

REPORT

Purification and characterization of Rpp25, an RNA-binding protein subunit of human ribonuclease P

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ABSTRACT

In HeLa cells, ribonuclease P (RNase P), the tRNA processing enzyme consists of an RNA subunit (H1 RNA) associated with at least nine protein subunits, Rpp14, Rpp20, Rpp21, Rpp29 (hPop4), Rpp30, Rpp38, Rpp40, hPop1, and hPop5 (18.8 kDa). We report here the cloning and immuno-biochemical analysis of Rpp25, another protein subunit of RNase P. Polyclonal rabbit antibodies raised against recombinant Rpp25 recognize their corresponding antigens in RNase P-containing fractions purified from HeLa cells, and they also precipitate active holoenzyme. Furthermore, this protein has general RNA binding properties.

Keywords: molecular cloning; UV crosslinking

INTRODUCTION

Ribonuclease P (RNase P) is an ubiquitous endoribonuclease that removes the 5' leader sequences of precursor tRNAs (ptRNAs) to generate their 5' mature termini (Altman & Kirsebom, 1999). In eubacteria, the RNA component of the enzyme is the catalytic subunit (Guerrier-Takada et al., 1983) and a single, small basic protein cofactor increases not only the catalytic activity but also the substrate range of the holoenzyme.

RNase P RNAs, although essential for enzyme activity in several classes of eukaryotes, such as fungi (Lee et al., 1996; Xiao et al., 2001), amphibians (Doria et al., 1991), zebrafish (Eder et al., 1996), and mammals (Bartkiewicz et al., 1989; Altman et al., 1993), have not been shown to be catalytic in vitro. The dependence of eukaryotic RNase P activity on protein components indicates that these subunits may be involved directly in the catalytic mechanism of the enzyme.

Among the several proteins that copurify with human RNase P activity (Rpp14, Rpp20, Rpp21, Rpp25, Rpp29 [hPop4], Rpp30, Rpp38, Rpp40, hPop1, hPop5; Lygerou

et al., 1996; Eder et al., 1997; Jarrous et al., 1998, 1999; Van Eenennaam et al., 1999, 2001), only Rpp25 has not been characterized to date. We now report the immuno-biochemical characterization, cloning, and expression of Rpp25, an RNA-binding protein that copurifies with RNase P activity.

RESULTS

Molecular cloning and expression of Rpp25

Human RNase P was purified from HeLa cells as described previously (Eder et al., 1997) and a protein with an apparent molecular weight of 25 kDa, which copurified with RNase P activity, was extracted from a preparative 12% polyacrylamide/SDS gel and subjected to peptide microsequencing analysis. One such peptide, QPEPGVADEDQTA, was used to search the GenBank Expressed Sequence Tag database (dbEST). Two EST clones (human fetus and pancreas) containing the relevant peptide were found and sequenced and the open reading frame (ORF) that codes for a theoretical polypeptide of 199 amino acid residues was identified (Fig. 1).

BLAST searches using the human Rpp25 amino acid sequence as the query revealed *Mus musculus*

