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Autoantigenic properties of some protein subunits of catalytically active complexes of human ribonuclease P

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ABSTRACT

At least six proteins co-purify with human ribonuclease P (RNase P), a tRNA processing ribonucleoprotein. Two of these proteins, Rpp30 and Rpp38, are Th autoantigens. Recombinant Rpp30 and Rpp38 are also recognized by Th sera from systemic sclerosis patients. Two of the other proteins associated with RNase P, Rpp20 and Rpp40, do not cross-react with Th sera. Polyclonal antibodies raised against all four recombinant proteins recognize the corresponding proteins associated with RNase P and precipitate active holoenzyme. Catalytically active RNase P holoenzyme can be separated from the nucleolar and mitochondrial RNA processing endoribonuclease, RNase MRP, even though these two enzymes may share some subunits.

Keywords: auto-antigens; human RNase P; nuclear localization sequences; RNase MRP

INTRODUCTION

Ribonuclease P (RNase P) is an ubiquitous ribonucleoprotein that processes the 5′ leader sequences of tRNA precursor (ptRNA) molecules (Altman, 1990). The RNA subunit of RNase P from *Escherichia coli*, M1 RNA, was shown to be catalytic under certain conditions in vitro (Guerrier-Takada et al., 1983). However, C5 protein, the protein subunit of *E. coli* RNase P, is required for catalysis in vivo (Altman, 1990; Altman et al., 1993). By contrast, none of the eukaryotic RNase P RNA subunits identified so far exhibit catalytic activity in vitro (Altman et al., 1993).

Human RNase P consists of one RNA species, H1 RNA (Bartkiewicz et al., 1989), and at least six proteins that co-purify with the enzymatic activity in our preparations (Eder et al., 1997). As judged by their electrophoretic mobilities in SDS-PAGE, these six putative subunits of RNase P have apparent molecular weights of 14, 20, 25, 30, 38, and 40 kDa. Accordingly, they were designated Rpp14, Rpp20, Rpp25, Rpp30, Rpp38, and Rpp40 (Eder et al., 1997). Another protein, Pop 1, may also be part of this complex in vivo (Lygerou et al., 1996b; see below).

Eukaryotic RNase P and the mitochondrial RNA processing endoribonuclease, RNase MRP (Chang & Clayton, 1987), apparently share some common protein subunits and structural features of their RNA subunits (Gold et al., 1988, 1989; Forster & Altman, 1990; Lygerou et al., 1994; Chu et al., 1997; Dichtl & Tollervey, 1997). In *Saccharomyces cerevisiae*, the *POP1* gene encodes a protein component (100 kDa) common to RNase P and RNase MRP (Lygerou et al., 1994, 1996a). A Pop1 homologue was characterized in human cells recently, and it was also shown that Pop1 is recognized by some sera from patients with autoimmune diseases (Lygerou et al., 1996b). A protein (22.6 kDa) that is encoded by the yeast *POP3* gene was also found to be a common component of both holoenzymes in yeast (Dichtl & Tollervey, 1997). Additionally, the product of the yeast *POP4* gene is associated with both yeast and human RNase P (Chu et al, 1997; and see below).

Sera from patients with different autoimmune diseases harbor antibodies that recognize and precipitate human RNase P (Gold et al., 1988). Specifically, sera from patients with systemic sclerosis (scleroderma) can precipitate RNase P RNA (Reddy et al., 1983; Gold et al., 1988) and immunodeplete its activity (Gold et al.,

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1988; Eder et al., 1997). Many of these autoantibodies react with a nuclear protein with apparent molecular weight of 38–40 kDa, known as the Th or To antigen (Hashimoto & Steitz, 1983; Reddy et al., 1983; Reimer et al., 1988). Some Th sera also harbor autoantibodies against another protein, Rpp30, that co-purifies with RNase P (Gold, 1988; Eder et al., 1997) and also against Pop 1 (Lygerou et al., 1996b). The Th antigen also binds to RNase P RNA as well as to RNase MRP RNA (Yuan et al., 1991).

We show here that *recombinant* Rpp38 is recognized by Th sera from various scleroderma patients, and that some sera also harbor autoantibodies against *recombinant* Rpp30. The Rpp38 autoantigen forms dimers and possesses features found in nuclear RNA-binding proteins. Moreover, through the use of polyclonal rabbit antibodies that were raised against the recombinant proteins, we show that Rpp30 and Rpp38 cDNAs code for proteins associated with catalytic RNase P complexes from HeLa cells. Human Rpp30 exhibits 91% identity to a mouse homologue and has notable homology also to a gene from *S. cerevisiae* (Stolc & Altman, 1997).

Two proteins that do not cross-react with Th sera, Rpp20 and Rpp40, also co-purify with human RNase P. Polyclonal rabbit antibodies raised against recombinant Rpp20, Rpp30, Rpp38, and Rpp40 precipitate catalytically active RNase P. The possible roles of these four protein components in RNase P function and their relationship to RNase MRP are addressed.

RESULTS

Molecular cloning of Rpp20 and Rpp40 cDNAs

Human RNase P from HeLa cells was purified as described previously (Eder et al., 1997; see Materials and Methods). Proteins of apparent molecular weight of 20 and 40 kDa that were shown to co-purify with human RNase P (Eder et al., 1997) were isolated from preparative SDS-PAGE gels for microsequencing analysis of peptide fragments.

The GenBank Expressed Sequence Tag database (dbEST) was searched for a peptide sequence (AVE AELDPVEY) corresponding to that obtained from the 20-kDa protein and a corresponding cDNA clone was identified. The sequence of this clone, designated *Rpp20* cDNA, is presented in Figure 1A. *Rpp20* cDNA has one open reading frame that codes for a polypeptide of 140 amino acids. The predicted molecular weight of the polypeptide is 15.6 kDa and it has a theoretical pI of 8.7.

Molecular cloning of Rpp40 cDNA is described in detail in Materials and Methods. *Rpp40* cDNA has an open reading frame that codes for a protein of 302 amino acids, with a predicted molecular weight of 34.5 kDa (Fig. 1B). In contrast to Rpp20, Rpp40 protein is acidic and has a theoretical pI of 5.3. The sequences of the peptide fragments that were derived from the 20-kDa and 40-kDa proteins were found in the two polypeptides encoded in the cDNA clones (see Fig. 1) that were designated *Rpp20* and *Rpp40*, respectively.

Expression of recombinant Rpp20, Rpp30, Rpp38, and Rpp40 proteins

The cDNAs of Rpp20, Rpp40, as well as Rpp30 and Rpp38 (Eder et al., 1997) were subcloned in pHTT7K and overexpressed in *E. coli* as histidine-tagged proteins. Each overexpressed polypeptide was purified on a nickel-charged resin column and was solubilized in 7 M urea. The apparent molecular weights of the recombinant, tagged proteins were determined in 12% polyacrylamide–SDS gels (Fig. 2A,B,C,D). Although the cDNAs of Rpp20, Rpp38 (Eder et al., 1997), and Rpp40 predicted recombinant proteins of 18.6, 35, and 37.5 kDa (including 2–3 kDa of the histidine tag), respectively, the recombinant proteins migrated in SDS-PAGE (Fig. 2A,C,D) with apparent molecular weights of about 22, 41, and 43 kDa, respectively. By contrast, no anomalous migration was seen for Rpp30, a tagged protein of 32 kDa (Fig. 2B). Thus, the cDNAs for Rpp20, Rpp30, Rpp38, and Rpp40 code for recombinant proteins with electrophoretic mobilities similar to those of the authentic proteins that co-purify with human RNase P from HeLa cells (Eder et al., 1997; see below) after taking into account the size of the histidine tags.

