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hPop1: an autoantigenic protein subunit shared by the human RNase P and RNase MRP ribonucleoproteins

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The eukaryotic endonucleases RNase P and RNase MRP require both RNA and protein subunits for function. Even though the human RNase P and MRP RNAs were previously characterized, the protein composition of the particles remains unknown. We have identified a human and a *Caenorhabditis elegans* sequence showing homology to yPop1, a protein subunit of the yeast RNase P and MRP particles. A cDNA containing the complete coding sequence for the human protein, hPop1, was cloned. Sequence analysis identifies three novel sequence motifs, conserved between the human, *C.elegans* and yeast proteins. Affinity-purified anti-hPop1 antibodies recognize a single 115 kDa protein in HeLa cell nuclear extracts. Immunoprecipitations with different anti-hPop1 antibodies demonstrate an association of hPop1 with the vast majority of the RNase P and MRP RNAs in HeLa cell nuclear extracts. Additionally, anti-hPop1 immunoprecipitates possess RNase P enzymatic activity. These results establish hPop1 as the first identified RNase P and MRP protein subunit from humans. Anti-hPop1 antibodies generate a strong nucleolar and a weaker homogeneous nuclear staining in HeLa cells. A certain class of autoimmune patient serum precipitates *in vitro*-translated hPop1. hPop1 is therefore an autoantigen in patients suffering from connective tissue diseases.

Keywords: autoantigen/homology/nucleolus/nucleus/RNA processing

Introduction

The eukaryotic nucleus contains a number of ribonucleoprotein complexes, known as small nuclear ribonucleoproteins (snRNPs), most of which are involved in the processing of mRNA, rRNA and tRNA precursors synthesized in the nucleus. Although many snRNPs might be part of the catalytic complexes required for RNA processing, only two have been shown to possess enzymatic activity on an RNA substrate *in vitro*: RNase P and RNase MRP.

RNase P endonucleolytically cleaves precursor tRNA molecules to remove the 5′ leader sequences and generate the correct 5′ termini of the mature tRNAs (reviewed in Altman *et al.*, 1993a). RNase P has been identified in all the phylogenetic domains (Archae, Eubacteria and Eukarya) and in the mitochondria of some eukaryotic cells.

The eubacterial RNase P is the most extensively characterized (reviewed in Pace and Brown, 1995). It consists of two subunits: an RNA of 350–410 nucleotides and a basic protein of ~14 kDa. The demonstration that the RNA subunit alone can catalyse the cleavage reaction under certain conditions *in vitro*, identified the bacterial RNase P as a ribozyme (Guerrier-Takada *et al.*, 1983). The protein subunit is, however, absolutely required *in vivo* and greatly stimulates the efficiency and versatility of the enzyme *in vitro* (Gopalan *et al.*, 1995).

An RNase P-like enzymatic activity has been identified in the nucleus of various eukaryotic cells. In all cases examined, the nuclear RNase P is believed to contain both essential RNA and protein subunits, based on its sensitivity to nuclease and protease treatment and its buoyant density. Biochemical purification allowed the cloning of the RNase P RNA from *Schizosaccharomyces pombe* (Krupp *et al.*, 1986), HeLa cells (Bartkiewicz *et al.*, 1989), *Saccharomyces cerevisiae* (Lee and Engelke, 1989) and *Xenopus laevis* oocytes (Doria *et al.*, 1991). This facilitated the subsequent cloning of the RNase P RNAs from related species (Zimmerly *et al.*, 1990; Altman *et al.*, 1993b; Tranguch and Engelke, 1993). Comparative sequence analysis of the available RNase P RNAs revealed a very low primary sequence conservation but a number of shared secondary and tertiary structural features, many of which have counterparts in eubacterial RNase P RNAs. The eukaryotic RNase P RNAs have not been shown to be catalytic in the absence of proteins. Sedimentation studies indicate that protein constitutes a much larger proportion of the eukaryotic enzyme (~50–70% of the complex weight in eukaryotes versus ~10% for the bacterial enzyme). An involvement of the protein subunit(s) in substrate binding has been suggested for the *S.cerevisiae* enzyme (Nichols *et al.*, 1988).

Very little is known about the protein components of eukaryotic RNase P because purification of the enzyme has been hampered by its low abundance and its highly labile character. Only in the case of *S.pombe* has the nuclear RNase P been purified to apparent homogeneity (Zimmerly *et al.*, 1993). It appears to contain a single protein subunit with an apparent molecular weight of 100 kDa. The corresponding gene has not yet been cloned. A genetic approach in the yeast *S.cerevisiae* allowed us recently to clone the first gene encoding for a nuclear RNase P protein subunit (Lygerou *et al.*, 1994). This 100 kDa protein is referred to here as yPop1.

The eukaryotic RNase MRP was originally identified as an endonuclease able to cleave an RNA substrate

derived from the mitochondrial origin of DNA replication *in vitro* (Chang and Clayton, 1987b; for a review see Clayton, 1994). In some studies a fraction of the MRP RNA has been localized in cytoplasmic structures (Li *et al.*, 1994; Jacobson *et al.*, 1995; Matera *et al.*, 1995) that were identified as mitochondria (Li *et al.*, 1994). However, all studies agree that the bulk of this enzyme is localized in the nucleolus (Kiss and Filipowicz, 1992; Topper *et al.*, 1992; Li *et al.*, 1994; Jacobson *et al.*, 1995; Matera *et al.*, 1995). At least in the yeast *S.cerevisiae*, RNase MRP cleaves directly the ribosomal RNA precursor to allow the subsequent formation of the 5′ end of the major 5.8S rRNA species (Lygerou *et al.*, 1996). An involvement of RNase MRP in mitochondrial DNA replication has not yet been demonstrated *in vivo*.

The RNA subunit of the RNase MRP has been cloned from several vertebrates (Chang and Clayton, 1989; Topper and Clayton, 1990; Bennett *et al.*, 1992; Dairaghi and Clayton, 1993), plant (Kiss *et al.*, 1992) and yeast (Schmitt and Clayton, 1992; Paluh and Clayton, 1995) species. Secondary structure predictions based on phylogenetic comparisons (Schmitt *et al.*, 1993) suggest that the RNase MRP RNA has common structural features with the eukaryotic and prokaryotic RNase P RNAs (Forster and Altman, 1990). It has been proposed that both the eukaryotic RNase P and MRP enzymes are derived from an ancestral RNase P-like enzyme through gene duplication (Morrissey and Tollervey, 1995).

yPop1 was the first subunit of the yeast RNase MRP to be identified (Lygerou *et al.*, 1994). A second *S.cerevisiae* protein, Snm1p, was also identified by genetic means and shown to be associated with at least a subpopulation of the RNase MRP but not the RNase P RNA (Schmitt and Clayton, 1994). Despite the extensive study of RNase MRP from higher eukaryotes, no protein subunits have been identified.

Sera from patients suffering from autoimmune diseases, such as systemic lupus erythematosus (SLE) and scleroderma, often contain antibodies to various nuclear and cytoplasmic ribonucleoprotein complexes. Although the mechanisms eliciting the autoimmune response are not known, autoantibodies have often served as useful tools in studying the structure and function of their intracellular targets. A group of patient sera, referred to as Th (or To), immunoprecipitate two small RNAs from HeLa cell extracts (Hashimoto and Steitz, 1983; Reddy *et al.*, 1983). These RNAs, first called 7-2 and 8-2 RNAs, were later shown to be the RNA components of RNase MRP (Gold *et al.*, 1989) and RNase P (Gold *et al.*, 1988), respectively. The human RNase MRP RNA is also called Th RNA while the human RNase P RNA is also referred to as the H1 RNA. Purified RNAs are not precipitated by these sera, suggesting that the autoantibodies recognize either protein subunit(s) of these particles or an RNA–protein antigen (Hashimoto and Steitz, 1983).