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1/                               31/1
CAA AGC CGG CGG TCC CGG CGA CGA CGG CGC ATG GAG AAC TTC CGT AAG GTG CGC TCC GAA
                               M E N F R K V R S E
61/11                             91/21
GAG GCG CCA GCG GGG TGC GGG GCC GAG GGA GGC GGC CCG GGC TCC GGC CCC TTC GCA GAC
E A P A G C G A E G G G P G S G P F A D
121/31                             151/41
CTG GCG CCG GGC GCG GTG CAC ATG CGG GTC AAG GAA GGC AGC AAG ATC CGG AAC CTG ATG
L A P G A V H M R V K E G S K I R N L M
181/51                             211/61
GCC TTC GCC ACC GCC AGC ATG GCG CAG CCA GCC ACG CGC GCC ATC GTC TTC AGC GGC TGC
A F A T A S M A Q P A T R A I V F S G C
241/71                             271/81
GGC CGG GCC ACC ACC AAA ACC GTC ACG TGC GCC GAG ATC CTC AAG CGC CGC CTG GCG GGC
G R A T T K T V T C A E I L K R R L A G
301/91                             331/101
CTG CAC CAG GTC ACG CGG CTG CGC TAC CGG AGC GTA CGC GAG GTG TGG CAG AGC CTC CCG
L H Q V T R L R Y R S V R E V W Q S L P
361/111                             391/121
CCT GGG CCC ACG CAG GGT CAG ACG CCT GGC GAG CCG GCC GCT AGT CTC AGC GTA CTT AAG
P G P T Q G Q T P G E P A A S L S V L K
421/131                             451/141
AAC GTG CCC GGC CTC GCC ATC CTA CTT TCC AAG GAC GCG CTG GAT CCG CGA CAG CCC GGC
N V P G L A I L L S K D A L D P R Q P G
481/151                             511/161
TAC CAG CCC CCG AAT CCC CAT CCT GGT CCC TCG TCC CCG CCA GCC GCG CCA GCG TCC AAG
Y Q P P N P H P G P S S P P A A P A S K
541/171                             571/181
AGG AGC CTA GGG GAA CCC GCA GCT GGA GAA GGC TCC GCG AAG CGA TCG CAA CCC GAG CCA
R S L G E P A A G E G S A K R S Q P E P
601/191                             631/
GGG GTT GCG GAC GAG GAT CAG ACG GCC TGA aac tcc gga ctg tgg gcc acc tag tcc ggc
G V A D E D Q T A *

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FIGURE 1. cDNA sequence and translated polypeptide sequence for Rpp25. The nucleotide sequence is numbered from the start of the total sequence indicated. The amino acid sequence is numbered from the first methionine residue and is shown in boldface letters. The portions of the peptide sequences that correspond to the tryptic peptide fragment derived from authentic Rpp25 are underlined.

(GenBank accession ID: AJ293897) and *Drosophila melanogaster* (flybase accession ID: CGN9422) homologs. The length, predicted molecular weight (MW) and isoelectric point (pI) of the three Rpp25 homologs are: *Homo sapiens*, 199 amino acids, 20,632 Da, and 9.66; *M. musculus* 163 amino acids, 17,675 Da, and 10.48; and *D. melanogaster*, 205 amino acids, 23,015 Da, and 8.73. Although our searches identified Rpp25 homologs in several higher plants, we are intrigued by the failure of our database mining efforts to identify homologs in eukaryotes such as *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (data not shown). Although the protein sequences might have diverged significantly and therefore escaped identification during BLAST searches, it is also possible that Rpp25 is not a member of the common subset of proteins absolutely essential for activity of all eukaryotic RNase P (see Discussion).

ClustalW (Thompson et al., 1994) was used to align the amino acid sequences of the three Rpp25 homologs from *H. sapiens*, *M. musculus*, and *D. melanogas-*

ter. The alignment revealed that these sequences share approximately 20% identity and 30% similarity (Fig. 2). In addition to various hydrophobic and basic residues, several proline residues are highly conserved. The Pfam program was unable to identify already characterized domain architectures in proteins in the Rpp25 sequence.

The Rpp25 coding sequence was subcloned in pHTT7K to facilitate expression in *Escherichia coli* of an N-terminal histidine-tagged Rpp25 derivative. This fusion protein was purified on a Ni-charged resin column and the highly purified, recombinant Rpp25 protein was then used to raise polyclonal antibodies in rabbits (see Materials and Methods).

Immunochemical analysis of Rpp25

Human RNase P was purified through the glycerol gradient fractionation and Mono Q FPLC steps of the procedure described elsewhere (Eder et al., 1997). Fractions constituting the peak of enzyme activity after glycerol gradient and MonoQ chromatography were

