 Nucleic Acids Research, 1999, Vol. 27, No. 12 **2465–2472**

hPop4: a new protein subunit of the human RNase MRP and RNase P ribonucleoprotein complexes

Hans van Eenennaam, Ger J. M. Pruijn and Walther J. van Venrooij*

Department of Biochemistry, University of Nijmegen, PO Box 9101, NL-6500 HB Nijmegen, The Netherlands

Received March 17, 1999; Revised and Accepted April 23, 1999

ABSTRACT

RNase MRP is a ribonucleoprotein particle involved in the processing of pre-rRNA. The RNase MRP particle is structurally highly related to the RNase P particle, which is involved in pre-tRNA processing. Their RNA components fold into a similar secondary structure and they share several protein subunits. We have identified and characterised human and mouse cDNAs that encode proteins homologous to yPop4p, a protein subunit of both the yeast RNase MRP and RNase P complexes. The human Pop4 cDNA encodes a highly basic protein of 220 amino acids. Transfection experiments with epitope-tagged hPop4 protein indicated that hPop4 is localised in the nucleus and accumulates in the nucleolus. Immunoprecipitation assays using extracts from transfected cells expressing epitope-tagged hPop4 revealed that this protein is associated with both the human RNase MRP and RNase P particles. Polyclonal rabbit antibodies raised against recombinant hPop4 recognised a 30 kDa protein in total HeLa cell extracts and specifically co-immunoprecipitated the RNA components of the RNase MRP and RNase P complexes. Finally we showed that anti-hPop4 immunoprecipitates possess RNase P enzymatic activity. Taken together, these data show that we have identified a protein that represents the human counterpart of the yeast Pop4p protein.

INTRODUCTION

In eukaryotes the 5.8S, 18S and the 25S/28S rRNAs are transcribed as one long precursor by RNA polymerase I. The maturation of this precursor not only involves endo- and exonucleolytic cleavages, but also methylation and pseudouridinylation (1). Proper processing of the precursor rRNA requires a group of small nucleolar ribonucleoprotein particles (snoRNPs). These snoRNPs contain snoRNAs, which are heterogeneous in size, structural elements and protein association. Based on their structural elements the snoRNAs can be divided into three groups: Box C/D snoRNAs, Box H/ACA snoRNAs and RNase MRP/RNase P (2). While most Box C/D snoRNAs have been demonstrated to function in ribose methylation (3) and Box H/ACA in pseudouridinylation of the rRNAs (4), RNase MRP and RNase P function as endonucleases (5,6).

Originally the RNase MRP has been identified as an endoribonuclease able to cleave, *in vitro*, mitochondrial RNA that functions as a primer for mitochondrial DNA replication (7). Most of the RNase MRP is, however, not localised in the mitochondria but is found in the nucleolus of cells (8,9). Genetic and biochemical experiments in *Saccharomyces cerevisiae* have shown that the RNase MRP is involved in the formation of the short form of the 5.8S rRNA [5.8S(S)], by catalysing cleavage at site A3 in the Internal Transcribed Spacer 1 (ITS1) of pre-rRNA (10–13). An involvement of RNase MRP in mitochondrial DNA replication *in vivo* has not been demonstrated yet.

The sensitivity of RNase MRP function to both ribonucleases and proteases demonstrates a requirement for both RNA and protein subunits for catalytic activity (8). The RNA subunit of the RNase MRP has been cloned from several species, including human (14), mouse (15), rat (16), cow (17), toad (18), yeast (19), *Arabidopsis* and tobacco (20). The RNA subunit of RNase MRP is related to the RNA subunit of RNase P, an endoribonuclease involved in the processing of the 5'-end of precursor tRNAs (21,22) and also suggested to be involved in processing of the precursor rRNA in Internal Transcribed Spacer 2 (ITS2) (5). Both RNA components have similar secondary structure elements, in particular the so-called cage-shaped domain (23,24) which contains many conserved nucleotides, supporting the idea that the RNase MRP and RNase P RNAs have evolved from a common ancestor (25,26). The identification of these RNAs via anti-Th/anti-To sera from patients suffering from the connective tissue diseases systemic lupus erythematosus (SLE) and scleroderma, which immunoprecipitate both RNA components, has led to alternative names for these RNA components: RNase MRP is also referred to as Th or 7-2 RNA and RNase P RNA as H1 or 8-2 RNA (27–31).

The close relationship between RNase MRP and RNase P is also supported by the fact that both particles contain similar protein subunits. Recently, purification of the RNase P particle from *S.cerevisiae* led to the identification of nine protein subunits co-purifying with the RNase P RNA (32). All these proteins are encoded by genes essential for RNase P activity and for cell viability. Four of these protein subunits, Pop1p (12), Pop3p (33), Pop4p (34) and Rpp1p (35), had been identified before and all four appeared to be components of both the RNase P and RNase MRP particle. Other subunits shared by both particles are Pop5p, Pop6p, Pop7p/Rpp2p and Pop8p.

*To whom correspondence should be addressed. Tel: +31 24 3613656; Fax: +31 24 3540525; Email: w.vanvenrooij@bioch.kun.nl

Yeast RNase MRP and RNase P also contain at least one protein subunit specifically associated with each of these particles, which are designated Snm1p (36) and Rpr2p (32), respectively.

The human RNase P particle has also been purified from HeLa cells, which resulted in the cDNA cloning of four RNase P protein subunits (37,38). Rpp20, Rpp30, Rpp38 and Rpp40 have been characterised and sequence comparisons revealed that Rpp20 is the human homologue of Pop7p/Rpp2p while Rpp30 is the human homologue of Rpp1p (32,35,38,39). The first human protein subunit characterised was the hPop1 protein (40), the homologue of yeast Pop1p. For hPop1, Rpp30 and Rpp38 it has been established that they are subunits of both RNase MRP and RNase P (40,41) and this is likely to be the case for Rpp20 as well.