Different Th/To sera immunoprecipitate a number of proteins from HeLa cell extracts (Kipnis *et al.*, 1990; Rossmanith and Karwan, 1993), the most reproducibly observed being a polypeptide with an apparent molecular weight of 40 kDa. A polypeptide of 40 kDa can be UV-cross-linked to both the RNase MRP and RNase P RNAs incubated with HeLa cell extracts and the complex is immunoprecipitable by Th sera (Yuan *et al.*, 1991; Liu *et al.*, 1994). These data have been taken to indicate that the Th/To sera recognize a 40 kDa protein subunit common to the human RNase P and RNase MRP ribonucleoproteins, which is referred to as the 40 kDa Th antigen (Th40). However, immunoblotting experiments with different Th sera did not reveal a single immunoreactive polypeptide and the cloning of a cDNA encoding the Th antigen has not been accomplished.

We describe here the identification and characterization of a human protein, hPop1, which exhibits homology to the yeast yPop1 protein. We show that this 115 kDa, predominantly nucleolar protein is associated with both the human RNase P and MRP RNAs and is recognized by different autoimmune patient sera with the Th specificity.

Results

Identification of putative Pop1 homologues

We previously reported the identification and characterization of yPop1, a protein component of the yeast RNase P and RNase MRP RNP particles (Lygerou *et al.*, 1994). The yPop1 amino acid sequence was compared with protein and translated nucleic acid databases to identify possible homologous sequences. Two nucleic acid sequence entries, corresponding to a *Caenorhabditis elegans* and a human sequence were retrieved. These sequences contained open reading frames (ORFs) which could encode proteins exhibiting statistically significant homology to yPop1 over relatively short amino acid stretches. We refer to the putative proteins encoded by these ORFs as cPop1, for the *C.elegans* protein and hPop1 for the human protein.

The *C.elegans* sequence was determined as part of a genome sequencing project (Wilson *et al.*, 1994). The corresponding gene is transcribed and the predicted ORF has the potential to encode for a basic protein (pI=10.42) of 86.2 kDa (see Materials and methods). A number of putative nuclear localization sequences, but no other known protein motifs, could be identified.

The human sequence was determined as part of a project analysing randomly sampled cDNA clones from the human immature myeloid cell line KG1. A 4.27 kb cDNA had been sequenced which contained a long ORF and 1.6 kb of 3′ untranslated region. No in-frame stop codon was present upstream of the first methionine of the sequenced cDNA, suggesting that it was truncated at its 5′ end. This possibility was further supported by the comparison of the deduced protein sequence with yPop1 and cPop1.

Cloning of a full-length hPop1 cDNA

We have confirmed the sequence of the original cDNA by cloning and sequencing HeLa cell cDNAs encoding the same protein. We found a single nucleotide difference in the sequence of the cDNAs from the myeloid and HeLa cells, which corresponded to a silent substitution (see Materials and methods). To establish the complete protein coding sequence of hPop1, we recovered cDNA clones extending further 5′ of the known sequence. These clones extended the ORF by 121 amino acids. The DNA and deduced amino acid sequences not present in the original database entry are shown in Figure 1A. Most of the recovered clones had the 5′ untranslated region shown. In these cDNAs, the first in-frame ATG is preceded by a

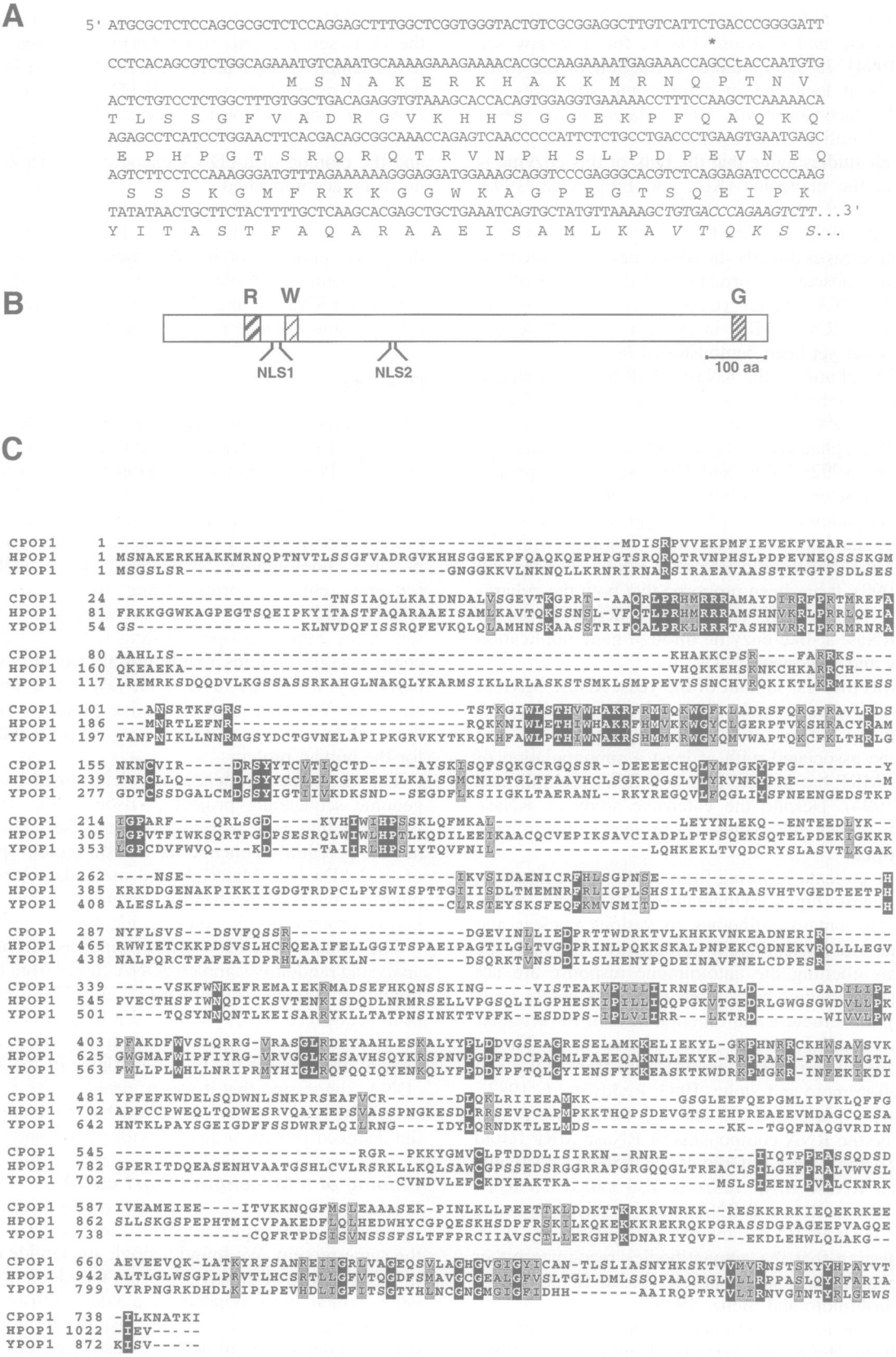

Fig. 1. Sequence of the hPop1 cDNA and structure of the Pop1 proteins. (**A**) Nucleotide sequence of the 5′ end of a hPop1 cDNA and deduced amino acid sequence. The 3′ end of the sequence that overlaps with the previously sequenced hPop1 cDNA (accession No. D31765) is shown in italics. A stop codon upstream of and in frame with the initiating ATG is underscored with an asterisk. The first three nucleotides correspond to the upstream ATG discussed in the text. (**B**) Schematic representation of the full-length hPop1 protein. The regions of homology to yPop1 and cPop1 are shown as boxes (marked R, W and G) while the positions of the putative nuclear localization sequences (NLS1 and 2) are indicated. (**C**) Alignment of the *C.elegans* (cPop1), human (hPop1) and yeast (yPop1) homologues. Amino acids absolutely conserved in the three proteins are marked by a black box while amino acids with a conserved character are marked with a grey box.