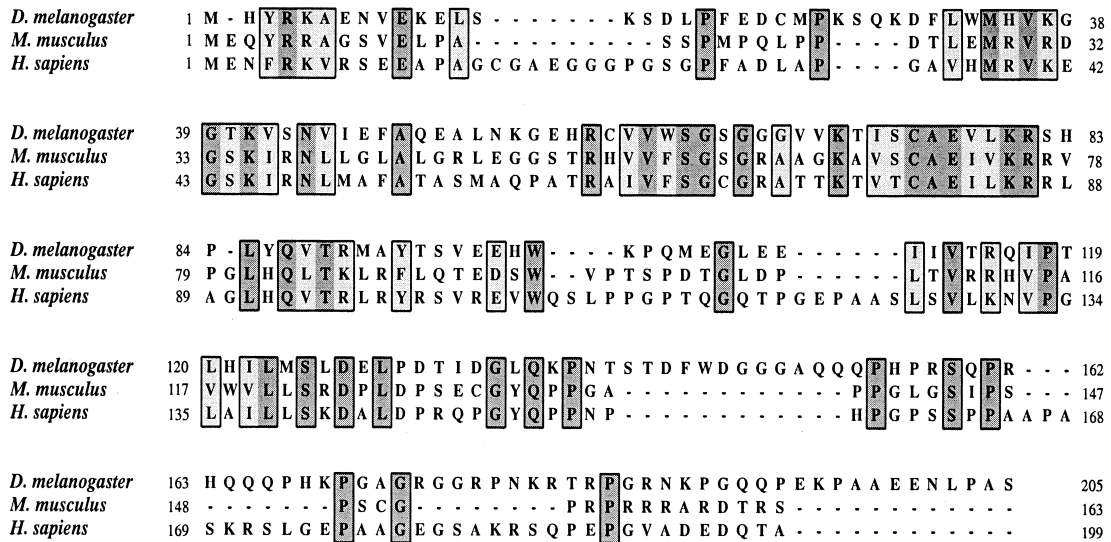


FIGURE 2. Sequence alignment of eukaryal Rpp25 homologs. A ClustalW-based alignment of the amino acid sequences of Rpp25 from *D. melanogaster*, *M. musculus*, and *H. sapiens* is depicted. Positions exhibiting identity and similarity are indicated in dark and light shaded boxes, respectively.

subjected to western blot analysis using anti-Rpp25 and Rpp38 antibodies. The finding that strong cross-reactivity to anti-Rpp25 antisera was observed only in the peak fractions confirmed that Rpp25 was associated with human RNase P activity (Fig. 3). Western blot analysis were also carried out using antibodies to Rpp20, Rpp21, Rpp29, Rpp30, Rpp40, and hPop1:

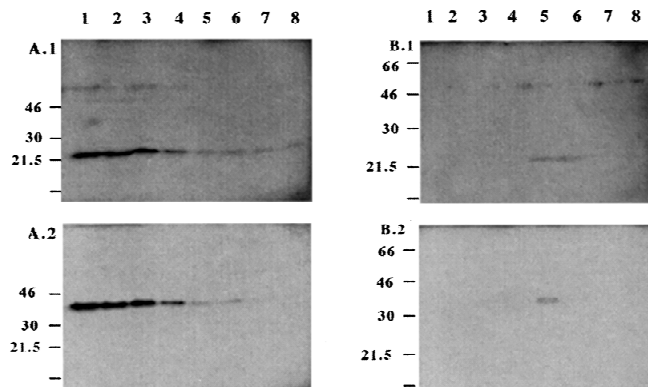


FIGURE 3. Western blot analysis. Rpp38 and Rpp25 copurify with RNase P activity. Aliquots from HeLa RNase P fractions purified through glycerol gradient step (**A**) [fractions 2 (lane 1), 4 (lane 2), 6 (lane 3), 8 (lane 4), 10 (lane 5), 12 (lane 6), 14 (lane 7), and 16 (lane 8)] and Mono Q step (**B**) [fractions 10 (lane 1), 14 (lane 2), 18 (lane 3), 20 (lane 4), 22 (lane 5), 24 (lane 6), 26 (lane 7), and 28 (lane 8)] were tested by western blot analysis for the presence of Rpp25 (**A.1** and **B.1**) and Rpp38 (**A.2** and **B.2**), respectively, using their corresponding polyclonal antibodies. Protein size markers are shown on the left-hand side of the figure. **A:** Fractions 2, 4, 6, and 8 off glycerol gradient correspond to RNase P activity peak (T. Jiang, pers. comm.). **B:** Fractions 22 and 24, off Mono Q column correspond to RNase P activity peak (T. Jiang, pers. comm.).

results confirmed the copurification of these proteins with RNase P activity (data not shown).