Recombinant Rpp30 and Rpp38 proteins are recognized by anti-Th sera

Anti-Th sera from scleroderma patients (a generous gift of Dr. J. Craft, Yale University School of Medicine) that were previously shown to react with the Rpp30 and Rpp38 proteins of human RNase P (Gold, 1988; Eder at al., 1997) were tested for their ability to recognize the recombinant proteins. ThFi and ThLo sera cross-reacted with both recombinant Rpp30 (Fig. 2E,F, lane 2) and Rpp38 (Fig. 2E,F, lane 3). ThFi serum reacted with Rpp30 and Rpp38 of human RNase P in a positive control (Fig. 2E, lane 1). ThLo serum has been shown previously to react with both proteins (Eder et al., 1997). By contrast, ThHE and ThMi sera cross-reacted with recombinant Rpp38 (Fig. 2G,H, lane 3), but not with Rpp30 (Fig. 2G,H, lane 2). The titers of the autoantibodies against Rpp38 were lower in ThHE and ThMi than ThFi and ThLo sera. ThFi and ThLo sera were also more efficient in precipitating human RNase P activity than were ThMi (Eder et al., 1997) and ThHE (data not shown). Neither recombinant Rpp20 (Fig. 2F,G,H, lane 1) nor Rpp40 (Fig. 2E,F, lane 4) cross-reacted with the four autoimmune sera tested in this study. Thus, four sera from scleroderma patients defined as Th positive recognized recombinant Rpp38, and two of these sera, ThFi and

A

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1                                                   31
GCG CGG GCA GGG CCG CAC GAG CTG GCT GGC TGC TTG CAC CCA CAT CCT TCT TTC TCT GGG
61                                                  91
ACC TGG GGT CGC GGT TAC TTG GGC TGG CCG GCG AAC CCT TGA GTG GCC TGG CGG GGA GCG
121                                                 151
GGC CTC GCG CGC CTG GAG GGC CCT GTG GAA CGA AGA GAG GCA CAC AGC ATG GCA GAA AAC
                                                                M   A   E   N
181/5                                               211/15
CGA GAG CCC CGC GGT GCT GTG GAG GCT GAA CTG GAT CCA GTG GAA TAC ACC CTT AGG AAA
R   E   P   R   G   A   V   E   A   E   L   D   P   V   E   Y   T   L   R   K
241/25                                              271/35
AGG CTT CCC AGC CGC TTG CCC CGG AGA CCC AAT GAC ATT TAT GTC AAC ATG AAG ACG GAC
R   L   P   S   R   L   P   R   R   P   N   D   I   Y   V   N   M   K   T   D
301/45                                              331/55
TTT AAG GCC CAG CTG GCC CGC TGC CAG AAG CTG CTG GAC GGA GGG GCC CGG GGT CAG AAC
F   K   A   Q   L   A   R   C   Q   K   L   L   D   G   G   A   R   G   Q   N
361/65                                              391/75
GCG TGC TCT GAG ATC TAC ATT CAC GGC TTG GGC CTG GCC ATC AAC CAC GCC ATC AAC ATC
A   C   S   E   I   Y   I   H   G   L   G   L   A   I   N   H   A   I   N   I
421/85                                              451/95
GCG CTG CAG CTG CAG GCG GGC AGC TTC GGG TCC TTG CAG GTG GCT GCC AAT ACC TCC ACC
A   L   Q   L   Q   A   G   S   F   G   S   L   Q   V   A   A   N   T   S   T
481/105                                             511/115
GTG GAG CTT GTT GAT GAG CTG GAG CCA GAG ACC GAC ACA CGG GAG CCA CTG ACT CGG ATC
V   E   L   V   D   E   L   E   P   E   T   D   T   R   E   P   L   T   R   I
541/125                                             571/135
CGC AAC AAC TCA GCC ATC CAC ATC CGA GTC TTC AGG GTC ACA CCC AAG TAA TTG AAA AGA
R   N   N   S   A   I   H   I   R   V   F   R   V   T   P   K   *
601                                                 631
CAC TCC TCC ACT TAT CCC CTC CGT GAT ATG GCT CTT CGC ATG CTG AGT ACT GGA CCT CGG
661                                                 691
ACC AGA GCC ATG TAA GAA AAG GCC TGT TCC CTG GAA GCC CAA AGG ACT CTG CAT TGA GGG
721                                                 751
TGG GGG TAA TTG TCT CTT GGT GGC CCA GTT AGT GGG CCT TCC TGA GTG TGT GTA TGC GGT
781                                                 811
CTG TAA CTA TTG CCA TAT AAA TAA AAA ATC CTG TTG CAC TAG TGT CCT GCC AAA AAA AAA
841                                                 871
AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AAA AA
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B

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1                                                   31
CGG ACG GAG GAA AAG TGG CGT TGA AAA GGC AAG AGC CAG GTT CCC AGC CAC TGG GAC AGT
61/21                                               91
GAA TTT ACA GCT TCA GAC ATC CAC CAA AGA GCG GCT GCG TGG GCT CCT AAG AAG TCT AAG
121                                                 151
AAC TGC TCA GAA ATC CAG CTC CGC CAT CCC CAC TCC ACC GCC GCC AGG TCA CAG CAG CGT
181                                                 211
GTT TCA TTT CTC ATT CCT GAA TGT GGG ATA CTA TCG GAA GAA CTG AAA AAC CTG GTC ATG
                                                                            M
241/2                                               271/12
AAC ACT GGA CCC TAT TAC TTT GTG AAG AAT TTA CCT CTT CAT GAA TTA ATT ACA CCT GAA
N   T   G   P   Y   Y   F   V   K   N   L   P   L   H   E   L   I   T   P   E
301/22                                              331/32
TTC ATC AGT ACC TTT ATA AAG AAA GGT TCT TGC TAT GCA CTA ACA TAC AAT ACA CAT ATT
F   I   S   T   F   I   K   K   G   S   C   Y   A   L   T   Y   N   T   H   I
361/42                                              391/52
GAT GAA GAT AAT ACT GTT GCC CTG CTA CCA AAT GGG AAA TTA ATT TTG TCA CTG GAT AAA
D   E   D   N   T   V   A   L   L   P   N   G   K   L   I   L   S   L   D   K
421/62                                              451/72
GAC ACT TAT GAA GAA ACT GGA CTT CAG GGT CAT CCA TCT CAG TTT TCT GGC AGA AAA ATT
D   T   Y   E   E   T   G   L   Q   G   H   P   S   Q   F   S   G   R   K   I
481/82                                              511/92
ATG AAA TTT ATT GTT TCC ATT GAT TTG ATG GAA TTA TCC TTA AAC TTG GAT TCT AAG AAG
M   K   F   I   V   S   I   D   L   M   E   L   S   L   N   L   D   S   K   K
541/102                                             571/112
TAT GAA AGA ATA TCT TGG TCT TTC AAA GAA AAG AAG CCA TTG AAA TTT GAT TTT CTT TTG
Y   E   R   I   S   W   S   F   K   E   K   K   P   L   K   F   D   F   L   L
601/122                                             631/132
GCT TGG CAT AAA ACA GGT TCA GAA GAA TCG ACA ATG ATG TCA TAT TTT TCC AAG TAC CAA
A   W   H   K   T   G   S   E   E   S   T   M   M   S   Y   F   S   K   Y   Q
661/142                                             691/152
ATT CAG GAG CAC CAG CCA AAA GTA GCA CTG AGC ACG TTG AGA GAT CTC CAG TGC CCA GTG
I   Q   E   H   Q   P   K   V   A   L   S   T   L   R   D   L   Q   C   P   V
721/162                                             751/172
CTG CAG AGC AGC GAG CTG GAG GGA ACG CCA GAG GTG TCC TGC CGG GCT CTG GAG CTC TTC
L   Q   S   S   E   L   E   G   T   P   E   V   S   C   R   A   L   E   L   F
781/182                                             811/192
GAC TGG CTC GGC GCC GTC TTC AGT AAT GTC GAC CTA AAT GAG CCT AAT AAT TTC ATA TCA
D   W   L   G   A   V   F   S   N   V   D   L   N   E   P   N   N   F   I   S
841/202                                             871/212
ACC TAT TGC TGT CCT GAG CCA AGC ACA GTG GTG GCA AAA GCT TAT TTG TGT ACA ATC ACT
T   Y   C   C   P   E   P   S   T   V   V   A   K   A   Y   L   C   T   I   T
901/222                                             931/232
GGC TTC ATA CTT CCA GAG AAG ATC TGT CTC CTA TTG GAA CAT CTC TGT CAC TAC TTT GAT
G   F   I   L   P   E   K   I   C   L   L   L   E   H   L   C   H   Y   F   D
961/242                                             991/252
GAA CCG AAG TTA GCT CCA TGG GTT ACA CTG TCC GTT CAA GGC TTT GCA GAC AGC CCT GTT
E   P   K   L   A   P   W   V   T   L   S   V   Q   G   F   A   D   S   P   V
1021/262                                            1051/272
TCT TGG GAA AAA AAT GAA CAT GGT TTT CGA AAA GGA GGA GAA CAT TTA TAT AAC TTT GTG
S   W   E   K   N   E   H   G   F   R   K   G   G   E   H   L   Y   N   F   V
1081/282                                            1111/292
ATT TTT AAT AAT CAG GAC TAT TGG CTT CAG ATG GCT GTT GGG GCA AAT GAT CAC TGT CCA
I   F   N   N   Q   D   Y   W   L   Q   M   A   V   G   A   N   D   H   C   P
1141/302                                            1171
CCA TAA AAA ATA AAA ATT AAA AAT CGT GTT TAC TTA CAA AAA AAA AAA AAA AAA
P   *
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FIGURE 1. cDNA sequence and corresponding polypeptide sequence for *RPP20* (**A**) and *RPP40* (**B**). Nucleotide sequences are numbered from the start of each total sequence. Amino acid sequences are numbered from the first methionine residue and are shown in bold. Portions of the peptide sequences that correspond to tryptic peptide fragments derived from HeLa Rpp20 and Rpp40 are underlined. GenBank accession number for RPP20, Z46005; for RPP40, U94317.