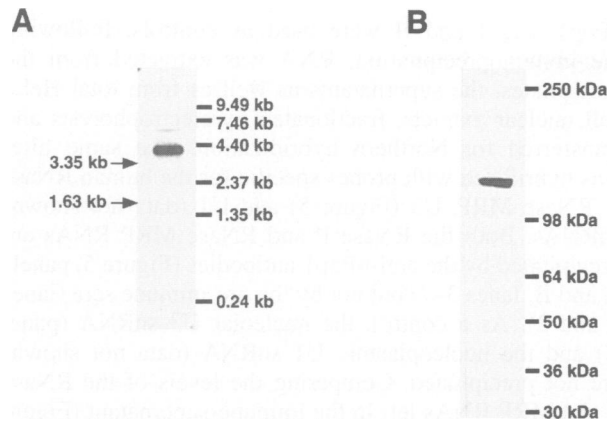

Fig. 2. hPop1 is expressed in HeLa cells. (**A**) Analysis of hPop1 mRNAs. Poly(A)$^+$ RNAs (1 μg) from HeLa cells were fractionated on a formaldehyde–agarose gel, transferred to nitrocellulose and hybridized with an antisense riboprobe deriving from the hPop1 locus. The sizes and positions of migration of RNA molecular weight markers (yeast ribosomal RNAs and RNA ladder from GIBCO/BRL) are indicated. (**B**) Western blot analysis with affinity-purified anti-hPop1 antibodies. Rabbit antibodies were raised against a fragment of hPop1 (amino acids 508–835) expressed as a GST fusion in *E.coli* and were affinity purified as described in Materials and methods. Proteins of a HeLa cell nuclear extract (Dignam *et al.*, 1983) were separated on a 6% to 15% gradient SDS–polyacrylamide gel and transferred to a nitrocellulose filter. After incubation with the affinity-purified anti-hPop1 antibody, detection was performed using peroxidase-conjugated goat anti-rabbit IgGs and the ECL detection system (Amersham). On the right, the corresponding sizes of a protein molecular weight marker are indicated.

stop codon in the same frame, indicating that the complete ORF has been identified. An upstream ATG is present at the extreme 5′ end of these cDNAs but it is probably too close to the mRNA cap to be used for translation initiation (see Figure 1). We also identified a second type of cDNA clone that has an identical sequence in the protein coding region but a different 5′ untranslated region (data not shown). These clones probably represent unspliced or partially spliced pre-mRNAs because they contain a consensus 3′ splice site at the point of divergence with the previous class of cDNAs as well as multiple out-of-frame ATG upstream of the main ORF. The main ORF was still delimited by an upstream in-frame stop codon in these cDNAs (data not shown).

We analysed the transcripts deriving from the hPop1 locus by hybridizing poly(A)$^+$ RNA extracted from HeLa cells with a labelled antisense RNA probe derived from the coding region. As shown in Figure 2A, this probe hybridizes to a major mRNA of ~4.1 kb and a minor larger mRNA of ~6 kb. These two transcripts could represent alternatively initiated, spliced and/or polyadenylated mRNAs. The total cDNA sequence amounts currently to 4.6 kb but lacks a poly(A) tail at its 3′ end. Therefore, it probably represents a 3′ truncated fragment of the 6 kb transcript. A putative polyadenylation signal is located in the 3′ UTR (position 3542 in the database entry). Its usage would generate a transcript of ~4.0 kb, consistent with the size of the major mRNA detected by Northern hybridization.

The complete hPop1 ORF has the potential to encode a basic protein (pI=9.86) of 1024 amino acids, with a predicted molecular weight of 114.7 kDa. A schematic diagram of hPop1 is depicted in Figure 1B. A number of putative nuclear localization sequences (NLS) can be identified: a bipartite NLS (NLS1) close to the N-terminus of the protein (amino acids 182–198) is near a couple of putative bipartite NLSs of suboptimal spacing, while an SV40-type NLS (NLS2) is found towards the middle of the protein (amino acids 382–387).

The availability of the full-length hPop1 protein sequence allowed us to align and compare the yeast, human and *C.elegans* proteins in more detail (Figure 1C). Despite the low overall similarity (22–27% identity in pairwise comparisons), three short conserved sequence blocks are apparent. The first region of homology (amino acids 133–159 of the human protein) spans 26 amino acids close to the N-terminus. We refer to this region as the R box, because it contains several arginine residues (five out of the 10 absolutely conserved amino acids are arginines). Due to its positively charged character this region might be involved in interactions with RNA. The W-box, also found close to the N-terminus (amino acids 200–222 of the human protein), is a block of 22 amino acids of which 11 are identical in the three proteins and eight additionally correspond to conservative amino acid substitutions. Three of the absolutely conserved amino acids are tryptophans, which could play a role in protein–RNA interactions by stacking on RNA bases. At the C-terminus (amino acids 963–985 of the human protein), a third region of homology, referred to as the G box, is present. It consists of five conserved glycine residues and seven amino acids with a conserved hydrophobic character, distributed over 22 residues. We could not find other proteins harbouring any one of these domains in protein or translated nucleic acid databases (Swissprot, Trembl, EMBL). The presence of these conserved sequence blocks dispersed along the length of the three proteins suggested that, despite the low overall conservation, these proteins might be orthologues. We therefore undertook a characterization of the human protein.

hPop1 is a nucleolar–nucleoplasmic protein

Three different regions of the hPop1 coding sequence (see Materials and methods) were expressed in *Escherichia coli* and used to immunize rabbits. The sera obtained precipitated *in vitro*-translated hPop1 fragments (data not shown). Specific anti-hPop1 antibodies from the most reactive serum were purified by affinity selection with a recombinant fragment of the hPop1 protein. The affinity-purified antibodies recognize a single, high molecular weight band in HeLa cell nuclear extracts (Figure 2B), with an apparent molecular weight consistent with the predicted 114.7 kDa. After longer migrations, the reacting band was resolved in a closely spaced doublet (data not shown) which could be due to protein modifications.

To assess the cellular localization of hPop1, the affinity-purified anti-hPop1 antibodies were used for immunolocalization experiments in HeLa cells. Anti-hPop1 antibodies generate a strong nucleolar and weak, homogeneous nucleoplasmic staining (Figure 3, panel B). No signal was detected in the cytoplasm above the background staining observed with the pre-immune serum (Figure 3, panel D). However, we cannot definitively exclude that a small fraction of the hPop1 protein is found in the cytoplasm.