Polyclonal antibodies raised against recombinant Rpp25 were also tested for their ability to precipitate catalytically active RNase P complexes from partially purified preparations of RNase P. Immunoprecipitation of active holoenzyme was observed using anti-Rpp25 antibodies. Similar results were obtained using anti-Rpp21 antibodies, whereas preimmune sera failed to immunodeplete RNase P activity (Fig. 4). These results led us to investigate whether the purified, his-tagged Rpp25 could bind to H1 RNA, the RNA subunit of human RNase P.

Interactions of Rpp25 with the RNA subunit of human RNase P and ptRNA substrates

Purified recombinant Rpp25 protein, as well as Rpp20 and Rpp40, were incubated with ³²P-labeled H1 RNA, and a fragment of H1 RNA, the first 74 nts, containing the P3 domain (Chen & Pace, 1997; Jiang et al., 2001; referred to here as Sty RNA) and the mixture was subjected to short-wave UV irradiation. The products of the reaction were analyzed on a 9% SDS/polyacrylamide gel (Fig. 5A). Specific complexes with Sty RNA can be seen in Figure 5A, lane 1; higher molecular weight complexes can be seen in lane 4, with glycerol gradient enzyme, indicating that other protein subunits also interact with the holoenzyme. No interaction was observed with Rpp20 (Fig. 5A, lane 2), nor Rpp40 (Fig. 5A, lane 3). Using H1 RNA as the RNA species, with purified Rpp25 the only complex observable migrates near the stacking/

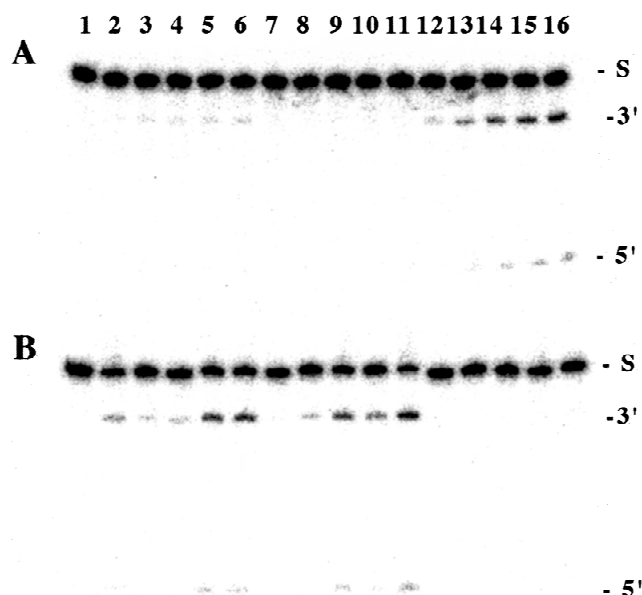


FIGURE 4. Immunoprecipitation of HeLa RNase P. HeLa RNase P purified through the glycerol gradient step was subjected to immunoprecipitation analysis using Protein A beads coupled with sera obtained from rabbits immunized with recombinant Rpp25 and Rpp21, as well as preimmune sera, as described in Materials and Methods. Supernatant fraction (top panel) and immunoprecipitated fraction (bottom panel) were assayed using ^{32}P labeled pSupS1 (Krupp et al., 1985) as substrates. Aliquots were taken after 2, 4, 6, 8, and 10 min incubation, and the products of the reaction were analyzed on an 8% denaturing polyacrylamide gel. Lane 1: pSupS1 incubated alone, for 10 min; lanes 2–6: samples treated with anti-Rpp25 antibodies; lanes 7–11: samples treated with anti-Rpp21 antibodies; lanes 12–16: samples treated with preimmune sera. The position of the reaction products are indicated: S = pSupS1; 5' = 5' leader sequence; 3' = tRNA moiety.

separation gel boundary (data not shown). A similar result is seen with an RNase P fraction purified through the glycerol gradient step (data not shown). Accordingly, Rpp25 interactions with several RNAs were then assayed by performing gel shift assays.