In this report we describe the identification of the human and mouse homologues of yeast Pop4p. A complete cDNA encoding the human homologue of yPop4 was cloned and characterised. This cDNA encodes a novel nucleolar 30 kDa protein that is associated with both RNase MRP and RNase P. Polyclonal rabbit antibodies were raised against hPop4 and used to confirm the association of hPop4 with these two ribonucleoprotein complexes.

MATERIALS AND METHODS

Accession number

The hPop4 cDNA described in this report has been deposited in the EMBL database under accession no. Y18863.

cDNA cloning and sequence analysis

Database searches were done using the BLASTN 2.0.5 program (42). The accession nos of the overlapping human Expressed Sequence Tags (ESTs) are N40691, AA134865, W74573, AA308539, AA132996 and AA576911. The accession nos of the overlapping mouse ESTs are W66853, W46039, AA1990967, AA929907 and W15729.

Oligonucleotides were designed based on the human EST sequences to amplify the open reading frame (ORF) of hPop4: pop41, 5'-GCG-GAT-CCC-TCG-AGA-TGA-AGA-GTG-TGA-TCT-ACC-ATG-CAT-TG-3'; pop42, 5'-GCG-GAT-CCC-CCG-GGT-CAT-CTA-GAC-AGG-TCA-ATC-GTT-CCC-TTC-GC-3'. PCR was performed on 200 ng denatured DNA from λgt11 human placenta (Clontech) and teratocarcinoma cDNA libraries (43). The amplified fragments were ligated in the PCR-II-TOPO vector (Invitrogen) and sequenced using the dideoxynucleotide chain termination method.

Transfection constructs

Vesicular stomatitis virus G epitope (44) (VSV-G)-tagged (hereafter referred to as VSV-tagged) cDNAs were constructed as follows. The VSV–55k and 55k–VSV cDNA constructs, as described (45), contain a *Xho*I and a *Xba*I site, respectively, between the 55k ORF and the VSV tag sequence, which is positioned either at the N-terminal or at the C-terminal side of the ORF. Digestion by either *Xho*I/*Sma*I or *Xho*I/*Xba*I results in release of the 55k cDNA from these plasmids. The VSV-tagged constructs of hPop4 were constructed by isolation of the hPop4 ORF from the hPop4/PCR-II-TOPO construct by either *Xho*I/*Sma*I or *Xho*I/*Xba*I digestion and ligation into *Xho*I/*Sma*I- or *Xho*I/*Xba*I-digested VSV–55k or 55k–VSV constructs. The integrity of the resulting constructs was checked by DNA sequencing. The pCI-neo plasmid (Promega), which has been used previously to prepare VSV–55k and 55k–VSV, was used as a control in the transfection experiments.

Transient transfection of HeLa cells

HeLa monolayer cells were grown to 80% confluency by standard tissue culture techniques and subsequently 3×10^6 cells were transfected with 10 µg plasmid DNAs in a total volume of 400 µl of Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Electroporation was performed at 276 V and a capacity of 950 µF with a Gene Pulser II (Bio-Rad). After electroporation, cells were resuspended in 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal calf serum and grown overnight either on coverslips or in flasks.

Cells grown on coverslips were washed twice with phosphate-buffered saline (PBS), fixed with methanol (5 min at –20°C) and used for immunofluorescence assays.

Cells grown in flasks were harvested, washed once with PBS and used to prepare extracts for immunoprecipitation assays.

Immunofluorescence

Indirect immunofluorescence assays were performed on hPop4-VSV-transfected HeLa cells. Fixed cells were incubated with affinity-purified rabbit anti-hPop1 antibodies (diluted 1:100 in PBS) (40) and affinity-purified mouse anti-VSV tag antibodies (diluted 1:50 in PBS; Boehringer) for 1 h at room temperature, washed with PBS and subsequently incubated with swine anti-rabbit antibody coupled to FITC (diluted 1:50 in PBS) and rabbit-anti-mouse antibody coupled to TRITC (diluted 1:50 in PBS) for 1 h at room temperature. Cells were mounted with PBS/glycerol containing Mowiol and bound antibodies were visualised by confocal microscopy.

Preparation of HeLa cell extracts

Extracts of HeLa cells were prepared by resuspending cell pellets in buffer A [25 mM Tris–HCl pH 7.5, 100 mM KCl, 1 mM dithioerythritol (DTE), 2 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride, 0.05% NP-40] and lysis by sonification using a Branson microtip (three times for 20 s). Insoluble material was removed by centrifugation (12 000 *g*, 15 min) and supernatants were used directly for immunoprecipitations.

Anti-hPop4 antiserum

To raise a polyclonal anti-hPop4 antiserum, the hPop4 protein was expressed as a fusion protein with gluthatione *S*-transferase (GST) in *Escherichia coli* and purified as described previously (46). Rabbits were immunised with this material according to standard procedures (47). For each immunisation 200 µg of GST–hPop4 was used.

Western blot analysis

For western blot analysis the anti-hPop4 and pre-immune sera were used in a 500-fold dilution in the presence of 1% normal goat serum. Detection was performed using horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako Immunoglobulins) as secondary antibody and visualisation by chemiluminescence.

Immunoprecipitation and pre-tRNA processing assay

Monoclonal anti-VSV tag (Boehringer) and anti-fibrillarin ASWU1 (a kind gift of Dr M. Monestier) antibodies, patient anti-Th/To serum and rabbit anti-hPop4, anti-hPop1, anti-Ro52 and pre-immune serum from the rabbit immunised with hPop4 protein were coupled to protein A–agarose beads (Biozym) in IPP500 (500 mM NaCl, 10 mM Tris–HCl pH 8.0, 0.05% NP-40) by incubation for 2 h at room temperature. Beads were washed twice with IPP500 and once with IPP150 (150 mM NaCl, 10 mM Tris–HCl pH 8.0, 0.05% NP-40). For each immunoprecipitation cell extract was incubated with the antibody-coupled beads for 2 h at 4°C. Subsequently, beads were washed three times with IPP150.