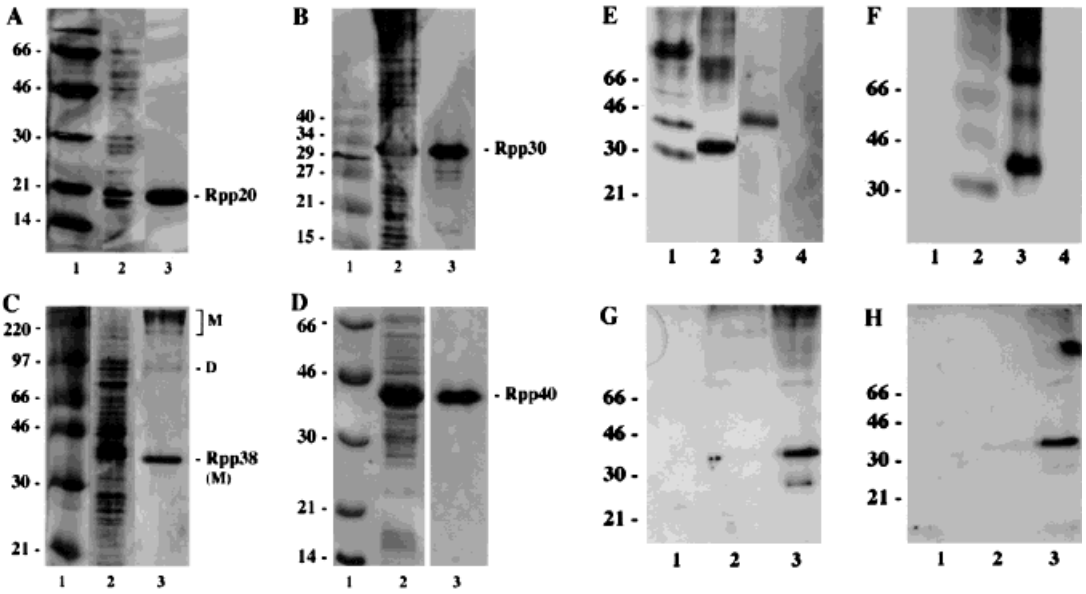

FIGURE 2. Molecular weights of recombinant RNase P proteins and recognition of recombinant Rpp30 and Rpp38 by Th sera from patients with systemic sclerosis. Histidine-tagged Rpp20 (**A**), Rpp30 (**B**), Rpp38 (**C**), and Rpp40 (**D**) were overexpressed in *E. coli* (see Materials and Methods) and affinity-purified using His-bind resin for Rpp20 (A) or Hi-Trap FPLC column for Rpp30, Rpp38, and Rpp40 (B,C,D). The chelating column was charged with 50 mM nickel sulfate, and protein was eluted using a gradient of imidazole (20–200 mM). Lane 1 in A–D shows the positions of protein size markers in kDa. Lane 2 in A–D shows the protein composition of cell lysates. Lane 3 in A–D shows silver stains of the eluted protein after gel electrophoresis. Dimers (D) and multimers (M) of Rpp38 are indicated in C. Panel D is a composite of two different gel runs. Autoimmune Th sera from four different systemic sclerosis patients, ThFi (**E**), ThLo (**F**), ThHE (**G**), and ThMi (**H**), were used in western blot analyses of recombinant Rpp20 (lane 1 in F–H), Rpp30 (lane 2 in E–H), Rpp38 (lane 3 in E–H), and/or Rpp40 (lane 4 in E and F). Five to ten micrograms of recombinant protein were used for each blot. Lane 1 in panel E contained RNase P pooled from several fractions from the DEAE-sepharose step. Panel E is a composite of lanes from the same gel.

ThLo, also harbored autoantibodies against recombinant Rpp30. These findings show that Rpp38 is identical to the Th/To antigen (Reimer et al., 1988; Yuan et al., 1991).

Polyclonal antibodies raised against recombinant Rpp20, Rpp30, Rpp38, and Rpp40 immunoprecipitate HeLa RNase P

Rabbit polyclonal antibodies raised against recombinant Rpp20, Rpp30, Rpp38, and Rpp40 were tested for their ability to precipitate catalytically active RNase P complexes directly from HeLa cell lysates or from partially purified preparations of RNase P. Immunoprecipitation of active holoenzyme was observed using anti-Rpp20 (Fig. 3E, lane 5; see below for data concerning western blots). Similar results were obtained for RNase P purified from the glycerol gradient step for anti-Rpp30 (Fig. 3F, lane 6), anti-Rpp38 (Fig. 3G, lane 6), and anti-Rpp40 (Fig. 3H, lane 4) antisera and for RNase P purified through the Mono Q step (see below). Additionally, ThFi autoimmune serum was able to bring down active RNase P (Fig. 3E, lane 6). The four preimmune sera, by contrast, failed to precipitate RNase P activity (Fig. 3E, lane 4; Fig. 3F, G: compare lanes 3 and 4; Fig. 3H: compare lanes 5 and 6). Therefore, Rpp20, Rpp30, Rpp38, and Rpp40 are associated with catalytically active RNase P complexes.