Rpp25 and RNA samples were incubated for 10 min and then loaded directly on a 5% nondenaturing gel (see Materials and Methods). The RNAs tested included H1 RNA and an RNase P substrate, the precursor to *E. coli* tRNA^{Tyr} (pTyr; Guerrier-Takada et al., 1989). These experiments were also carried out with Rpp25 in which the His-tag was removed. The interaction of Rpp25 with the various RNAs tested is not influenced by the absence or presence of a His-tag in Rpp25 (Fig. 5B). As can be seen, multiple complexes are detected; these complexes are still present even in the presence of 5 mM DTT in the reaction mix (data not shown). We have also observed complexes of Rpp25 with M1 RNA (the RNA subunit of *E. coli* RNase P), and with an non-RNase P-related RNA fragment (data not shown).

It has recently been reported (Jarrous et al., 2001) that Rpp14 and Rpp21, two protein subunits of human

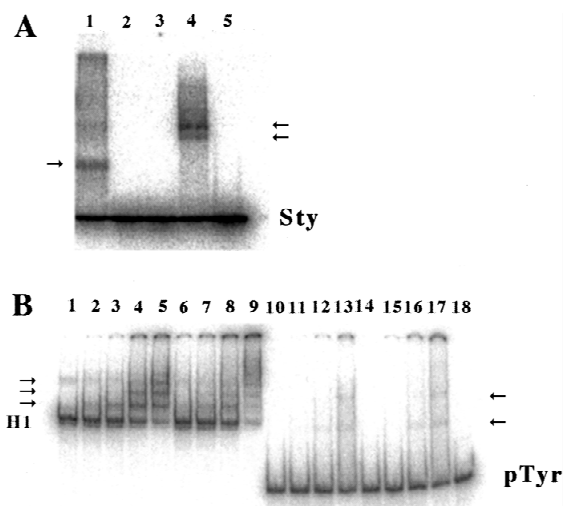


FIGURE 5. UV-crosslinking and gel shift assays. **A:** 9% polyacrylamide/SDS gel. ^{32}P -labeled Sty RNA was incubated alone (lane 5), with recombinant Rpp25 (lane 1), Rpp20 (lane 2), Rpp40 (lane 3), or RNase P fraction purified through the glycerol gradient step (lane 4), as described in Materials and Methods. After UV irradiation for 4 min at room temperature, the samples were loaded onto a 9% polyacrylamide/SDS gel. The gel was dried and exposed to autoradiography. Specific complexes can be seen in lanes 1 and 4. **B:** 5% nondenaturing polyacrylamide gel. ^{32}P -labeled H1 RNA (0.04 pmol) was incubated for 10 min in the presence of increasing concentration of Rpp25 (0, 0.3, 0.6, 1.2, and 2.5 pmol), without (lanes 1–5), or with (lanes 6–9) the His tag. ^{32}P -labeled pTyr (0.5 pmol) was incubated for 5 min in the presence of increasing concentration of Rpp25 (0.6, 1.2, 2.5, and 5 pmol), without (lanes 10–13) or with (lanes 14–17) the His tag. After incubation, the samples were loaded on a 5% polyacrylamide gel as described in Materials and Methods. Multiple complex bands can be seen, as indicated by the arrows.

RNase P, also bind to precursor tRNA. However, we do not know yet how, nor to which regions of the precursor tRNA, these three proteins bind.