To analyse co-precipitating RNAs, the RNA was isolated by phenol/chloroform extraction and ethanol precipitation. RNAs were resolved on a denaturing polyacrylamide gel and blotted to a Hybond-N membrane (Amersham). Northern blot hybridisations with riboprobes specific for human RNase P, RNase MRP, U3 RNA and U1 RNA were performed as previously described (48).

To assay for RNase P enzymatic activity in the immunoprecipitates, an internally ^{32}P-labelled pre-tRNA substrate (*Schizosaccharomyces pombe* tRNA^{Ser} SupS1; 49), a kind gift of Dr B. Séraphin, was transcribed *in vitro* and gel purified. This 110 nt long substrate contains a 5'-end extension of 28 nt in comparison with the mature tRNA. The immunoprecipitates were incubated with equal amounts of substrate in assay buffer (20 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTE, 50 mM KCl, 50 mg/ml BSA, 60 U/ml RNasin) for 10 min at 37°C under constant agitation. RNA was subsequently isolated by phenol/chloroform extraction and ethanol precipitation and analysed by denaturing polyacrylamide gel electrophoresis and autoradiography.

RESULTS

Identification of putative human and mouse Pop4p homologues

Recently, the cDNA cloning and characterisation of Pop4p from *S.cerevisiae* has been described (34). This protein was shown to be a subunit of both the yeast RNase MRP and RNase P particles and to be essential for 5.8S rRNA and pre-tRNA processing. The ORF of the yeast Pop4 cDNA encodes a protein comprised of 279 codons with a predicted molecular weight of 33 kDa.

The amino acid sequence of yeast Pop4p, hereafter designated yPop4, was compared with protein and 'translated nucleic acid' sequence databases to identify homologous sequences that might represent mammalian homologues of yPop4. Six overlapping nucleic acid sequence entries, corresponding to human ESTs, were retrieved. Using these ESTs a cDNA sequence could be constructed of 1133 nt, containing an ORF encoding a protein of 220 amino acids. For reasons documented below this protein will be referred to as hPop4.

Besides the human ESTs retrieved from the sequence databases using the yPop4 amino acid sequence, five mouse ESTs were selected. The combination of these five ESTs also allowed the derivation of a cDNA sequence, which in this case was comprised of 1433 nt, containing an ORF encoding a protein of 221 amino acids. The latter protein will be referred to as mPop4, since its amino acid sequence is highly homologous to that of hPop4 (see below).

No in-frame stop codon was found upstream of the first ATG in the human cDNA sequence, which might implicate that the cDNA did not represent the complete mRNA. However, since such an in-frame stop codon is present upstream of the first ATG in the mPop4 cDNA sequence and since the amino acid sequences derived from the hPop4 and mPop4 cDNA are highly homologous, it is reasonable to assume that the first ATG in the human cDNA sequence represents the start codon of the hPop4 mRNA.

Cloning of hPop4 cDNA

To clone a cDNA encoding the complete ORF of hPop4, two oligonucleotides were designed based on the cDNA sequence derived from the human ESTs. These oligonucleotides were used as PCR primers to amplify the hPop4 ORF using DNA from both human placenta and teratocarcinoma cDNA libraries as template. Sequencing of several clones resulting from this procedure revealed that clones derived from both cDNA libraries were completely identical, thereby ruling out the introduction of PCR artifacts. Nevertheless, in these cDNA clones minor differences were found in comparison to the sequence derived from the human ESTs. Nucleotides 100–104 of the cDNA sequence (numbering according to EMBL/Genbank accession no. Y18863) are 5'-GCGGG-3', while the EST sequence contains an additional nucleotide in this segment, 5'-GC**G**GGG-3'. The resulting frameshift is restored by the presence of an additional C residue at position 139 in the cDNA. Finally, a C residue was found at position 162 of the cDNA rather than a G at this position in the EST sequence, resulting in an alanine codon instead of a glycine at position 46 in the amino acid sequence. Although we cannot exclude the possibility that these differences represent genetic polymorphisms, it is more likely that they are due to sequencing errors, which are known to occur relatively frequently in EST sequences. In the complete hPop4 cDNA sequence the coding sequence corresponds to the sequence determined in this study while the UTRs are derived from the EST sequences. At position –20 relative to the start of the poly(A) tail a putative polyadenylation signal can be found. In conclusion, the combined cDNA is 1133 nt long and encodes a protein of 220 amino acids, with a predicted molecular weight of 25.4 kDa and a predicted pI of 10.9.

To investigate whether the size of the hPop4 cDNA was in accordance with the size of the mRNA, the latter was analysed by northern blot hybridization using total RNA extracted from a human melanoma cell line. A probe derived from nucleotides 100–590 of the cDNA sequence hybridised to a single mRNA species of ~1.3 kb, which is in good agreement with the length of the cDNA (data not shown).