To localize more precisely hPop1 in the nucleolus, double immunostaining with a monoclonal anti-fibrillarin

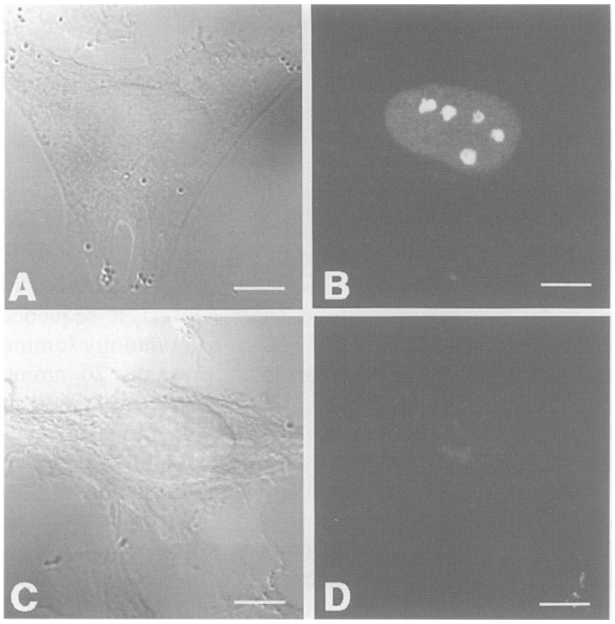

Fig. 3. Immunocytochemical localization of hPop1. HeLa cells were fixed, permeabilized and stained with affinity-purified anti-hPop1 antibodies (panel **B**) or the corresponding pre-immune serum (panel **D**) as described in Materials and methods. Antibody–antigen complexes were detected with fluorescein-labelled goat anti-rabbit IgGs and visualized with confocal microscopy. An image of the same cell with Nomarski optics is shown in panels **A** and **C** respectively. The size bars correspond to 10 μm. Identical contrast and brightness settings are shown for panels B and D.

antibody was performed (Figure 4, panels A–C). Fibrillarin is associated with many small nucleolar RNAs and is localized in the fibrillar compartment of the nucleolus (Reimer *et al.*, 1987). Superimposition of the two images shows that the nucleolar hPop1 largely co-localizes with fibrillarin (Figure 4, panel C). hPop1 is therefore present in the fibrillar compartment of the nucleolus.

Many snRNAs, snoRNAs and associated proteins are present in a nuclear organelle termed the coiled body (for a review see Lamond and Carmo-Fonseca, 1993). To assess whether hPop1 is found in the coiled body, a monoclonal antibody against p80 coilin, a coiled body component, was used. As shown in Figure 4, panels D–F, hPop1 does not appear to accumulate in coiled bodies.

Nucleoplasmic snRNPs often show accumulation in subnuclear compartments termed nuclear speckles. We did not observe an apparent accumulation of hPop1 in nuclear speckles, above the homogeneous nucleoplasmic staining, in co-localization experiments with anti-U2B″ monoclonal antibodies (data not shown).

In conclusion, hPop1 is expressed in human cells, localizes to the nucleus and strongly accumulates in the nucleolus.

hPop1 is associated with both the human RNase P and RNase MRP RNAs

To investigate the association of hPop1 with cellular RNAs, we performed immunoprecipitation experiments from HeLa cell nuclear extracts using the anti-hPop1 antibodies. Four different anti-hPop1 sera (I–IV) raised against different regions of the hPop1 protein, as well as affinity-purified anti-hPop1 antibodies from serum II, were used. The pre-immune sera corresponding to the anti-hPop1 sera I and II were used as controls. Following the immunoprecipitation, RNA was extracted from the precipitates, the supernatants as well as from total HeLa cell nuclear extracts, fractionated by electrophoresis and transferred for Northern hybridization. The same filter was hybridized with probes specific for the human RNase P, RNase MRP, U3 (Figure 5) and U1 (data not shown) snRNAs. Both the RNase P and RNase MRP RNAs are precipitated by the anti-hPop1 antibodies (Figure 5, panels A and B, lanes 3–7) but not by the pre-immune sera (lanes 1 and 2). As a control, the nucleolar U3 snRNA (panel C) and the nucleoplasmic U1 snRNA (data not shown) are not precipitated. Comparing the levels of the RNase P and MRP RNAs left in the immune-supernatant (Figure 5, panels A and B, lanes 8–14) with the total RNase P and MRP RNAs present in HeLa cell nuclear extracts (lanes 15 and 16) reveals that most of the anti-hPop1 antibodies very efficiently precipitate both the RNase P and MRP RNAs: for instance, <5% of these RNAs appear to remain in the immune-supernatant using the affinity-purified anti-hPop1 antibodies (the RNAs loaded in the supernatant and total lanes correspond to one-quarter of the immunoprecipitates).

The co-immunoprecipitation experiments show that hPop1 is associated with both the human RNase P and RNase MRP snRNAs and is therefore the human homologue of the yeast yPop1.

Anti-hPop1 antibodies immunoprecipitate the RNase P enzymatic activity

Having demonstrated that the anti-hPop1 antibodies specifically precipitate the RNase P RNA, we tested whether the RNase P enzymatic activity would be enriched in the anti-hPop1 immunoprecipitate. Aliquots of the immunoprecipitates, analysed for their RNA content in Figure 5, were incubated with an internally labelled pre-tRNA substrate. The products of the reaction were then resolved on a polyacrylamide gel and visualized by autoradiography. As shown in Figure 6, all the anti-hPop1 immunoprecipitates efficiently process the pre-tRNA precursor (lanes 3–7). Even though the anti-hPop1 immunoprecipitates of the various sera were not limiting in this assay, no activity could be detected with the corresponding amount of immunoprecipitates from the pre-immune sera (Figure 6, lanes 1 and 2), demonstrating that the RNase P activity association with hPop1 is highly specific. The cleavage is endonucleolytic and maps to the correct nucleotide for an RNase P enzymatic activity (data not shown).

hPop1 is therefore associated with an active RNase P enzyme and the anti-hPop1 antibodies generated can be used to study the human RNase P enzymatic activity.

hPop1 is recognized by autoimmune patient sera with the Th specificity

Autoimmune patient sera with the Th specificity immunoprecipitate RNase P and RNase MRP RNAs from HeLa cell extracts and are believed to contain autoantibodies directed against a protein associated with both RNAs. The autoantigenic protein(s) recognized by these sera, which is referred to as the Th antigen, has not been identified.

To investigate whether hPop1 might be such an autoimmune antigen, different patient sera with the Th specifi-

