deficient cells as determined at 16°C, 30°C and 37°C (data not shown).

3.5. RNase H(70) is localized in the nucleus

Considering the enzyme activity and the potential nuclear localization signals (KKKK at aa 44 or 45, and at aa 178; KKRR at aa 19; KKPR at aa 462, or KPRK at aa 463) of RNase H(70), we looked where the enzyme was located within living cells. For that purpose we employed transformation of yeast cells with an expression vector containing *RNH70* fused to the gene for the EGFP as described in Section 2. These experiments, shown in Fig. 3, revealed a fluorescent signal exclusively in the nucleus (panel A), as was confirmed by co-staining of DNA (panel B). Control experiments using the expression vector alone showed a uniform distribution of fluorescence within the cells (not shown).

4. Discussion

In the work presented here, we have reported the purification to homogeneity of an enzyme, RNase H(70) of *S. cerevisiae*, which we had discovered more than 15 years ago [14]. Several obstacles severely impeded our efforts. (i) The polypeptide is highly sensitive to proteolysis which results in breakdown products, some of which retain enzyme activity. Therefore, only after the final purification step could we unambiguously prove enzyme activity in the in situ renaturation gel assay, and discriminate RNase H(70) from other yeast RNases H. (ii) RNase H(70) is expressed in only very low amounts during the whole cell cycle (data not shown). (iii) During purification a high proportion of the enzyme is lost by proteolysis whereas a copurifying polypeptide of similar molecular mass, the poly(A) binding protein, obviously is more stable and therefore is enriched in comparison to RNase H(70). Finally, however, an improved purification procedure enabled us to obtain a small amount of enzyme free of impurities, which could be used for isolation and sequencing of several tryptic peptides. Together these peptides were found in one ORF of the *S. cerevisiae* genome, namely in YGR276c on the left arm near the telomere of chromosome VII.

YGR276c, renamed *RNH70*, encodes a polypeptide of calculated molecular mass 62 849. At present we do not know the reason for the difference of this molecular mass from the observed protein band in SDS-PAGE at about 70 kDa; the difference may be due to post-translational modifications which have to be elucidated in further studies. During chromatofocusing on Mono P a p*I* of around 5 was observed, whereas the p*I* calculated from the amino acid sequence deduced from ORF YGR276c is 6.75. Thus, phosphorylation might be the reason for the acidic p*I* as well as for the observed molecular weight. Not unexpectedly, RNase H(70) could be identified as a nuclear protein.

We were surprised by our result that complete removal of

gene *RNH70* did not lead to an abnormal phenotype of the deletion mutants, at least not under the conditions tested. Recently we had discovered another RNase H of yeast, RNase H(35) [8]. Deletion of the coding region of that enzyme also did not entail a severely pathological phenotype but a significant decrease of total RNase H activity in the cell extract of the deletion mutant. Still another yeast gene encoding a protein with RNase H activity is known, *RNH1* [7], the deletion of which again does not have a dramatic impact on the affected cells. Strangely enough, even cells harboring combined deletions of the as yet known RNase H genes are viable (manuscript in preparation). Hence, different proteins possessing RNase H activity evidently are able to substitute for each other, and additional such protein(s) must exist in yeast cells. The only candidate we are at present aware of is Rad27p/FEN1 [29].

Another remarkable finding is the fact that the amino acid sequence of neither RNase H(35) nor RNase H(70) of *S. cerevisiae* is evolutionarily related to that of the 'prototype' RNases H, the RNase HI of *E. coli* and the RNase H domain of retroviral reverse transcriptase. Unexpectedly, selective hydrolysis of the RNA strand from a RNA-DNA hybrid can be carried out by polypeptides of completely unrelated primary structures. A homology search with the yeast RNase H(70) amino acid sequence revealed the GOR protein as the closest relative. This chimpanzee (and human) autoimmune antigen elicits an autoimmune response in a high proportion of hepatitis C patients [30]. The currently incurable disease is caused by the single (+) stranded RNA hepatitis C virus, it has enormous medical impact, as it may finally lead to cirrhosis and/or hepatocellular carcinoma. Although the complete sequence of the GOR protein is still unknown we suspect that it may be the human homolog of *S. cerevisiae* RNase H(70). In this connection another vertebrate RNase H should be mentioned: about 10 years ago a bovine RNase H was described [31] which resembled yeast RNase H(70) in several respects but seemed to be different from bovine RNases HI and II. The 78 kDa protein was, like yeast RNase H(70), shown to be strongly inhibited by *N*-ethylmaleimide and to prefer Mg^{2+} over Mn^{2+} as divalent cation. Furthermore it stimulated in vitro enzyme activity of DNA polymerase α [31]. Whether this bovine enzyme might be the homolog of yeast RNase H(70), or even of the so-called GOR protein, remains to be determined. Another human relative of yeast RNase H(70) is the interferon stimulated ISG20 protein. Together with PML (promyelocytic leukemia protein) and SP100 (an autoantigen of patients suffering from biliary cirrhosis), it is found in nuclear matrix associated multiprotein complexes, the so-called PML nuclear bodies, which might play a role during viral infections [32].