In Figure 1 an alignment is shown of the amino acid sequences derived from the human, mouse and yeast Pop4 cDNAs. The homology between human and mouse Pop4 proteins is high (83% identity, 90% similarity), while the homology between human and yeast Pop4 is much lower, but still significant (29% identity, 49% similarity). The amino acid conservation between mammalian and yeast Pop4 proteins is most extensive in three blocks in the C-terminal half of the Pop4 protein.

```
hPOP4     1 ---MKSVIYHALSQKEANDSD-------------VQPSGA------QRAEAFVRAFLKRS
mPOP4     1 ---MKAAIYHAFSHKEAKDHD-------------VQELGS------QRAEAFVRAFLKQS
yPOP4     1 MDRTQTFIKDCLFTKCLEDPEKPFNENRFQDTLLLLPTDGGLTSRLQRQQRKSKLNLDNL

hPOP4    39 TPRMSPQAREDQLQR----------KAVVLEYFTRHK-RKEKKKKAKGLSARQRRELRLF
mPOP4    39 IPHMSQEDCESHLQR----------KAVILEYFTRLKPRPRPKKKSKGLSAKQRRDMRLF
yPOP4    61 QKVSQLESADKQLEKRDYQRINKNSKIALREYINNCKKNTKKCLKLAYENKITDKEDLLH

hPOP4    88 DIKPEQ-------QRYSLFLPLH-ELWKQYIRDLCSGLK--PDTQPQMIQAKLLKADLHG
mPOP4    89 DIKPEQ-------QRYSLFLPLH-ELWKQYIRDLCNGLK--PDTQPQMIQAKLLKADLHG
yPOP4   121 YIEEKHPTIYESLPQYVDFVPMYKELWINYIKELLNITKNLKTFNGSLALLKLSMADYNG

hPOP4   138 AIISVTKSKCPSYVGITGILLQETKHIFKIITKE---DRLKVIPKLNCVFTVET------
mPOP4   139 AIISVTKSKCPSYVGVTGILLQETKHVFKIITRE---DHLKVIPKLNCVFTIEI------
yPOP4   181 ALLRVTKSKNKTLIGLQGIVIWDSQKFFIMIVKGNIIDEIKCIPKKGTVFQFEIPISDDD

hPOP4   189 DGFISY-IYGSKFQLRSSERSAKKFKAKGTIDL------
mPOP4   190 DDFISY-IYGSKFQLRASERSAKKFKAKGSIDL------
yPOP4   241 DSALRYSILGDRFKYRSVDRAGRKFKSRRCDDMLYYIQN
```

Figure 1. Alignment of amino acid sequences derived from human, mouse and yeast Pop4 cDNAs. Amino acids that are identical in at least two of three proteins are marked by a black box, while amino acids with a conserved character are marked with a grey box. Dashes indicate the absence of corresponding amino acids.

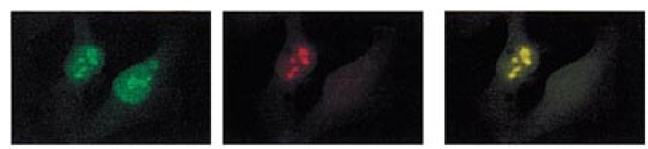
In the hPop4 protein sequence a clustering of basic amino acids is evident between residues 53 and 85, which may contain a functional bipartite nuclear localisation sequence (NLS), as in this region three partly overlapping elements matching the bipartite NLS consensus sequence can be discerned.

hPop4 accumulates in the nucleoli

To investigate the subcellular localisation of hPop4, a VSV-G tag sequence (44) was fused to either the 5'- or the 3'-end of the hPop4 cDNA and cloned in the mammalian expression vector pCI-neo. The resulting constructs (VSV–hPop4 and hPop4–VSV) were used to transfect HeLa cells and after overnight culturing the localisation of the VSV-tagged hPop4 protein was determined via indirect fluorescence confocal microscopy, using a monoclonal anti-VSV tag antibody. The results, which were identical for both constructs, showed a strong nucleolar and a weak, homogeneous nucleoplasmic staining pattern (Fig. 2B). Since the anti-VSV tag antibody in non-transfected cells generated no signal above background, the observed staining appeared to be specific for the hPop4–VSV protein expressed in transfected cells. To confirm that the nuclear regions most intensely stained by the anti-VSV tag antibodies represented nucleoli and to investigate whether hPop4 co-localised with hPop1, double immunostaining with affinity-purified anti-hPop1 antibodies (40) was performed (Fig. 2A–C). Note that hPop1 is also an RNase MRP and RNase P subunit and therefore co-localisation of hPop1, which has previously been shown to accumulate in the nucleolus, and hPop4–VSV would be consistent with an association of hPop4–VSV with these ribonucleoprotein complexes. Indeed a full co-localisation of hPop4–VSV and hPop1 was observed (Fig. 2C). In conclusion, transiently expressed hPop4–VSV localises to the nucleus and strongly accumulates in the nucleolar compartment.

hPop4 is associated with both the RNase MRP and RNase P particles

To determine whether the hPop4 protein is indeed a subunit of the human RNase MRP and the related RNase P particle, immunoprecipitation experiments were performed with epitope-tagged hPop4 protein. HeLa cells were transfected with both VSV-tagged hPop4 constructs and the corresponding empty vector (pCI-neo) as a control. Cell extracts prepared from these cells were used for immunoprecipitation with anti-VSV antibodies, anti-fibrillarin (a protein associated with Box C/D snoRNPs) antibodies and a patient serum known to immunoprecipitate both RNase MRP and RNase P complexes (anti-Th/To). RNAs were extracted from immunoprecipitates and from total cell extracts, fractionated by gel electrophoresis and analysed by northern blot hybridisation using probes specific for RNase MRP, RNase P, U3 RNA (a Box C/D snoRNA) and U1 RNA. As is shown in Figure 3 (lane 3), the RNase MRP and RNase P RNAs are both precipitated by the anti-VSV tag antibody from a cell extract containing VSV-tagged hPop4 protein. Identical results were obtained for both VSV-tagged hPop4 constructs. The specificity of this result was established by the lack of co-precipitation of U1 and U3 RNA and by the inability of the anti-VSV tag antibody to co-precipitate RNase MRP and RNase P RNA from extracts of control cells (transfection with pCI-neo vector; Fig. 3, lane 7). As expected, the anti-Th/To patient serum immunoprecipitated both the RNase MRP and RNase P RNAs from both types of cell extracts (lanes 2 and 6). The specific immunoprecipitation of U3 RNA by the anti-fibrillarin antibodies (lanes 4 and 8)



A B C

Figure 2. Subcellular localisation of hPop4. Double immunofluorescence and confocal microscopy was performed on HeLa cells, which were transiently transfected with hPop4–VSV. Cells were fixed with methanol and stained with affinity-purified anti-hPop1 (40) (**A**) and anti-VSV (**B**) antibodies. (**C**) superimposition of (A) and (B).