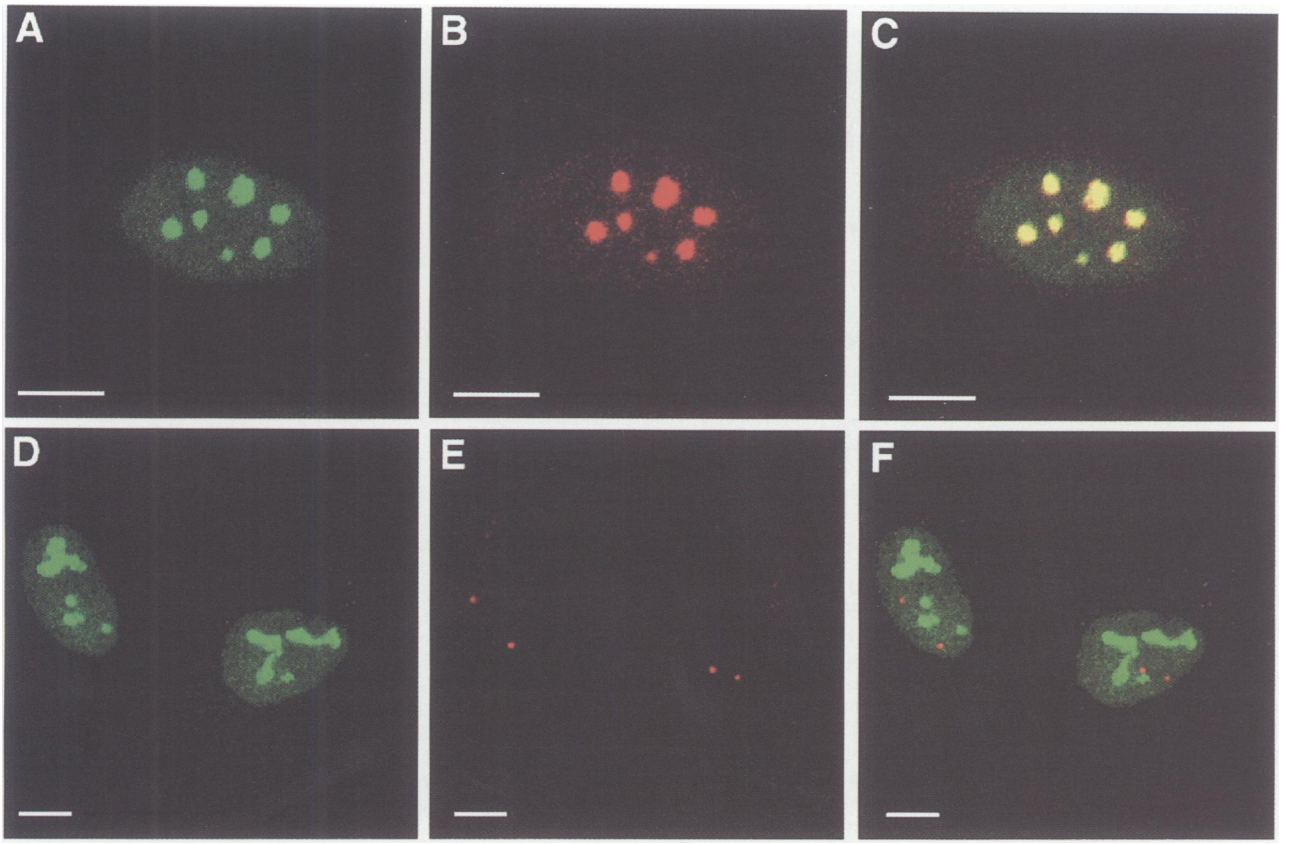

Fig. 4. Localization of hPop1 in subnuclear compartments. Double immunofluorescence was performed on fixed and permeabilized HeLa cells using affinity-purified rabbit anti-hPop1 antibodies and either a mouse monoclonal anti-fibrillarin antibody (panels A–C) or a mouse monoclonal anti-coilin antibody (panels D–F). Antibodies were detected with fluorescein-labelled goat anti-rabbit IgGs and Texas Red-labelled goat anti-mouse IgGs and visualized with confocal microscopy. Panels **A** and **D**: hPop1 localization (green). Panels **B** and **E**: fibrillarin and coilin respectively (red). Panels **C** and **F**: superimposition of panels A–B and D–E respectively, regions of co-localization appear as yellow. The size bars correspond to 10 μm. The weak cytoplasmic staining detected with affinity-purified rabbit anti-hPop1 appears to be non-specific background staining as it is also detected with monoclonal anti-coilin antibody (panel E), preimmune serum and DAPI (data not shown).

city were tested for their ability to precipitate the hPop1 protein. Thirty-five different patient sera were selected based on their ability to precipitate the RNase P and MRP RNA from HeLa extracts (Table I). Seven of these sera precipitated the human La protein, a protein associated with all newly synthesized RNA polymerase III transcripts. Since both the RNase P and MRP RNAs are transcribed by RNA polymerase III, immunoprecipitation of these RNAs by these seven sera could be due to their association with the La protein. These sera were therefore not analysed further. At least 26 of the remaining 28 sera precipitate both RNase P and MRP RNAs and are therefore of the Th specificity (Table I). These 28 sera, as well as control sera, were used to immunoprecipitate full-length hPop1 protein produced by *in vitro* translation in rabbit reticulocyte lysates. Typical results of such an immunoprecipitation experiment are depicted in Figure 7A. In this experiment, four out of the six anti-Th sera tested (lanes 2–7) immunoprecipitate the *in vitro*-translated hPop1. The precipitation is specific, since neither normal human serum (lane 8), nor autoimmune patient sera with different specificities (lanes 9, 10) precipitate hPop1. The collective results from all the sera tested are shown in Table I. Of the 28 sera tested, 13 precipitated efficiently the full-length hPop1 protein. hPop1 is therefore recognized by ~50% of the autoimmune patient sera with the Th specificity.

To localize further the autoimmune epitope recognized by the Th sera, subclones containing different regions of the hPop1 coding sequence were constructed. The different *in vitro*-translated hPop1 fragments tested and their precipitability by Th sera are depicted in Figure 7C. A fragment spanning amino acids 214–512 in the middle of the hPop1 protein is precipitated by the same 13 Th sera that efficiently precipitate the full-length protein (Table I). An example of the immunoprecipitation of *in vitro*-translated hPop1(214–512) fragment is shown in Figure 7B. Three Th sera (lanes 2–4) precipitate hPop1(214–512). This precipitation is specific, since control human sera fail to precipitate this protein fragment (lanes 5–7) and the other hPop1 fragments tested are not precipitated by the same sera (data not shown).

Different autoimmune patient sera with the Th specificity are therefore directed against hPop1 and the autoimmune epitope resides within a 298 amino acid-long fragment in the middle of hPop1.

Discussion

The protein composition of the human RNase P and MRP RNP enzymes was previously unknown. We have identified a human protein, hPop1, which exhibits homology to the yeast yPop1. We demonstrate that hPop1 is a subunit of the human RNase P and MRP RNPs. We show

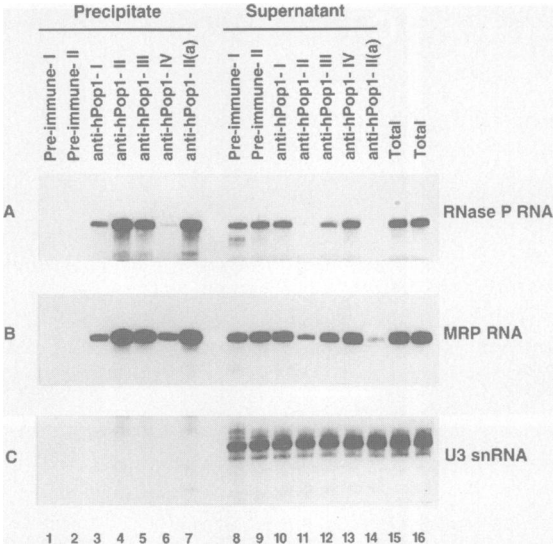

Fig. 5. Co-precipitation of the human RNase P and MRP RNAs with hPop1. Immunoprecipitation with HeLa cell nuclear extracts using rabbit sera raised against different regions of the hPop1 protein (anti-hPop1 I-IV; see Materials and methods), affinity-purified anti-hPop1 antibodies from serum II [anti-hPop1-II(a)] and the preimmune sera corresponding to anti-hPop1 sera I and II (pre-immune I and II). RNAs were extracted from the immunoprecipitates (lanes 1–7), the supernatants (lanes 8–14) and total nuclear extract (lanes 15 and 16), resolved by polyacrylamide gel electrophoresis and transferred to a nylon filter. Specific antisense riboprobes were used to detect the human RNase P, MRP and U3 snRNAs as indicated on the right. The amount of RNA loaded in lanes 8 to 16 corresponds to one-quarter of the amount loaded in lanes 1 to 7. The anti-hPop1 serum IV precipitates low levels of the U1 snRNA (data not shown).