We note also that the four polyclonal sera, when first tested to verify that each recognized its corresponding recombinant protein did, indeed, react with a major band in western blots that correspond to the monomer form of each of the tagged Rpp20, Rpp30, Rpp38, and Rpp40 proteins, respectively (anti-Rpp20: Fig. 3A, lane 1; anti-Rpp30: Fig. 3B, lane 2; anti-Rpp38: Fig. 3C, lane 2; and anti-Rpp40: Fig. 3D, lane 2). However, even though Rpp20 and Rpp38 were denatured in urea and boiled before loading on the denaturing gel, these two proteins yielded species, presumably dimers, of apparent molecular weights 44 kDa (Fig. 3A, lane 1) and 82 kDa (Fig. 3C, lane 2), respectively. Recombinant Rpp38 also forms multimers (Fig. 2C, lane 3; Fig. 3C, lane 2). Polyclonal anti-Rpp38 serum harbored antibodies that recognized recombinant Rpp20 in monomer and multimer forms (Fig. 3C, lane 1), but did not recognize recombinant Rpp30 (Fig. 3C, lane 3) or Rpp40 (data not shown).

Rpp20, Rpp30, Rpp38, and Rpp40 coexist in highly purified RNase P complexes

Human RNase P that was purified through the MonoQ anion exchange step (see Materials and Methods) was

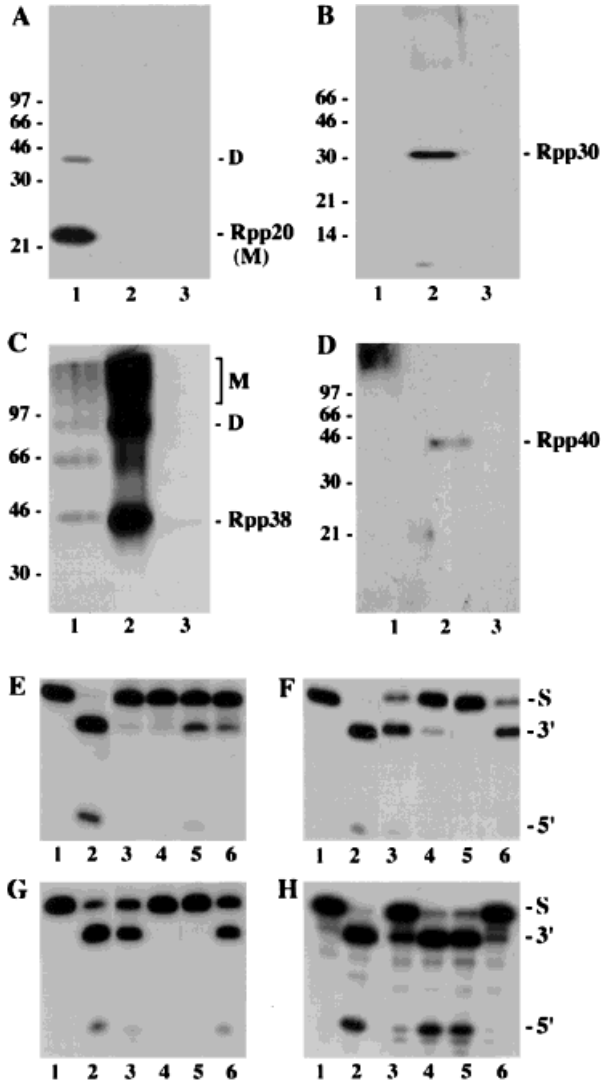

FIGURE 3. Polyclonal rabbit antibodies against recombinant Rpp proteins and immunoprecipitation of RNase P activity. Polyclonal antibodies were raised in rabbits against recombinant Rpp20 (**A**), Rpp30 (**B**), Rpp38 (**C**), and Rpp40 (**D**) and tested for their ability to recognize the corresponding endogenous HeLa proteins in western blot analysis. Lane 1, panels A–D, recombinant Rpp20. Lane 2, panels A, B and lane 3, panel C, recombinant Rpp30. Lane 3, panels A, B, D and lane 2, panel C, recombinant Rpp38. Lane 2, panel D, recombinant Rpp40. Protein dimers and multimers of Rpp20 and Rpp38 are indicated. Positions of protein size markers, in kDa, are shown to the left. **E,F,G,H:** Immunoprecipitates were tested for RNase P activity. Lane 1, Incubation of substrate ptRNA^{Ser} alone; lane 2, purified RNase P added. Nuclear extract (50 μL) from HeLa S3 cells (E) was incubated with protein A Sepharose beads alone (lane 3) or coupled to the preimmune serum (lane 4), Rpp20 antiserum (lane 5), or ThFi serum (lane 6). Aliquots of 1 μL from a glycerol gradient fraction enriched with RNase P activity were incubated with protein A sepharose beads coupled to preimmune or immune sera against recombinant Rpp30 (F), Rpp38 (G), and Rpp40 (H). After overnight incubation, the beads were centrifuged and, after supernatants were removed, resuspended in 1 $\times$ PA buffer to obtain immunoprecipitates, as described in Materials and Methods. Immunoprecipitates with preimmune (F and G, lane 4; H, lane 6) and immune (F and G, lane 6; H, lane 4) sera, as well as their supernatants using the preimmune (F and G, lane 3; H, lane 5) and immune (F and G, lane 5; H, lane 3) sera were all assayed for RNase P activity. Reaction time was for 20–30 min, and cleavage products, mature tRNA (3′), and 5′ leader sequence (5′), were separated on 8% polyacrylamide–7 M urea gel.

tested for the presence of Rpp20, Rpp30, Rpp38, and/or Rpp40 using rabbit polyclonal antibodies. Both Rpp30 and Rpp38 were detected in fractions that contained the peak of enzymatic activity (F22–F24: Fig. 4A,D). A similar correlation was also seen between RNase P activity and Rpp40, which was detected concurrently with Rpp30 (Fig. 4B). When the nitrocellulose filter used in Figure 4A was also probed with anti-Rpp20 antibodies, Rpp20 was found in F23 as well (Fig. 4C). An association of these proteins with holoenzyme activity was seen after further purification of RNase P on an S-12 size-exclusion FPLC column, precisely as has been shown previously (Eder et al., 1997). However, some Rpp38 is found in fractions other than those containing RNase P activity in eluates both from MonoQ and MonoS columns (data not shown), an indication that either this protein is more loosely bound to the

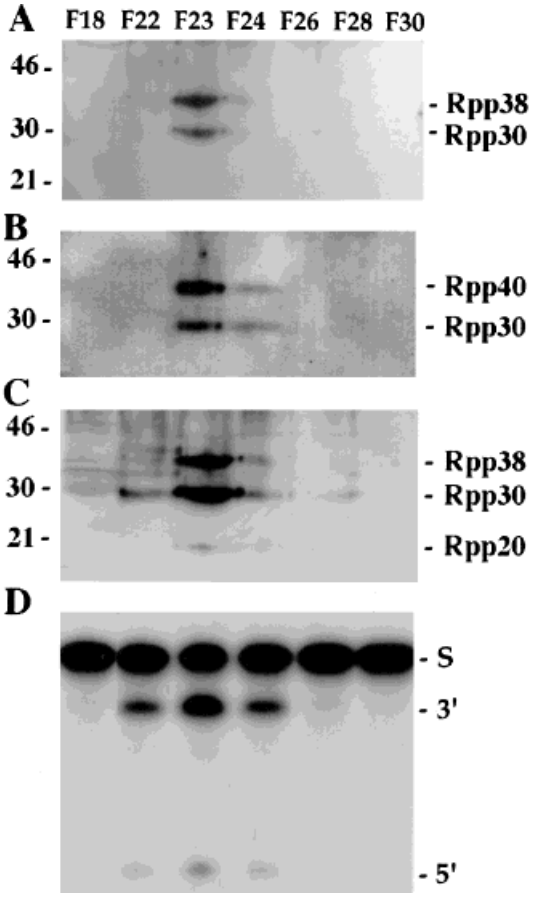

FIGURE 4. Rpp20, Rpp30, Rpp38, and Rpp40 coexist in highly purified RNase P. Aliquots (0.5 mL) from fractions eluted from a MonoQ column were tested by western blot analysis for the presence of Rpp30 and Rpp38 (**A**), Rpp30 and Rpp40 (**B**), and Rpp20, Rpp30, and Rpp38 (**C**) using their corresponding polyclonal antibodies (see Materials and Methods). Protein size markers are shown on the left-hand side of the figure. **D:** RNase P assay. Aliquots (2 μL) from MonoQ fractions were tested for RNase P activity using ^{32}P-labeled ptRNA^{Ser} as substrate (S). Reaction time was for 5 min, and cleavage products, mature tRNA (3′), and 5′ leader sequence (5′), were separated in an 8% polyacrylamide–7 M urea gel.