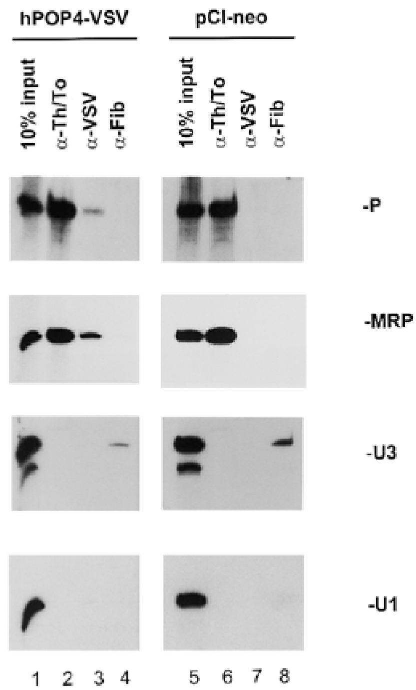


Figure 3. VSV-tagged hPop4 associates with RNase MRP and RNase P. HeLa cells were transfected with hPop4–VSV cDNA construct and the empty vector as a negative control. After overnight culturing, cell extracts were used for immunoprecipitation with anti-Th/To (α-Th/To), anti-VSV tag (α-VSV) and anti-fibrillarin (α-Fib) antibodies. RNAs were isolated from the immunoprecipitates (lanes 2–4 and 6–8) and from the total cell extracts (lanes 1 and 5) and analysed by northern blot hybridisation using specific probes for RNase P, RNase MRP, U3 RNA and U1 RNA, as indicated on the right. Lanes 1–4, material from cells expressing hPop4–VSV; lanes 5–8, material from control cells transfected with pCI-neo. The amounts of the RNAs analysed in lanes 1 and 5 corresponds to 10% of the amounts of extracts used for immunoprecipitations (10% input). The faint U1 snRNA band observed in lane 3 is due to some non-specific co-precipitation with the anti-VSV antibody.

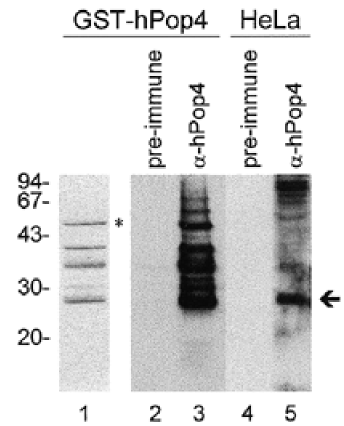


Figure 4. Western blot analysis of rabbit antiserum raised against GST–hPop4. Rabbit antisera were raised against recombinant GST–hPop4 fusion protein expressed in *E.coli.* GST–hPop4 protein (lane 1 shows the recombinant protein preparation stained with Coomassie Brilliant Blue) was separated by 13% SDS–PAGE and transferred to nitrocellulose filters. After incubation with anti-hPop4 antiserum (lane 3) or pre-immune serum (lane 2), bound antibodies were visualised with horseradish peroxidase-conjugated goat anti-rabbit antibodies and chemiluminescence. The reactivity of the antiserum with proteins from a total HeLa extract was analysed also by western blotting: anti-hPop4 (lane 5) and pre-immune serum (lane 4). On the left, the molecular weights of protein markers are indicated. The full-length GST–hPop4 protein band is indicated with an asterisk, while the arrow points to the HeLa hPop4 protein.

and the lack of immunoprecipitation of U1 RNA (lanes 2–4 and 6–8) further substantiated the specificity of the assay. Taken together, these results indicate that the VSV-tagged hPop4 protein associates with both the RNase MRP and RNase P particles.

Anti-hPop4 antibodies immunoprecipitate both RNase MRP and RNase P particles

To exclude the possibility that association of hPop4–VSV with the RNase MRP and RNase P particles was due to overexpression of the protein in the transiently transfected HeLa cells, a polyclonal antiserum was raised against recombinant hPop4 to study the endogenous non-tagged hPop4 protein. The hPop4 protein was expressed as a fusion protein with GST in *E.coli*, designated GST–hPop4. After purification using gluthathione–Sepharose 4B beads, GST–hPop4 (Fig. 4, lane 1) was used to immunise rabbits. Western blot analysis showed that the resulting rabbit antisera, in contrast to the corresponding pre-immune sera, recognised not only the recombinant GST–hPop4, but also the hPop4 protein expressed in HeLa cells (Fig. 4). Besides the band representing the GST–hPop4 protein, some faster migrating bands in the recombinant material were recognised by the anti-hPop4 antisera as well. These bands are most probably due to proteolytic degradation of the GST–hPop4 protein during the purification and may in part be stained due to anti-GST activity in the sera. Note that the endogenous hPop4 protein of HeLa cells migrated at ~30 kDa in SDS–PAGE gels.

To investigate whether hPop4 is a subunit of both the endogenous RNase MRP and RNase P particles, immunoprecipitations were performed with this anti-hPop4 antiserum using total HeLa cell extracts. The co-precipitating RNAs were isolated and analysed by northern blot hybridisation using probes specific for RNase MRP, RNase P and U3 RNA. As depicted in Figure 5 (lane 4), the anti-hPop4 antiserum efficiently immunoprecipitated both the RNase MRP and RNase P RNA components, while the pre-immune serum did not immunoprecipitate any of the RNAs analysed (Fig. 5, lane 5). The specificity of the antiserum was substantiated by the observations that a control polyclonal rabbit antiserum (lane 3) did not detectably precipitate RNAs, while a patient anti-Th/To antiserum as well as rabbit anti-hPop1 antiserum did co-immunoprecipitate the RNase MRP/RNase P RNAs (lanes 2 and 6).

In conclusion, these results fully support previous findings that hPop4 is a component of both RNase MRP and RNase P.

Anti-hPop4 antibodies immunoprecipitate RNase P enzymatic activity

Having demonstrated that the anti-hPop4 antibodies specifically immunoprecipitate the RNase MRP and RNase P RNAs from total HeLa cell extracts, we analysed whether the immunoprecipitates contained any RNase P enzymatic activity.