DISCUSSION

We show here that Rpp25 is part of the human RNase P complex. This protein is present in purified RNase P enzyme preparations as determined by its immunochemical properties and, previously, its biochemical purification. Fluorescent staining also indicates that Rpp25 is located in the nucleus and nucleosomes, as with other subunits of the enzyme (data not shown; pictures available on request). Furthermore, Rpp25 binds to various RNAs, some of which are not part of the RNase P complex. Whether it plays a specific role in the interaction with the RNA subunit of RNase P and/or the precursor tRNA substrates remains to be proven, although these RNAs do form complexes in native gels with Rpp25. Although the binding mediated by Rpp25 appears to be nonspecific, the overall affinity for an RNA ligand might be enhanced by Rpp25 when it acts in concert with an RNA-binding domain present in one of the other protein subunits of RNase P. Although

there are no RGG boxes in human Rpp25, the non-specific binding exhibited by Rpp25 is reminiscent of the characteristics of RBPs like the RGG box-containing proteins (Burd & Dreyfuss, 1994). Moreover, such a functional role might prove redundant in some instances and could help rationalize the absence of Rpp25 homologs in some eukaryotes.

Some of the protein subunits of human RNase P were shown to interact with the cognate RNA subunit by using a three-hybrid assay and UV crosslinking (Jiang et al., 2001). As part of a complementary approach, experiments are now in progress to purify the various protein subunits of the human RNase P complex in soluble form and investigate if any of them can bind H1 RNA *in vitro*, either alone (like Rpp25) or in some combination with other factors.

MATERIALS AND METHODS

Molecular cloning of Rpp25

Human RNase P from HeLa S3 cells was purified through the Mono Q FPLC step as described (Eder et al., 1997). Fractions copurifying with RNase P activity were analyzed on preparative polyacrylamide/SDS gels, and a protein band of apparent MW of 25 kDa, visualized by Coomassie brilliant blue staining, was analyzed by the W. M. Keck Biotechnology Resource Center at Yale University. The tryptic peptide QPEP-GVADEDQTA was searched in the dbEST using the tblastn program. Two human EST clones containing the relevant peptide were sequenced. One of these clones, ATCC 1053991 (GenBank accession no. AA448932) was found to contain the complete ORF of Rpp25.

Purification of recombinant protein and preparation of polyclonal antibodies

Two primers, p25NDEF (CCATGGCATATGGAGAACTCCGTAAGG) and p25RIR (AAGCTTGAATTCAGGCCGTCTGATCC), were used for PCR using the FailSafe™ PCR PreMix selection Kit (Epicentre), and with the Rpp25 cDNA as the template. The PCR product was first cloned into the *Sma*I site of pUC19. A *Pst*I digest, followed by treatment with Klenow polymerase and by *Nde*I digest, generated an insert encompassing the complete ORF of Rpp25. This insert was purified and ligated with pHTT7K vector that had been (1) digested with *Sac*I, (2) treated with Klenow polymerase, and (3) digested with *Nde*I to generate the plasmid pHTT7K/Rpp25. DNA sequencing confirmed the sequence of the clone obtained.

BL21(DE3) *E. coli* was transformed with pHTT7K/Rpp25. Five hundred milliliters of LB-kanamycin (25 µg/mL) were inoculated with 5 mL overnight culture in the same medium (LB Kan), and the cells were incubated at 37°C until $A_{600} \sim 0.2$ – 0.4 . A 1-mL aliquot was withdrawn to be used as the uninduced control in SDS-PAGE analysis. Subsequently, IPTG from a freshly made solution was added to a final concentration of 1 mM. The culture was then incubated for another 3 h at 30°C. A 1-mL aliquot was withdrawn and analyzed on

SDS-polyacrylamide gel to ascertain induction of the desired protein. Cells were harvested at 7,000 rpm for 10 min at 4°C (GSA rotor), and frozen at -76°C until use. The cell pellet was resuspended with 18 mL B-PER™ (Pierce) containing two tablets of complete, Mini, EDTA-free (protease inhibitor from Roche Molecular Biochemicals). Two milliliters of 10% N-Lauroylsarcosine (Sigma) in B-PER were added, to allow Rpp25 to remain in the soluble fraction. The cell suspension was mixed gently for 10 min at room temperature, and then centrifuged at $16,000 \times g$ for 20 min at 4°C. The supernatant was passed through a 0.45 µm membrane to prevent clogging of the column; 1/3 vol of 4× binding buffer (80 mM Tris-HCl, pH 7.9, 2 M NaCl, 20 mM imidazole) was added, and the sample loaded onto a His-Bind column equilibrated with binding buffer. After loading the sample, the column was washed with buffers containing 5 mM (binding buffer), 60 mM, and 100 mM imidazole; the protein was then eluted with a gradient from 100 mM to 400 mM imidazole; some protein was eluted with the 100 mM imidazole wash (data not shown). The purity of the preparation was determined by SDS-PAGE analysis.