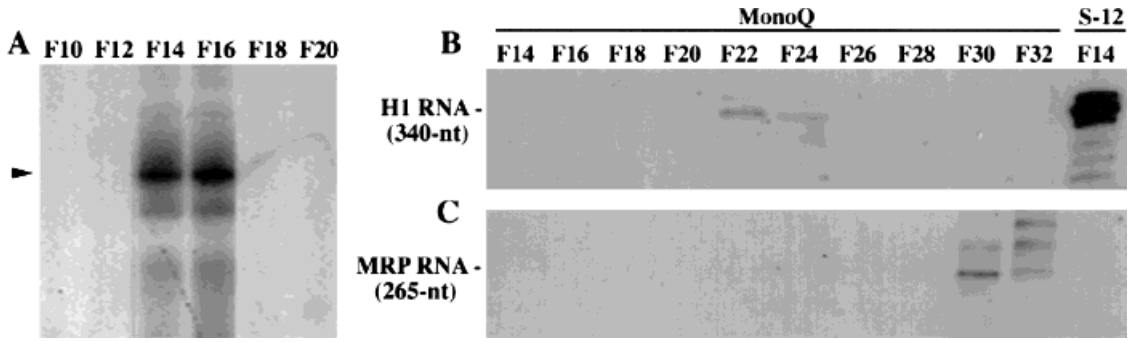

FIGURE 5. Rpp20, Rpp30, Rpp38, and Rpp40 are associated with H1 RNA. RNA was extracted from aliquots (0.1–0.2 mL) from MonoQ (**B, C**) or S-12 (**A,B,C**) fractions as indicated. RNA was labeled at its 3′ terminus with ^{32}P-pCp (A) or was subjected to northern blot analysis using 5′-end labeled oligonucleotide probes complementary to H1 RNA or human RNase MRP RNA (B,C). RNA was separated in an 6–8% polyacrylamide–7 M urea gel. The position of marker H1 RNA is indicated by an arrow in A. Positions of H1 RNA and RNase MRP RNA are shown in B and C. ^{32}P-labeled H1 RNA and RNase MRP RNA were used as size markers.

holoenzyme complex than others and/or that it is also a subunit of another separable enzyme.

We note also that RNase P purified through the DEAE step was tested for the presence of Th antigens with affinity-purified antibodies. Only Rpp30 and Rpp38 were detected by western blot analysis in these preparations (data not shown).

Rpp20, Rpp30, Rpp38, and Rpp40 are associated with H1 RNA

RNA extracted from eluates of the MonoQ (Fig. 4A) and the S-12 (see below) columns that contained RNase P activity was labeled with ^{32}P-pCp at its 3′ terminus in order to identify the RNA species associated with the catalytic activity and the protein subunits of the enzyme. An RNA (~340 nt) that corresponds in size to H1 RNA of human RNase P was the predominant high molecular weight RNA species that co-purified with Rpp20, Rpp30, Rpp38, and Rpp40 (data not shown), as well as with the peak of activity of human RNase P (cf. Figs. 5A, 6B) from the S-12 column (see below).

RNA from the MonoQ fractions was subjected to northern blot analysis that utilized an oligonucleotide probe complementary to the 3′-terminal 21 nt of the H1 RNA sequence. Full-length H1 RNA that co-purified with RNase P in fractions F22–F24 was detected in these and no other fractions (Fig. 5B). The presence of RNase MRP RNA was searched for with a probe complementary to the 3′-terminal 20 nt of human RNase MRP RNA. This RNA was found in fractions F28–F32 (Fig. 5C), from which H1 RNA was absent (Fig. 5B). Moreover, RNase MRP RNA, which is essential for RNase MRP activity, was not detected in fraction F14 of the S-12 column, but H1 RNA was readily observable (Fig. 5B,C, rightmost lane). These results show that Rpp20, Rpp30, Rpp38, and Rpp40 are associated with H1 RNA in catalytically active complexes of RNase P that can be separated from RNase MRP. In addition to studying the RNA content of fractions 14 and 16 from the MonoQ column, we checked these fractions for RNAse MRP enzymatic activity using the mouse mitochondrial RNA primer of DNA replica-

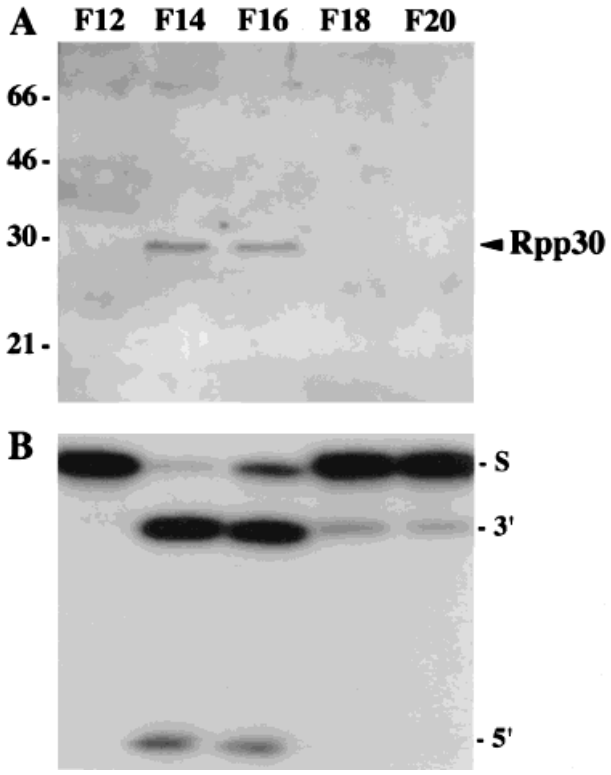

FIGURE 6. TSG15 (mouse Rpp30) is the mouse homologue of human Rpp30. **A:** Western blot analysis using anti-TSG15 antibodies. Fractions from the S-12 column were separated by SDS-PAGE and subjected to western blot analysis using affinity-purified anti-TSG15 antibodies. The position of human Rpp30 is shown. **B:** RNase P assay. Fractions from the S-12 column were assayed for human RNase P activity using ptRNA^{Ser} (S). Cleavage products, mature tRNA (3′), and 5′ leader sequence (5′) were separated as indicated in Materials and Methods.

tion as a substrate. No detectable RNase MRP activity was found in the fractions from the MonoQ column that contained RNase P activity. A low level of RNase MRP activity was found in those fractions, 30 and 32, that contained the RNase MRP RNA (data not shown), but these fractions contained no RNase P activity and no signal could be detected in western blots with polyclonal antibodies against Rpp38 (data not shown).