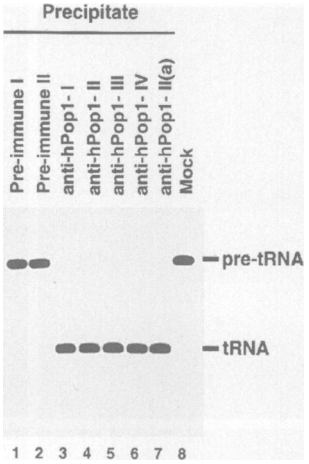

Fig. 6. Anti-hPop1 immunoprecipitates possess RNase P enzymatic activity. Four anti-hPop1 rabbit sera (lanes 3–6, sera I–IV), affinity-purified anti-hPop1 antibodies from serum II (lane 7) and the preimmune sera corresponding to anti-hPop1 sera I and II (lanes 1 and 2) were used for immunoprecipitation experiments with HeLa cell nuclear extracts. An aliquot of each immunoprecipitate, corresponding to 4 μl of extract, was incubated with an internally labelled pre-tRNA substrate. Following RNA extraction and polyacrylamide gel electrophoresis, the labelled RNAs were visualized by autoradiography. Lane 8: substrate incubated in the presence of buffer alone. The positions of the pre-tRNA substrate and the mature tRNA generated by RNase P cleavage are indicated on the right. With more dilute immunoprecipitates the level of RNase P activity reflected the difference in immunoprecipitation efficiency of the various sera.

further that hPop1 is an autoimmune antigen in humans suffering from connective tissue diseases.

Proteins homologous to yPop1 exist in very divergent eukaryotic species: identification of conserved sequence motifs

Database searches identified two putative open reading frames from *C.elegans* and from human that could encode proteins homologous to the yeast yPop1. We recovered cDNAs encoding the putative human homologue, hPop1. These cDNAs most likely contain the full-length protein coding sequence because the first in-frame ATG is preceded by a stop codon in the same frame. Additionally, the size of the polypeptide recognized by affinity-purified anti-hPop1 antibodies is consistent with the molecular weight predicted for the identified ORF (114.7 kDa) and the homology with yPop1 and cPop1 suggests that no region of hPop1 is missing. Our analysis of the hPop1 transcripts suggests the presence of alternative mRNAs in HeLa cells.

We have shown that the hPop1 protein is associated with both the human RNase P and MRP RNAs and is therefore an orthologue of yPop1. The *C.elegans* protein probably represents another orthologue, since it contains the same conserved domains. The low overall conservation of the three sequences agrees with our inability to identify yPop1 homologues by Southern hybridization or by complementation of the Pop1-1 temperature-sensitive yeast strain using expression libraries from different species (our unpublished observations). Furthermore, we were

Table I. Collective immunoprecipitation results using autoimmune patient sera

No. of sera	MRP RNA	P RNA	La	hPop1	hPop1 fragment (214–512)
12	+	+	–	+	+
1	+	nd	–	+	+
2	+	+	–	+/–	–
12	+	+	–	–	–
1	+	–	–	–	–
7	+	nd	+	nd	nd
Total					
35	35	26	7	13–15	13

unable to detect immunoprecipitation of RNase P enzymatic activity using the anti-hPop1 antibodies with extracts from organisms other than humans, and the affinity-purified anti-hPop1 antibodies did not cross-react with a single band in extracts from even closely related species (data not shown). No sequence similarity could be detected between any of the three eukaryotic proteins identified and the eubacterial RNase P protein (Gopalan *et al.*, 1995) or the mitochondrial RNase P protein from yeast (Dang and Martin, 1993), except for the presence of numerous basic residues.

Sequence analysis did not reveal the presence of known protein motifs in yPop1 or its homologues (apart from the presence of putative nuclear localization sequences). Phylogenetic comparisons could reveal regions involved in evolutionarily conserved functions of the protein, such as RNA binding or interactions with other conserved

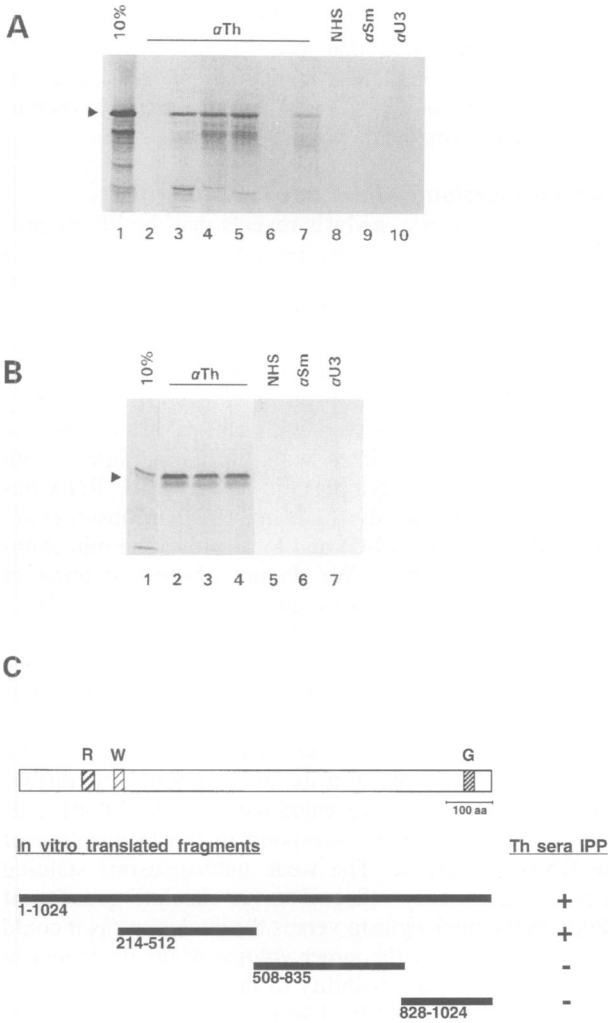

Fig. 7. Th sera immunoprecipitate *in vitro*-translated hPop1. (**A**) [^{35}S]methionine-labelled hPop1 protein was generated by *in vitro* translation. Following immunoprecipitation with different human sera, proteins from the immunoprecipitates were analysed by SDS–polyacrylamide electrophoresis and visualized by autoradiography. Lane 1, input lysate corresponding to 10% of the amount used for immunoprecipitations; lanes 2–7, different patient sera with the Th specificity; lane 8, normal human serum; lane 9, patient serum with anti-Sm specificity; lane 10, patient serum with anti-U3 specificity. (**B**) Precipitation of a labelled polypeptide corresponding to amino acids 214–512 of hPop1, generated by *in vitro* translation, by different human sera. Lane 1, 10% of input lysate; lanes 2–4, immunoprecipitates using different Th patient sera; lane 5, normal human serum; lane 6, anti-Sm patient serum; lane 7, anti-U3 patient serum. (**C**) Mapping the major autoantigenic epitope on hPop1. A schematic representation of the full-length hPop1 protein is shown above. The extents of the different *in vitro*-generated polypeptides are shown below, while their immunoprecipitability by Th sera is indicated by + or – on the right.

protein subunits. Three short, highly conserved amino acid regions were identified, termed the R, W and G boxes in the Pop1 homologues (see Results). The two N-terminal boxes (R and W), which are rich in basic residues and possess conserved aromatic residues, could be important for interactions with RNA molecules, either the RNase P and MRP RNAs or the RNA substrates of the enzymes. These conserved regions could be used to design oligonucleotides or to raise antipeptide antibodies which might facilitate the identification of other eukaryotic Pop1 homologues.

hPop1 is the first identified protein subunit of the human RNase P and MRP particles

We show that anti-hPop1 antibodies immunoprecipitate both the RNase P and MRP RNAs.