A remarkable homolog of RNase H(70) is also protein XPMC2 of *Xenopus laevis*, which is able to suppress certain mutations leading to mitotic catastrophe in the fission yeast *Schizosaccharomyces pombe* [33]. Interestingly, the use of PSI-Blast homology searches uncovered even sequence parts of prokaryotic DNA polymerases I and III, and RNases T as more distant relatives of RNase H(70). We hypothesize that these related proteins (or protein domains) might comprise a new superfamily of evolutionarily conserved nucleases.

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References

[1] Kornberg, A. and Baker, T.A. (1992) DNA Replication, Freeman, New York.
[2] Wintersberger, U. (1990) Pharmacol. Ther. 48, 259–280.
[3] Hughes, S.H., Arnold, E. and Hostomsky, Z. (1998) in: Ribonucleases H (Crouch, R.J. and Toulmé, J.J., Eds.), pp. 195–224, Editions INSERM, Paris.
[4] Johnson, M.S., McClure, M.A., Feng, D.F., Gray, J. and Doolittle, R.F. (1986) Proc. Natl. Acad. Sci. USA 83, 7648–7652.
[5] Kogoma, T. (1997) Microbiol. Mol. Biol. Rev. 61, 212–238.
[6] Itaya, M. (1990) Proc. Natl. Acad. Sci. USA 87, 8587–8591.
[7] Itaya, M., McKelvin, D., Chatterjie, S.K. and Crouch, R.J. (1991) Mol. Gen. Genet. 227, 438–445.
[8] Frank, P., Braunshofer-Reiter, C. and Wintersberger, U. (1998) FEBS Lett. 421, 23–26.
[9] Filippov, V., Filippova, M. and Gill, S.S. (1997) Biochem. Biophys. Res. Commun. 240, 844–849.
[10] Frank, P., Braunshofer-Reiter, C., Wintersberger, U., Grimm, R. and Büsen, W. (1998) Proc. Natl. Acad. Sci. USA 95, 12872–12877.
[11] Wu, H., Lima, W.F. and Crooke, S.T. (1998) Antisense Nucleic Acid Drug Dev. 8, 53–61.
[12] Cerritelli, S.M. and Crouch, R.J. (1998) Genomics 53, 300–307.
[13] Frank, P., Braunshofer-Reiter, C., Pöltl, A. and Holzmann, K. (1998) Biol. Chem. 379, 1407–1412.
[14] Karwan, R., Blutsch, H. and Wintersberger, U. (1983) Biochemistry 22, 5500–5502.
[15] Sachs, A.B., Bond, M.W. and Kornberg, R.D. (1986) Cell 45, 827–835.
[16] Karwan, R., Kühne, C. and Wintersberger, U. (1986) Proc. Natl. Acad. Sci. USA 83, 5919–5923.
[17] Valenta, B. (1993) PhD Thesis, University of Vienna.
[18] Sherman, F. (1991) Methods Enzymol. 194, 3–21.
[19] Frank, P., Cazenave, C., Albert, S. and Toulmé, J.J. (1993) Biochem. Biophys. Res. Commun. 196, 1552–1557.
[20] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[21] Laemmli, U.K. (1970) Nature 227, 680–685.
[22] Merril, C.R., Goldman, D. and VanKeuren, M. (1984) Methods Enzymol. 104, 441–447.
[23] Litman, R. (1968) J. Biol. Chem. 243, 6222–6233.
[24] Vandekerckhove, J. and Rasmussen, H.H. (1994) in: Cell Biology: A Laboratory Handbook (Celis, J.E., Ed.), pp. 359–368, Academic Press, San Diego, CA.
[25] Fernandez, J., De Mott, M., Atherton, D. and Mische, S.M. (1992) Anal. Biochem. 201, 255–264.
[26] Rothstein, R. (1991) Methods Enzymol. 194, 281–301.
[27] Corpet, F. (1988) Nucleic Acids Res. 16, 10881–10890.
[28] Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. (1997) Nucleic Acids Res. 25, 3389–3402.
[29] Shen, B., Nolan, J.P., Sklar, L.A. and Park, M.S. (1997) Nucleic Acids Res. 25, 3332–3338.
[30] Mishiro, S. et al. (1990) Lancet 336, 1400–1403.
[31] Hagemeier, A. and Grosse, F. (1989) Eur. J. Biochem. 185, 621–628.
[32] Gongora, C., David, G., Pintard, L., Tissot, C., Hua, T.D., Dejean, A. and Mechti, N. (1997) J. Biol. Chem. 272, 19457–19463.
[33] Su, J.Y. and Maller, J.L. (1995) Mol. Gen. Genet. 246, 387–396.