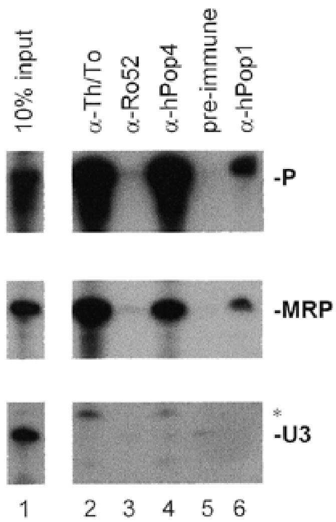


Figure 5. Anti-hPop4 antiserum co-immunoprecipitates the RNA components of RNase MRP and RNase P. RNPs were immunoprecipitated from a total HeLa cell extract using anti-Th/To (lane 2), anti-Ro52 (lane 3), anti-hPop4 (lane 4), pre-immune (lane 5) and anti-hPop1 (lane 6) antisera. Co-precipitating RNAs and RNA from total extract were isolated, resolved by denaturing polyacrylamide gel electrophoresis and analysed by northern blotting using riboprobes specific for human RNase P, RNase MRP and U3 RNA, as indicated on the right. The signals marked with an asterisk are due to incomplete removal of the RNase P probe prior to the incubation with the U3 RNA probe.

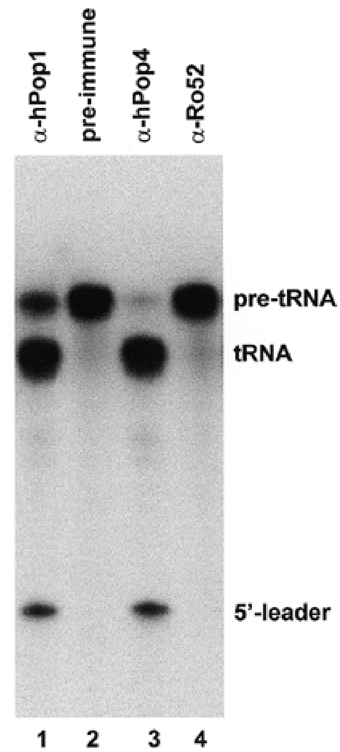


Figure 6. Anti-hPop4 antibodies immunoprecipitate enzymatically active RNase P. Anti-hPop1 (lane 1), pre-immune (lane 2), anti-hPop4 (lane 3) and anti-Ro52 (lane 4) sera were used for immunoprecipitations from total HeLa cell extract. Immunoprecipitates were assayed for RNase P enzymatic activity by incubating with a ^{32}P-labelled pre-tRNA substrate. Subsequently, the RNAs were isolated and resolved by denaturing polyacrylamide gel electrophoresis and visualised by autoradiography. On the right, the positions of the pre-tRNA, the mature tRNA and the 5'-leader sequence are indicated.

Immunoprecipitates were incubated with a ^{32}P-labelled pre-tRNA. The products of this reaction were resolved on a denaturing polyacrylamide gel and visualised using autoradiography. The results in Figure 6 show that the anti-hPop4 antibodies are indeed able to immunoprecipitate enzymatically active RNase P complexes, as the pre-tRNA is specifically cleaved into mature tRNA and the 5'-leader. The capability to immunoprecipitate this activity was indistinguishable from that of the anti-hPop1 antiserum, which was used as a positive control. In contrast, the pre-tRNA incubated with the immunoprecipitate of either the pre-immune serum or a control antiserum was not processed (lanes 2 and 4). We conclude that the hPop4 protein is associated with a catalytically active form of RNase P.

DISCUSSION

We have identified and cloned a new subunit of the human RNase MRP particle, which is also associated with the evolutionarily related RNase P particle. The new subunit exhibits homology to the yeast Pop4 protein and was therefore designated hPop4. We showed that the subcellular localisation of hPop4 is primarily nucleolar and that this protein is associated with catalytically active RNase P particles.

Amino acid sequence of hPop4

While a high degree of amino acid sequence conservation was observed between the human and putative mouse Pop4 polypeptides, the human and yeast Pop4 sequences are only moderately homologous (29% identity), which is, however, slightly higher than the degree of sequence conservation observed for other RNase MRP/RNase P proteins, like hPop1/Pop1 (22% identity; 40), Rpp30/Rpp1 (23% identity; 35) and Rpp20/Rpp2 (14% identity; 39). Sequence analysis did not reveal the presence of known protein sequence motifs in the mammalian Pop4 polypeptides, apart from putative NLSs. The most conserved regions are found in the C-terminal half of the protein. Within this region an evolutionarily conserved cluster of basic amino acids is found near the C-terminus. Recently, Chamberlain *et al.* (32) identified an element consisting of two contiguous Lys residues preceded or followed by an additional Lys at a distance of three to eight intervening residues, which is found in all known RNase P proteins, except Rpp1p (32,35). Several sequences corresponding to this element are present in hPop4 and mPop4 and a few such elements are found at an equivalent position in yeast. This element is also found in some ribosomal proteins and might be involved in protein–RNA association or protein–protein interactions.

Interestingly, Phe207 of yPop4, which has been demonstrated to be mutated in the yeast strain that led to the identification of this protein (34), is not conserved in the human and mouse Pop4 proteins. The substitution of the Phe by either a Leu or a Ser in yPop4 enabled the protein to suppress the rrp2-2 phenotype, which is due to a mutation in the RNA component of the RNase MRP. The authors suggested that this amino acid substitution somehow compensated for the functional defect of the base substitution in the RNA component of RNase MRP. The proposed direct interaction between the yPop4 protein and the RNase MRP RNA may not be found for the human RNase MRP RNA and hPop4, as the region of the RNase MRP RNA that is mutated in the rrp2-2 strain is not present in the human RNase MRP RNA (10).