Are RNase P and MRP RNAs present in a single complex of which hPop1 is a component? The consistent co-precipitation of RNase P and MRP RNAs by different autoimmune patient sera, and their apparent co-fractionation upon initial steps of biochemical purification, led to the speculation that the two RNAs might co-exist in cells in a single snRNP, named the Th/To ribonucleoprotein (Karwan, 1993; Rossmanith and Karwan, 1993). Additionally, the possibility of overlapping, redundant functions for RNase P and RNase MRP has been previously suggested (Clayton, 1994), given the capacity of purified RNase P to cleave RNase MRP substrates *in vitro* (Potuschak *et al.*, 1993; Lygerou *et al.*, 1996). The available data—even though not conclusive—do not lend support to these models. An association of the two RNAs is not required for *in vitro* enzymatic activity and is not tight, as purified active RNase P contains no RNase MRP RNA (Bartkiewicz *et al.*, 1989) and vice versa (Chang and Clayton, 1987a; Lygerou *et al.*, 1996). Human RNase P and MRP are not only separated during biochemical purification but also behave differently upon cellular fractionation: RNase P is mostly recovered with the cytoplasmic fraction, while the majority of RNase MRP is tightly associated with the nucleus (Reddy *et al.*, 1983). The separated RNase P and MRP particles are still immunoprecipitable by Th sera. At least in the yeast *S.cerevisiae*, RNase P and MRP do not seem to have overlapping functions or significantly affect each other's activities *in vivo*: both RNase P and MRP RNAs are essential for cell viability (Lee *et al.*, 1991; Schmitt and Clayton, 1992); a mutant in the RNase P RNA which affects tRNA maturation (RPR1 cDNA dimer; Lee *et al.*, 1991) does not affect rRNA processing (D.Tollervey, personal communication) and a mutant in the RNase MRP RNA (rrp2-2), shown to have rRNA processing defects (Lindahl *et al.*, 1992; Chu *et al.*, 1994; Lygerou *et al.*, 1994), does not affect tRNA processing (D.Tollervey, personal communication). Co-precipitation of the RNase P and MRP RNAs by the anti-hPop1 antibodies is therefore unlikely to be due to the presence of a single RNase P/MRP particle of which hPop1 is a subunit, since the majority of RNase P and MRP appear to exist as independent particles with distinct functions in the cell.

Do RNases P and MRP share a common protein or immunologically related polypeptides? Co-precipitation of the RNase P and MRP RNAs by anti-hPop1 antibodies and Th/To autoimmune antibodies could be due to the presence of distinct but related proteins on the two particles which share common epitopes. A similar situation is observed for the U1A and U2B″ protein components of the U1 and U2 snRNPs, respectively (Habets *et al.*, 1985). Our results argue that this is not the case for hPop1. Four different rabbit antibodies, raised against different regions of the hPop1 polypeptide, immunoprecipitate the human RNase P and MRP RNAs. Furthermore, these antibodies recognize a single, closely spaced protein doublet on a

Western blot of total HeLa cell proteins (Figure 2B and data not shown) suggesting that a single, post-translationally modified protein is present in both the RNase P and MRP particles. This is consistent with our results from yeast: purified RNase P and purified RNase MRP are each associated with yPop1 (Lygerou *et al.*, 1996).

Is hPop1 transiently associated with RNase P and RNase MRP? The hPop1 protein is associated with the vast majority of the RNase P and MRP RNAs present in HeLa cell nuclear extracts: at least 95% of these RNAs are precipitated by affinity-purified anti-hPop1 antibodies. It is therefore unlikely that hPop1 is only transiently associated with the two RNAs as part of their maturation or intracellular transport (in contrast to the La protein, which binds transiently to the newly transcribed RNase P and MRP RNAs; reviewed by van Venrooij *et al.*, 1993). Furthermore, the anti-hPop1 immunoprecipitates possess RNase P enzymatic activity, showing that hPop1 is associated with the active pool of RNase P.

Our results therefore strongly suggest that hPop1 is a subunit of the mature and active RNase P and MRP particles. Difficulties in biochemical purification did not previously permit a characterization of the protein composition of the human RNase P and MRP RNPs. Very little information is therefore available. Human RNase P has a sedimentation velocity of ~15S (Bartkiewicz *et al.*, 1989). An antibody raised against the *E.coli* RNase P protein was shown to cross-react with a 40 kDa band on immunoblots from a partially purified HeLa RNase P preparation (Mamula *et al.*, 1989). Further characterization of this protein has not been reported. Human RNase MRP sediments in two peaks on a glycerol gradient: a 15–20S peak which probably represents the RNase MRP monoparticle and a higher order complex of 65–80S, possibly corresponding to a fraction of RNase MRP associated with ribosomal precursors (Kiss *et al.*, 1992, 1996). About 10 polypeptides ranging in size from 10 to 100 kDa could be detected in a partially purified RNase MRP monoparticle preparation (Karwan *et al.*, 1991) but it is unclear whether they represent RNase MRP components. A polypeptide of 40 kDa can be cross-linked to both the human RNase P and MRP RNAs incubated with HeLa cell extracts (Yuan *et al.*, 1991; Liu *et al.*, 1994). The binding site for this non-characterized protein has been mapped to a region close to the 5′ end, which is conserved between the RNase P and MRP RNAs. Th autoimmune patient sera immunoprecipitate multiple bands from HeLa cell extracts, ranging in size from 20 to 120 kDa (Kipnis *et al.*, 1990; Rossmanith and Karwan, 1993; H.Pluk and W.J.van Venrooij, unpublished results). Which of these proteins constitute RNase P or MRP components is however unclear, as autoimmune sera often contain more than one type of autoantibody. The anti-hPop1 antibodies will hopefully facilitate biochemical purification and allow a better characterization of the human RNase P and MRP enzymes. None of the four anti-hPop1 antibodies tested causes dissociation of the RNase P complex, as in all cases high levels of enzymatic activity co-precipitate with hPop1. These antibodies can therefore be used to purify active RNase P and, by extrapolation, MRP particles. It should be noted that the function of the human RNase MRP remains obscure. We recently demonstrated that yeast RNase MRP directly cleaves the ribosomal RNA precursor *in vitro* (Lygerou *et al.*, 1996). It would be interesting to investigate whether the human enzyme has a similar function.

Cellular location of the hPop1 protein

Immunofluorescence analysis reveals that the hPop1 protein is localized in the nucleolus and nucleoplasm of HeLa cells, as expected for an RNase MRP and RNase P subunit, respectively. The RNase MRP RNA has been localized predominantly in the nucleolus both by *in situ* hybridization (Li *et al.*, 1994; Matera *et al.*, 1995) and by fluorescent RNA cytochemistry (Jacobson *et al.*, 1995). The strong nucleolar staining observed with anti-hPop1 antibodies, which largely co-localizes with fibrillarin, agrees with these studies. Some cytoplasmic RNase MRP RNA has been detected previously (Li *et al.*, 1994; Jacobson *et al.*, 1995; Matera *et al.*, 1995) and localized to the mitochondria (Li *et al.*, 1994). We do not observe staining of HeLa cells in the cytoplasm, above background levels, in agreement with biochemical fractionation studies which could not detect a significant portion of the MRP RNA in the mitochondria of HeLa cells (Kiss and Filipowicz, 1992). The subcellular localization of the RNase P RNA has not been carefully examined. Transfer RNA processing is believed to take place in the nucleoplasm. The diffuse nucleoplasmic staining revealed with the anti-hPop1 antibodies could therefore correspond to the localization of the RNase P particle. The weak nucleoplasmic staining does not necessarily reflect a lower absolute quantity of hPop1 in the nucleoplasm versus the nucleolus, as it could be accounted for by the larger volume of the nucleoplasm or by differential accessibility to the antibodies.

Nucleoplasmic snRNPs, like the spliceosomal U2, U4/U6 and U5, often reveal a speckled pattern and concentrate in coiled bodies (reviewed in Lamond and Carmo-Fonseca, 1993). Coiled bodies also contain the nucleolar snRNP protein fibrillarin (Raska *et al.*, 1991). Anti-hPop1 antibodies, however, do not stain similar structures. Matera *et al.* (1995) recently described a subnuclear region, termed the perinucleolar compartment (PNC) where RNase P, RNase MRP and other polymerase III-transcribed RNAs appear to accumulate. No staining of PNCs with Th autoimmune patient sera was detected in this study. In agreement with this, we did not observe accumulation of hPop1 in a similar structure.

hPop1 is an autoantigen

Multiple sera from patients suffering from certain rheumatic diseases (e.g. scleroderma) immunoprecipitate the RNase P and MRP RNAs from HeLa cell nuclear extracts. The autoantigenic protein recognized by these sera—which often is referred to as the Th antigen—was previously unknown.