Rpp30 is a conserved subunit of eukaryotic RNase P

Cloning of the cDNA for human Rpp30 (Eder et al., 1997) facilitated a search for eukaryotic homologues of this protein. A mouse EST clone (designated TSG15; GenBank accession no. X61814) was found that shared homology with the human cDNA clone for Rpp30 (Eder et al., 1997). The mRNA corresponding to the TSG15 cDNA clone was approximately 1 kb in size and was expressed at high levels in mouse testis, predominantly in pachytene spermatocytes and round haploid cells. The same mRNA was also expressed at low levels in other mouse tissues (Hoog, 1991; Starborg et al., 1992). TSG15 has a cDNA insert of about 0.6 kb that exhibits a high identity to the 3′ proximal coding sequence of human Rpp30 cDNA. A polypeptide that was translated from this TSG15 partial cDNA clone was used to raise polyclonal antibodies in rabbits (C. Hoog, unpubl. data).

Fractions across the peak of catalytically active human RNase P from the S-12 step were analyzed by western blotting using affinity-purified anti-TSG15 antibodies. A protein of apparent molecular weight of 30 kDa that corresponds in size to Rpp30 was detected in fractions F14 and F16 using antibodies against the recombinant TSG15 polypeptide (Fig. 6A). The intensity of the signal was strongest in fractions F14–F16, the same fractions that exhibit maximal activity of RNase P (Fig. 6B). The anti-TSG15 antibodies also precipitated active human RNase P purified from the S-12 column step (data not shown). Thus, we presumed that the TSG15 protein is the mouse homologue of human Rpp30. To confirm this presumption, another mouse EST clone (GenBank accession no. W71337) with homology to the human Rpp30 cDNA and that contained a cDNA insert of about 1 kb was also sequenced. The amino acid composition, predicted from the single open reading frame found, showed 91% identity to human Rpp30. The full-length mouse TSG15 protein (mouse Rpp30; GenBank accession no. U95123) contains 268 amino acids, and has a theoretical pI of 9.2, as does human Rpp30.

Both the human Rpp30 and mouse TSG15 sequences showed a 23% identity of amino acid sequence to the Rpp30 orthologue from *S. cerevisiae*, Rpp1 (Stolc & Altman, 1997). Together, these data indicate that Rpp30 is a broadly conserved subunit of eukaryotic RNase P.

DISCUSSION

RNase P belongs to a growing family of ribonucleoproteins involved in nuclear RNA processing in eukaryotes. Although the function of several of these ribonucleoproteins is known, their protein subunits and composition remain largely uncharacterized. We have shown previously that at least six proteins co-purify with human RNase P activity (Eder et al., 1997). The large number of proteins that co-purify with RNase P suggests that they may have a variety of roles, such as in catalysis, nuclear localization, assembly and/or regulation of holoenzyme activity. In this study, we provide evidence that four proteins, Rpp20, Rpp40, and the Th antigens, Rpp30, and Rpp38, co-purify with the catalytically active complex of human RNase P. The specific role of each of these proteins in catalysis remains to be elucidated.

Two more putative protein subunits of human RNase P currently under study, namely Rpp14 (GenBank accession no. BankIt 110124 AF001175) and Rpp25 (Eder et al., 1997), are also associated with H1 RNA. Moreover, peptide microsequence analyses of the 30-kDa protein that co-purified with RNase P activity and that resulted previously in the identification of the Rpp30 autoantigen (Eder et al., 1997) also revealed the existence of an additional tryptic peptide that was not found in the Rpp30 sequence. A complete cDNA sequence that coded for this latter tryptic peptide was identified and designated Rpp29 (GenBank accession no. BankIt 110146 AF001176; N. Jarrous, P.S. Eder, C. Guerrier-Takada, & S. Altman, unpubl. data). Rpp29 comigrates with human Rpp30 and has a homologue in yeast, now known as Pop4 (Chu et al., 1997).

Protein subunits of catalytically active complexes of human RNase P

The purification of RNase P from HeLa cells includes velocity sedimentation, ion exchange, and size-exclusion FPLC columns. Through the several steps of purification, Rpp20, Rpp30, Rpp38, and Rpp40 remain part of the RNase P holoenzyme. In velocity sedimentaton analysis using glycerol gradients, other small ribonucleoprotein particles co-sediment with human RNase P (Bartkiewicz et al., 1989; Lee et al., 1996). Yet, Rpp20, Rpp30, Rpp38, and Rpp40 are detectable by western blotting in fractions enriched with RNase P activity and are mainly associated with 15S particles rather than with RNase MRP activity or larger ribonucleoprotein structures (data not shown). As the enzymatic activity is further purified on MonoQ and S-12 FPLC columns, these proteins still remain tightly bound to the holoenzyme. Furthermore, after the S-12 size-exclusion column step, H1 RNA is the only large RNA species that co-purifies with RNase P activity as well as with Rpp20, Rpp30, Rpp38, and Rpp40.

Rpp30 exhibits high identity to a mouse homologue (TSG15 or mouse Rpp30) that is differentially expressed at high levels in mouse testes (Hoog, 1991; Starborg et al., 1992). In addition, human Rpp30 shows significant homology to a yeast Rpp30 orthologue. Thus, Rpp30 is a conserved protein subunit of eukaryotic RNase P and is essential for holoenzyme function in vivo (Stolc & Altman, 1997). Indeed, in a strain of *S. cerevisiae* in which the endogenous Rpp30 gene was replaced with a metabolically regulated Rpp30 gene, reducing the level of expression of Rpp30 led to accumulation of precursor tRNAs.

An orthologue of Rpp20 has also been found in *S. cerevisiae* (V. Stolc & S. Altman, unpubl.). Positively charged amino acids comprise 20.7% of Rpp20, a protein that can form dimers in denaturing gels (Fig. 3A). A zipper-like motif that may facilitate dimerization of the protein is found in positions 15–36 of the amino acid sequence of Rpp20 (Fig. 1A). By contrast with Rpp20, Rpp30, and Rpp38, Rpp40 is an acidic polypeptide, an indication that it may have quite a different function in the holoenzyme complex in comparison with the other proteins.

Rpp38, the Th antigen

Rpp38 cDNA codes for a recombinant protein that is a major target for autoantibodies found in Th sera from patients with systemic sclerosis. Rabbit polyclonal antibodies against recombinant Rpp38 immunoprecipitate RNase MRP RNA in addition to RNase P RNA in crude extracts of HeLa cells, as has been found in earlier studies with some Th sera (Gold et al., 1988, 1989). These findings show that Rpp38 is identical to, or shares an antigenic determinant with, the major Th/To antigen (Reimer et al., 1988; Yuan et al., 1991; Eder et al., 1997). These polyclonal sera also bind to an antigen, presumably Rpp38, in both the nucleolus and nucleoplasm of mammalian cells (Li et al., 1994; Jacobson et al., 1995; Matera et al., 1995; Lee et al., 1996). Because Rpp30 is also recognized by some of the sera that cross-react with Rpp38, both the recombinant autoantigens may be useful in defining diagnostic markers for autoimmune diseases after further large-scale analysis of patient sera.

Rpp38 has a combination of structural features also found in some other RNA-binding proteins and in nuclear-targeted proteins. Lysine, arginine, and histidine residues constitute 20% of the polypeptide's amino acid residues. In attempts to fractionate human RNase P on a cation exchange FPLC column (MonoS), we noticed, consistent with previous work (Eder et al., 1997), that Rpp38 was depleted in fractions that contained H1 RNA. These fractions contained very low levels of unstable RNase P activity (data not shown). This protein, therefore, may be essential for the physical stability of the holoenzyme complex.