The hPop4 sequence described in the present study is further supported by a recent paper, in which the cDNA sequence of a protein called Rpp29 has been described. Rpp29 has been isolated as a protein subunit of the human RNase P particle, which is homologous to yeast Pop4p (50). Sequence comparison revealed that Rpp29 is almost identical to hPop4, as might be anticipated by their mutual homology with yPop4. Only two differences were observed between the hPop4 and Rpp29 protein sequences. Human Pop4 contains a Ser38 and a Leu76, while in the sequence of Rpp29 these amino acids are substituted by a Thr and a Phe, respectively. The presence of the Ser and the Leu at these positions in hPop4 is supported by the identity of the equivalent amino acids in the predicted sequence for mPop4, which are also a Ser and a Leu (Fig. 1). Based upon both their sequences (the hPop4 and Rpp29 cDNAs differ only by 6 nt) and biochemical characteristics it is highly likely that hPop4 and Rpp29 represent the same protein. At present, it is unclear whether the small sequence differences between hPop4 and Rpp29 are due to imperfect cDNA sequences or to a genetic polymorphism.

Nucleolar accumulation of hPop4

The nucleolar accumulation observed for VSV-tagged hPop4 is in agreement with the association of the hPop4 protein with the RNase MRP and RNase P particles. Previously, it had been proposed that accumulation of proteins in the nucleoli is a two-step process (51–53). First, a nucleolar protein is transported from the cytoplasm to the nucleoplasm, which is dependent on an NLS, and subsequently functional domains that interact specifically with other nucleolar components mediate nucleolar entry. The subcellular localisation of the RNase MRP particle has been determined to be primarily nucleolar, while only a minority of RNase MRP complexes have been reported to reside in the mitochondria (8,9,54). We could, however, not detect any staining above background in the cytoplasm of HeLa cells, in agreement with biochemical fractionation data which did not show a significant portion of the RNase MRP RNA in the mitochondria of HeLa cells (9). RNase P has been reported to be localised in both the nucleoplasm and nucleolus and recent studies indicate that the majority of RNase P is localised in the nucleolus (55), strongly suggesting that at least some aspects of pre-tRNA processing occur in the nucleolar compartment.

hPop4 is shared by RNase MRP and RNase P

In yeast, Pop1p, Pop3p, Pop4p, Pop5p, Pop6p, Pop7p/Rpp2p, Pop8p and Rpp1p are protein subunits associated with both RNase MRP and RNase P (32,39). In addition, two yeast proteins have been identified which are specifically associated with either RNase MRP (Snm1p) (36) or RNase P (Rpr2p) (32). Presently such particle-specific proteins have not been identified in mammals yet.

In agreement with the association of yeast Pop4 with both particles, we showed that its human counterpart, hPop4, is also a subunit of both RNase MRP and RNase P and is associated with catalytically active RNase P. Also, the Rpp29 protein, which is most likely identical to hPop4, has recently been reported to be associated with (catalytically active) RNase P (50). The capability to immunoprecipitate enzymatically active RNase P has been reported before for antibodies directed against the hPop1, Rpp20, Rpp30, Rpp38 and Rpp40 protein subunits of RNase P (37,38,40).

The hPop4 protein is probably not directly bound to the RNase MRP and RNase P RNA components, since recent immunoprecipitation experiments using the anti-hPop4 antiserum failed to detect a radiolabelled polypeptide co-migrating on SDS–PAGE with hPop4 after UV crosslinking of RNase MRP particles reconstituted with radiolabelled RNase MRP RNA (unpublished observations). With the latter type of experiments we recently identified three human proteins with apparent molecular weights of 20, 25 and 40 kDa that directly interact with the RNase MRP RNA (41). These data suggest that the association of the hPop4 protein with these ribonucleoprotein particles might be mediated by protein–protein interactions. Further studies will be required to elucidate the molecular interactions that determine RNase MRP architecture.

ACKNOWLEDGEMENTS

We thank Drs Helma Pluk and Wiljan Hendriks (Department of Cell Biology, University of Nijmegen) for useful discussions and suggestions and Ben de Jong and Rolf Janssen for technical assistance. We are grateful to Dr Winfried Degen and Carla Onnekink for providing the northern blot with total human RNA. The patient sera were kindly provided by Dr Frank van den Hoogen (Department of Rheumatology, University of Nijmegen). We thank Dr Bertrand Séraphin (EMBL, Heidelberg) for providing us with the pre-tRNA substrate and Dr Marc Monestier (Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia) for the anti-fibrillarin monoclonal antibody ASWU1. This work was supported by the Netherlands Foundation for Chemical Research (NWO-CW) with financial aid from the Netherlands Organization for Scientific Research (NWO).

REFERENCES

1. Eichler,D.C. and Craig,N. (1994) *Prog. Nucleic Acid Res. Mol. Biol.*, **49**, 197–239.
2. Tollervey,D. and Kiss,T. (1997) *Curr. Opin. Cell Biol.*, **9**, 337–342.
3. Bachellerie,J.P. and Cavaille,J. (1997) *Trends Biochem. Sci.*, **22**, 257–261.
4. Peculis,B. (1997) *Curr. Biol.*, **7**, R480–R482.
5. Chamberlain,J.R., Pagan-Ramos,E., Kindelberger,D.W. and Engelke,D.R. (1996) *Nucleic Acids Res.*, **24**, 3158–3166.
6. Tollervey,D. (1996) *Mol. Biol. Rep.*, **22**, 75–79.
7. Chang,D.D. and Clayton,D.A. (1987) *EMBO J.*, **6**, 409–417.
8. Chang,D.D. and Clayton,D.A. (1987) *Science*, **235**, 1178–1184.
9. Reimer,G., Raska,I., Scheer,U. and Tan,E.M. (1988) *Exp. Cell Res.*, **176**, 117–128.
10. Chu,S., Archer,R.H., Zengel,J.M. and Lindahl,L. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 659–663.
11. Lygerou,Z., Allmang,C., Tollervey,D. and Séraphin,B. (1996) *Science*, **272**, 268–270.
12. Lygerou,Z., Mitchell,P., Petfalski,E., Séraphin,B. and Tollervey,D. (1994) *Genes Dev.*, **8**, 1423–1433.
13. Schmitt,M.E. and Clayton,D.A. (1993) *Mol. Cell. Biol.*, **13**, 7935–7941.