Polyclonal rabbit antibodies raised against recombinant Rpp25 were obtained from the Pocono Rabbit Farm (Candens, Pennsylvania).

Western blot analysis

Proteins in fractions obtained after glycerol gradient and MonoQ FPLC fractionation procedures were separated on 12% polyacrylamide/SDS gel, electrotransferred to a nitrocellulose membrane, and immunoblotted with a 1:100 dilution of sera as described (Jiang et al., 2001). As a secondary antibody, a 1:5,000 dilution of anti-rabbit mouse IgG antibody (Vector Laboratories) was made in the same buffer and incubated for 60 min. Blots were washed with TNT and TN (Jiang et al., 2001) and antibody-antigen complexes were visualized using the ECL-Plus detection system (Amersham-Pharmacia), following the manufacturer's instructions.

Immunoprecipitation and assay of RNase P activity

Polyclonal rabbit antibodies (40 µL of sera) raised against Rpp25 and Rpp21, as well as preimmune sera were mixed with 5 mg Protein A Sepharose CL-4B (Pharmacia) equilibrated with 500 µL NET-2 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% NP-40). Coupling was achieved by nutating the beads overnight at 4°C, followed by four washes with NET-2, and two washes with RNase P reaction buffer (52 mM Tris-HCl, pH 7.5, 100 mM NH₄Cl, 35 mM NaCl, 10 mM MgCl₂, 0.1 mM EDTA, 1 mM 2-mercaptoethanol, 0.01% Triton X-100) containing RNasin (10 U/mL). Three microliters from a glycerol gradient peak fraction, enriched in RNase P activity, were added to 160 µL of the same buffer, and 50 µL were added to each one of the samples. The beads were nutated at 4°C for about 2 h. The suspension was centrifuged for 2 min at 10,000 rpm (Eppendorf centrifuge) and the supernatant was collected for assays. The beads were washed four times with RNase P buffer, and finally resuspended with 20 µL RNase P buffer containing ³²P-labeled pSupS1 substrate. Ten microliters of the supernatant were mixed with

10 μ L substrate mix, and the reactions were incubated at 37 °C. The substrate concentration was 1 mM. Aliquots were withdrawn after 2, 4, 6, 8, and 10 min of incubation and analyzed on an 8% denaturing gel. Results were quantitated using a Fuji Phosphorimager.

UV crosslinking and gel shift assays

HeLa nuclear RNase P purified through the glycerol gradient step or purified recombinant Rpp25 were incubated at 37 °C in the presence of labeled H1 RNA, an H1 RNA fragment containing the P3 domain, or a precursor tRNA substrate in 20 mM HEPES-KOH, pH 8.0, 1 mM MgCl₂, 200 mM NaCl, 5% glycerol (HGMM buffer). After 5 min, the samples were either placed on a sheet of saran wrap over a short-wave UV transilluminator and irradiated for 4 min at room temperature (Jiang et al., 2001), or loaded onto a 5% polyacrylamide gel (20 × 20 × 0.1 cm) with 20 mM HEPES-KOH, pH 8.0, 1 mM MgCl₂ as the running buffer. Electrophoresis at 30 mA was carried out at 4 °C for 2 h, with continuous recirculation of the buffer. Results were quantitated using a Fuji Phosphorimager.

NOTE ADDED IN PROOF

While this manuscript was under review, two additional Rpp25 ESTs were deposited in the database. These new sequences indicate the presence of Rpp25 paralogs in both *Mus musculus* and *Homo sapiens*. The significance of this finding remains to be investigated.

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