Rpp38 forms dimers and multimers even in 7 M urea (Fig. 2C). Although we are aware that further direct functional analysis of Rpp38 must be performed, we note that two putative α-helical heptad repeats that can form coiled-coil domains are found at positions 74–102 and 108–121 (see Eder et al., 1997). In addition, a potential leucine zipper-like-motif may exist in positions 225–247, downstream from the second coiled-coil domain, forming a basic coiled-coil–leucine zipper motif. Such a motif was shown to facilitate binding of heterogenous nuclear ribonucleoprotein C1 dimers to precursor mRNA molecules (McAfee et al., 1996). Only Rpp29 of the other putative subunits of human RNase P possesses a similar coiled-coil–leucine zipper motif (N. Jarrous, P.S. Eder, C. Guerrier-Takada, & S. Altman, unpubl.).

Nucleolar localization of these ribonucleoprotein RNAs requires the *cis*-acting element (Jacobson et al., 1995) previously implicated in binding the Th/To antigen (Yuan et al., 1991). Stretches of basic residues in Rpp38 at positions 63–66, 241–245, and 275–281 resemble monopartite nuclear targeting sequences (Laskey & Dingwall, 1993; Gerace, 1995). These features of Rpp38 suggest a role for this protein in the nucleolar localization of RNase P and/or its assembly and are consistent with a similar role for Rpp38 in RNase MRP (see below). Rpp29, another protein that co-purifies with RNase P activity, has a theoretical pI of 10.1 and three bipartite nuclear localization consensus sequences of the kind found in nuclear targeted proteins (Robbins et al., 1991; Laskey & Dingwall, 1993), in addition to monopartite nuclear localization sequences (N. Jarrous, P.S. Eder, C. Guerrier-Takada, & S. Altman, unpubl.).

RNase P, RNase MRP, and the Pop proteins

Pop1 and Pop3 of yeast are associated with RNase P and RNase MRP RNAs in immunoprecipitates from crude cell extracts (Lygerou et al., 1994; Dichtl & Tollervey, 1997). Although we have yet to show that human Pop 1 protein is essential for catalytic activity of highly purified human RNase P in vitro, we cannot rule out an important role for this protein in the function of a complex that contains both RNase P and RNase MRP (see below) or, for that matter, in RNase P alone. Moreover, although it is reasonable to assume that Pop3 is a conserved protein in eukaryotes, as is the case for Rpp1 (Stolc & Altman, 1997), Pop1 (Lygerou et al., 1996b), and Pop4 (Rpp29; N. Jarrous, P.S. Eder, C. Guerrier-Takada, & S. Altman, unpubl.), this polypeptide of 22.6 kDa has no notable homology to Rpp14, Rpp20, Rpp25, Rpp29, Rpp30, Rpp38, or Rpp40, as judged in a comparison of their cDNA and amino acid sequences. Pop3, too, may very well be part of the human RNase P-RNase MRP complex. Pop4 from yeast

(Chu et al., 1997), however, is homologous with Rpp29 (see above).

RNase P, RNase MRP, and Rpp proteins

Our biochemical purification studies show that Rpp20, Rpp30, Rpp38, and Rpp40 are found associated with human RNase P. However, immunoprecipitates from S100 extracts of HeLa cells show that anti-Rpp20, anti-Rpp30, anti-Rpp38, and anti-Rpp40 antibodies bring down relatively small amounts of active RNase MRP RNA in addition to RNase P RNA (data not shown). Thus, some of these proteins may be common components of a complex that contains both endoribonucleases (Kiss et al., 1992; Lee et al., 1996). Moreover, genetic studies in *S. cerevisiae* reveal that a reduction in the level of Rpp1 (homologous to human Rpp30) inhibits the correct cleavage of the internal transcribed spacer I of rRNA near the A3 site, an indication that RNase MRP as well as RNase P function was disrupted (Stolc & Altman, 1997). The two RNases may coexist in a processing complex in vivo that must remain intact to carry out processing of rRNA precursors. Such a complex, in yeast and human cells, may contain the Pop proteins, and SNM1, a protein that is a component of RNase MRP (Schmitt & Clayton, 1994), in addition to homologues of some or all of the proteins we describe here. Nevertheless, RNase P and RNase MRP can be dissociated from the putative human complex and separated from each other by biochemical purification. Finally, differences may very well exist in the protein composition, structure, and function of human RNase P and RNase MRP and their counterparts in lower eukaryotes, e.g., in yeast.

MATERIALS AND METHODS

Purification of human RNase P

Purification of human RNase P was performed as described (Bartkiewicz et al., 1989; Eder et al., 1997). Briefly, HeLa S3 cells (Cell Culture Center, Minneapolis, Minnesota) were grown to 0.6×10^6/mL and cells from 60-L cultures were pelleted, disrupted, and the cell extract was centrifuged at $10{,}000 \times g$ followed by another centrifugation at $100{,}000 \times g$ (S100 extract). The extract was fractionated by DEAE-sepharose anion exchange chromatorgraphy and eluted fractions enriched with RNase P activity were concentrated and sedimented in 15–30% glycerol gradient. RNase P was purified further using a MonoQ HR-10/10 anion exchange and an S-12 (1.0×10 cm) size-exclusion FPLC columns (Pharmacia). Fractions obtained from the several steps of purification were kept in 25% glycerol at $-20\,^{\circ}$C. Protein concentration, RNase P specific activity, and purification factor (>2,300-fold) were determined as described (Eder et al., 1997).

Molecular cloning of *Rpp20* and *Rpp40* cDNAs

Fractions enriched with human RNase P that were eluted from S-12 size-exclusion FPLC columns were separated in a 12% polyacrylamide–0.1% SDS preparative gel and proteins were visualized by Coomassie brilliant blue staining (Eder et al., 1997). The two bands with molecular weights of 20 kDa and 40 kDa were extracted from the gel (30–100 pmol) and peptide fragments thereof were analyzed by the W.M. Keck Biotechnology Resource Center at Yale University.

The tryptic peptide sequence AVEAELDPVEY obtained from the 20-kDa protein was searched in the GenBank Expressed Sequence Tag database (dbEST) using the tblastn program (Altschul et al., 1990), which translates the DNA database in all six frames to search for a match to the submitted sequence. A clone (GenBank accession no. Z46005) isolated from a human fetal brain cDNA library was identified and provided to us courtesy of P. Soularue (Genethon).

Human cDNA that was amplified by PCR from a human fetal liver cDNA library in λgt10 (Clontech) was used in the PCR to identify a DNA fragment that encodes two of the peptide fragments from the Rpp40 protein. A 275-bp product corresponding to a fragment of the Rpp40 cDNA was obtained by a two-step, nested amplification. In the first reaction, the antisense-strand degenerate primer derived from the peptide QIQEHQPK was 5′-TT NGG (C/T)TG (A/G)TG (C/T)TC (C/T)TG (A/G/T)AT (C/T)TG-3′. The sense-strand degenerate primer derived from amino acids 4–10 of the peptide ILSLDKDATYEETGLQGHPSQR was 5′-TN GA(C/T) AA(A/G) GA(C/T) ACN TA(C/T) GA-3′. In the second PCR reaction, the same antisense-strand degenerate primer was used, but a new, nested sense-strand primer corresponding to amino acids 7–13 of the same peptide was used: 5′-GA(C/T) ACN TA(C/T) GA(A/G) GA(A/G) ACN GG-3′. The first PCR reaction included 35 cycles of denaturation at 94 °C for 1 min, annealing at 41 °C for 1 min, and extension at 72 °C for 1 min. An aliquot was removed for the second reaction, which was performed for 35 cycles that included denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min. A 275-bp product was isolated, subcloned into pBluescript II SK+ (Stratagene), and sequenced using a Sequenase kit (Amersham).