14. Topper,J.N. and Clayton,D.A. (1990) *Nucleic Acids Res.*, **18**, 793–799.
15. Chang,D.D. and Clayton,D.A. (1989) *Cell*, **56**, 131–139.
16. Yuan,Y., Singh,R. and Reddy,R. (1989) *J. Biol. Chem.*, **264**, 14835–14839.
17. Dairaghi,D.J. and Clayton,D.A. (1993) *J. Mol. Evol.*, **37**, 338–346.
18. Bennett,J.L., Jeong Yu,S. and Clayton,D.A. (1992) *J. Biol. Chem.*, **267**, 21765–21772.
19. Schmitt,M.E. and Clayton,D.A. (1992) *Genes Dev.*, **6**, 1975–1985.
20. Kiss,T., Marshallsay,C. and Filipowicz,W. (1992) *EMBO J.*, **11**, 3737–3746.
21. Altman,S., Kirsebom,L. and Talbot,S. (1993) *FASEB J.*, **7**, 7–14.
22. Chamberlain,J.R., Tranguch,A.J., Pagan Ramos,E. and Engelke,D.R. (1996) *Prog. Nucleic Acid Res. Mol. Biol.*, **55**, 87–119.
23. Forster,A.C. and Altman,S. (1990) *Cell*, **62**, 407–409.
24. Schmitt,M.E., Bennett,J.L., Dairaghi,D.J. and Clayton,D.A. (1993) *FASEB J.*, **7**, 208–213.
25. Morrissey,J.P. and Tollervey,D. (1995) *Trends Biochem. Sci.*, **20**, 78–82.
26. Reilly,T.H. and Schmitt,M.E. (1995) *Mol. Biol. Rep.*, **22**, 87–93.
27. Gold,H.A., Topper,J.N., Clayton,D.A. and Craft,J. (1989) *Science*, **245**, 1377–1380.
28. Gold,H.A., Craft,J., Hardin,J.A., Bartkiewicz,M. and Altman,S. (1988) *Proc. Natl Acad. Sci. USA*, **85**, 5483–5487.
29. Hashimoto,C. and Steitz,J.A. (1983) *J. Biol. Chem.*, **258**, 1379–1382.
30. Reddy,R., Tan,E.M., Henning,D., Nohga,K. and Busch,H. (1983) *J. Biol. Chem.*, **258**, 1383–1386.
31. Hardin,J.A., Rahn,D.R., Shen,C., Lerner,M.R., Wolin,S.L., Rosa,M.D. and Steitz,J.A. (1982) *J. Clin. Invest.*, **70**, 141–147.
32. Chamberlain,J.R., Lee,Y., Lane,W.S. and Engelke,D.R. (1998) *Genes Dev.*, **12**, 1678–1690.
33. Dichtl,B. and Tollervey,D. (1997) *EMBO J.*, **16**, 417–429.
34. Chu,S., Zengel,J.M. and Lindahl,L. (1997) *RNA*, **3**, 382–391.
35. Stolc,V. and Altman,S. (1997) *Genes Dev.*, **11**, 2926–2937.
36. Schmitt,M.E. and Clayton,D.A. (1994) *Genes Dev.*, **8**, 2617–2628.
37. Eder,P.S., Kekuda,R., Stolc,V. and Altman,S. (1997) *Proc. Natl Acad. Sci. USA*, **94**, 1101–1106.
38. Jarrous,N., Eder,P.S., Guerrier Takada,C., Hoog,C. and Altman,S. (1998) *RNA*, **4**, 407–417.
39. Stolc,V., Katz,A. and Altman,S. (1998) *Proc. Natl Acad. Sci. USA*, **95**, 6716–6721.
40. Lygerou,Z., Pluk,H., van Venrooij,W.J. and Séraphin,B. (1996) *EMBO J.*, **15**, 5936–5948.
41. Pluk,H., van Eenennaam,H., Rutjes,S.A., Pruijn,G.J.M. and van Venrooij,W.J. (1999) *RNA*, **5**, 512–524.
42. 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.
43. Sillekens,P.T., Habets,W.J., Beijer,R.P. and van Venrooij,W.J. (1987) *EMBO J.*, **6**, 3841–3848.
44. Kreis,T.E. (1986) *EMBO J.*, **5**, 931–941.
45. Pluk,H., Soffner,J., Luhrmann,R. and van Venrooij,W.J. (1998) *Mol. Cell. Biol.*, **18**, 488–498.
46. Frangioni,J.V. and Neel,B.G. (1993) *Anal. Biochem.*, **210**, 179–187.
47. Harlow,E. and Lane,D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
48. Verheijen,R., Wiik,A., De Jong,B.A., Hoier Madsen,M., Ullman,S., Halberg,P. and van Venrooij,W.J. (1994) *J. Immunol. Methods*, **169**, 173–182.
49. Krupp,G., Cherayil,B., Frendewey,D., Nishikawa,S. and Soll,D. (1986) *EMBO J.*, **5**, 1697–1703.
50. Jarrous,N., Eder,P.S., Wesolowski,D. and Altman,S. (1999) *RNA*, **5**, 153–157.
51. Girard,J.P., Bagni,C., Caizergues Ferrer,M., Amalric,F. and Lapeyre,B. (1994) *J. Biol. Chem.*, **269**, 18499–18506.
52. Schmidt Zachmann,M.S. and Nigg,E.A. (1993) *J. Cell Sci.*, **105**, 799–806.
53. Yan,C. and Melese,T. (1993) *J. Cell Biol.*, **123**, 1081–1091.
54. Li,K., Smagula,C.S., Parsons,W.J., Richardson,J.A., Gonzalez,M., Hagler,H.K. and Williams,R.S. (1994) *J. Cell Biol.*, **124**, 871–882.
55. Bertrand,E., Houser Scott,F., Kendall,A., Singer,R.H. and Engelke,D.R. (1998) *Genes Dev.*, **12**, 2463–2468.