The 275-bp fragment was used to screen a human fetal liver cDNA library to isolate larger clones of Rpp40. From a total of 7.5×10^5 plaques screened, a single positive clone was obtained with an insert of 1.1 kb. The insert was subcloned into pBluescript and sequenced. Sequence analysis predicted that the insert had the complete 5′ coding sequence, but was incomplete at the 3′ end. A variation of 3′ rapid amplification of cDNA ends (Eder et al., 1997) was used to identify the 3′ coding sequence and untranslated region. Briefly, poly(A)+ RNA from HeLa cells was used in a reverse transcription reaction with a T_{17}-adapter primer, 5′-CGACTCGAGTCGACATCGA$(T)_{17}$-3′. The second step was an Rpp40-specific linear amplification of the antisense cDNA, using only a sense-strand primer, 5′-CAT CCA TCT CAG TTT TCT GGC AGA-3′. The PCR included 35 cycles with denaturation at 94 °C for 1 min, annealing at 63 °C for 30 s, and extension at 72 °C for 1 min, using the primer 5′-TTC AGA AGA ATC GAC AAT GAT GTC-3′ (sense), and the adapter primer, 5′-CGA CTC GAG TCG ACA TCG-3′. A 700-bp product was obtained, of which 250 bp were overlapping coding sequence from the Rpp40 clone. The full-length Rpp40 coding sequence was amplified by PCR.

Overexpression and purification of recombinant proteins and preparation of polyclonal antibodies

Two primers, one encompassing the ATG translation initiation codon and upstream sequences of *RPP20* cDNA, and the other encompassing the translation stop codon and 35 bp downstream, were used for PCR with *RPP20* cDNA as a template. The PCR product was digested with *Nde* I and *Sac* I and then subcloned downstream from a T7 promoter and in-frame with the six histidine residues of pHTT7K (V. Gopalan & S. Altman, unpubl.) that had also been digested with *Nde* I and *Sac* I.

An *Eco*R I–*Bsm* I DNA fragment derived from *RPP30* cDNA and that has the entire open reading frame of the polypeptide (Eder et al., 1997) was made blunt and then subcloned with pHTT7K that had been digested with *Nde* I and *Bam*H I and filled-in. Subcloning of the *RPP30* coding sequence was in-frame with the histidine tag.

A *Pst* I–*Sac* I DNA fragment from *RPP38* cDNA (Eder et al., 1997) was ligated to an *Nde* I–*Pst* I fragment that encompassed the 5′ end of the sequence coding for the protein, and then subcloned into pHTT7K digested with *Nde* I and *Sac* I. The six histidine residues of the vector were in-frame with the Rpp38 open reading frame.

Two primers, one encompassing the ATG initiation codon of *RPP40* cDNA, and the other including the translation stop codon, were used for PCR with the *RPP40* cDNA as a template. The PCR product was digested with *Nde* I and *Bam*H I and then subcloned into pHTT7K digested with *Nde* I and *Bam*H I. The *RPP40* sequence was in-frame with the histidine tag.

Strain BL21 (DE3) of *E. coli* was transformed with pHTT7K plasmid that contained the desired cDNA insert. Cells were grown in 1 liter of LB broth to 0.6 OD_{600}. IPTG (0.8 mM) was then added and growth continued for an additional 2–3 h. Overexpression of the tagged Rpp20 (22 kDa), Rpp30 (33 kDa) and Rpp38 (35 kDa), Rpp40 (38 kDa) proteins was confirmed by analyzing cell lysates in 12% polyacrylamide–SDS gels, which were subsequently stained with Coomassie blue. All recombinant proteins in cell lysates or pellets were solubilized in 7 M urea. Hi-Trap metal chelating FPLC columns (Pharmacia), or histidine-bind resin (Novagen) columns charged with 50 mM nickel sulfate were used to purify the histidine-tagged proteins. The purity of the polypeptides was determined by SDS-PAGE followed by silver staining.

Polyclonal rabbit antibodies raised against recombinant Rpp20, Rpp30, Rpp38, and Rpp40 proteins were obtained from the Pocono Rabbit Farm (Canadensis, Pennsylvania).

Western blot analysis

Protein in eluates from the several chromatography columns, aliquots from glycerol gradient sedimentations, or recombinant proteins were separated in 12% polyacrylamide–0.1% SDS gels. Proteins were electrotransferred to nitrocellulose filters and immunoblotted with a 1:100 dilution of sera from patients with systemic sclerosis or from immunized rabbits, as described (Eder et al., 1997). A 1:1,500 dilution of goat anti-human or anti-rabbit IgG antibodies (Vector Laboratories) was used as the secondary antibody. Blots were washed (Eder et al., 1997) and bands were visualized using the LumiGLO chemiluminescent kit, following instructions of the manufacturer (KPL, Maryland).

Immunoprecipitation of active holoenzyme

Sera (40 μL) from either scleroderma patients or immunized rabbits were mixed with 5 mg of washed protein A–Sepharose CL-4B beads (Pharmacia) in NET-2 buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% NP-40) (Gold et al., 1988) by nutating over night at 4 °C. Beads were washed four times with NET-2 buffer and twice with 1 $\times$ PA binding buffer containing 0.1 U/μL RNasin. Unless otherwise indicated, 1:20 dilutions of fractions enriched with RNase P that were obtained from the various purification steps, were added to the beads. Immunoprecipitations were performed in 70–100 μL during overnight nutation at 4 °C. Beads were collected by a short centrifugation (7,000 rpm for 3 min), the supernatant was removed, and the beads were washed at least four times with 1 $\times$ PA. Supernatant and beads were assayed for enzymatic activity.

Analysis of RNA

RNA from 0.1–0.2 mL of MonoQ or S-12 fractions was extracted by phenol and phenol–chloroform, and then precipitated with ethanol (Gold et al., 1988). For 3′-terminal labeling, the dried RNA pellet was resuspended in 15 μL of a reaction mixture that contained 50 mM Hepes, pH 7.5, 20 mM $MgCl_2$, 3 mM DTT, 10% DMSO, 10 ng/μL BSA, 7.5 μM ATP, 5 units of T4 RNA ligase, and 30 μCi of ^{32}P-pCp (Amersham). The labeling reaction was performed overnight at 4 °C and stopped by adding loading dye that contained 9 M urea and phenol. Labeled RNA was separated in 5% polyacrylamide–7 M urea gels. *Msp* I-digested pBluescript SK(+/−) DNA end-labeled with $\alpha[^{32}P]$dCTP by Klenow enzyme was used as a size marker. RNA was also subjected to northern blot analysis (Guerrier-Takada et al., 1995), using DNA oligonucleotides complementary to positions 245–265 of the human RNase MRP RNA sequence (Topper & Clayton, 1990), and to positions 319–340 of human RNase P RNA (H1 RNA) (Bartkiewicz et al., 1989). These oligonucleotides were labeled at their 5′ termini with $\gamma[^{32}P]$ATP by T4 polynucleotide kinase.

Assay for RNase P activity

Cleavage of 5′ leader of the precursor to the yeast suppressor $tRNA^{Ser}$ (SupS1) by RNase P was performed in 20 μL of 1 $\times$ PA buffer (50 mM Tris-HCl, pH 7.5, 10 mg $MgCl_2$, and 30 mM NH_4Cl) at 37 °C. The substrate was uniformly labeled with $\alpha[^{32}P]$GTP (3,000 mCi/mmol, Amersham). Substrate (1 pmol) that contained 3,000 cpm was used per assay. Time of incubation was as indicated in each experiment. RNA cleavage products were separated in 8% polyacrylamide–7 M urea gels and bands were visualized by autoradiography.

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