We show here that 50% of the Th sera tested immunoprecipitate *in vitro*-translated hPop1. Human Pop1 is therefore recognized by anti-Th autoantibodies. In agreement with this, the intracellular localization of hPop1 closely resembles the staining observed with Th sera (Jacobson *et al.*, 1995). We localized the autoimmune epitope within 296 amino acids in the middle of the hPop1 protein. This region does not correspond to any of the conserved

sequence blocks identified by sequence analysis of the Pop1 homologues. The lack of conservation of the autoepitope agrees with the lack of immunoprecipitation of the yeast RNase P and MRP RNAs by different human Th sera (our unpublished results). Anti-hPop1 autoantibodies are unusual in this respect, since many autoimmune epitopes, like those recognized by anti-Sm and anti-fibrillarin antibodies, are conserved (Tollervey and Mattaj, 1987; Aris and Blobel, 1988).

It was so far believed that a 40 kDa protein of unknown sequence, called Th40, would be the autoantigen recognized by Th sera (see Introduction). What is the relation of hPop1 to Th40? Human Pop1 is expressed as a high molecular weight protein in HeLa cells (Figure 3B). It is therefore unlikely that the consistent observation of a 40 kDa polypeptide by different groups is due to protein degradation or *in vivo* processing of hPop1. Th40 might also be an autoimmune antigen, either sharing a common epitope with hPop1 or being recognized by different autoantibodies present in Th sera. Different proteins containing the same autoimmune epitope have been previously described. For example, at least three of the eight core proteins of the spliceosomal snRNPs are recognized by the Sm autoantibodies (Rokeach and Hoch, 1992). Additionally, autoimmune patient sera often contain more than one autoantibody activity, sometimes directed against different proteins on the same particle (Tan, 1989). It should be noted that half of the Th patient sera tested do not precipitate the *in vitro*-translated hPop1 protein. The autoantibodies present in these sera might still be directed against hPop1 but recognize an epitope containing modifications not present on the *in vitro*-translated polypeptide or an RNA–protein epitope. These sera, however, may also belong to a different class, directed against another RNase P and/or MRP protein subunit, which could be Th40.

Alternatively, Th40 might not be an autoantigen. In that case, its precipitation by Th sera might be due to its association with hPop1. Since Th sera do not recognize a single band on immunoblots, the proposition that Th40 is an autoantigen was based on immunoprecipitation experiments. A complete characterization of the proteins recognized by Th sera will probably have to await the cloning of the remaining protein components of the human RNase P and MRP RNPs.

Why do RNases P and MRP share common protein subunits?

We have identified Pop1 homologues from very divergent eukaryotic species (*S.cerevisiae*, *C.elegans* and human) and demonstrated that association with both the RNase P and MRP RNAs is conserved in yeast and humans. The presence of common proteins, shared by different snRNPs is not unusual: the spliceosomal snRNPs contain eight common proteins, collectively referred to as the Sm proteins, while most of the nucleolar snRNAs are associated with fibrillarin (reviewed in Mattaj *et al.*, 1993). In those cases, however, common proteins are believed to reflect the common subcellular localization and the involvement in the same RNA processing pathway of the respective snRNPs. RNase P and RNase MRP have been proposed to have a common evolutionary origin (Morrissey and Tollervey, 1995). If, however, they now have distinct functions in different cellular compartments, why would the presence of common protein subunit(s) be maintained? It is possible that RNase P and MRP follow a similar maturation pathway, which would be facilitated by the presence of common proteins. Alternatively (or additionally) it could be speculated that the presence of a common protein subunit allows coordinate regulation of the RNase P and MRP levels and/or activities. Processing of tRNAs and rRNAs, which are both components of the translation machinery, could thus be coordinated. In this respect, the observation of a slower-migrating form of hPop1 (data not shown), which could be due to protein modification, is intriguing.

Materials and methods

Cloning of a cDNA encoding the full-length hPop1 and sequence analysis

Database searches were done using the BLAST program (Altschul *et al.*, 1990). The accession number of the *C.elegans* genomic sequence coding for cPop1 is U00048. Sequences of two Expressed Sequence Tags (ESTs) overlapping this locus have accession numbers D37494, D34432, D27973 and D27974. The coding sequence showing homology to yPop1 spans a region of 3.57 kb and was predicted to contain 12 introns. Two of the putative introns, 8 and 12, are present in the sequenced cDNAs. These introns do not interrupt the reading frame, suggesting that the corresponding sequences are protein coding and were incorrectly predicted as intronic. We furthermore reassigned the predicted 3′ splice site of intron 9 to include a short peptide that improved the alignment of the deduced protein sequence to the hPop1.

The accession number of the human partial cDNA sequence coding for hPop1 is D31765. PCR primers were designed based upon this human DNA sequence and used to amplify three DNA fragments corresponding to amino acids 214–512, 508–835 and 828–1024 of the hPop1 protein, from a randomly primed HeLa cell cDNA library (a kind gift of T.Kreis). The amplified fragments were subcloned in vector pT7-7TT and sequenced using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The HeLa cDNA sequenced differed at a single position when compared with the KG1 cell cDNA sequence deposited in the data base (a C to T substitution at position 2138 of data base entry D31765), which did not change the encoded amino acid. To clone the missing 5′ end of the hPop1 cDNA, 5′ RACE was performed essentially as described by Schuster *et al.* (1992). Briefly, 2 μg of HeLa poly(A)$^+$ RNA and an antisense hPop1 primer (based upon the 5′ end of the partial cDNA sequence) were used for first strand cDNA synthesis. After RNase H treatment the cDNA was purified with Geneclean II (Bio101) and a homopolymeric G-tail was added by terminal deoxynucleotidyl transferase. 5 μl of the tailing reaction were directly used for PCR with a nested antisense hPop1 and a C-tail primer (AAGGAATT(C)$_{13}$). The resulting PCR products were analysed by Southern blot hybridization, and hybridizing bands were re-amplified. The resulting PCR products were purified from gel using Wizard^{TM} PCR Preps (Promega) and cloned into the pGEM-T vector. The sequence common to three clones with slightly different 5′ ends is presented in Figure 1A (accession number X99302). These three clones end within four nucleotides of each other, suggesting that they are full-length. Some other amplification products contained the same coding sequence but a different 5′ untranslated region probably originating from partially spliced pre-mRNA (see text). Our cDNA sequence is further supported by the sequence of a partial cDNA amplified from a HeLa cDNA library that overlaps the upstream stop codon and ATG presented in Figure 1A (data not shown).

A full-length hPop1 cDNA was constructed by subcloning hPop1 DNA fragments, corresponding to amino acids 214–510, 511–832 and 833–1024, in vector pT7-7TT resulting in a hPop1 cDNA construct coding for amino acids 214–1024. Subsequently, a 5′ RACE clone containing the translational start codon and 26 nt of 5′ UTR, coding for amino acids 1–213 was subcloned into this construct resulting in the full-length hPop1 cDNA. Northern hybridization was performed on poly(A)$^+$ RNA isolated from HeLa cells, using an antisense riboprobe corresponding to amino acids 508–835 of the hPop1 protein and following standard procedures (Sambrook *et al.*, 1989).

Antibody production and affinity purification

To raise polyclonal anti-hPop1 rabbit antibodies, three different polypeptides, corresponding to amino acids 214–512 (rabbit I), 508–835 (rabbits II and III) and 828–1024 (rabbit IV) of the hPop1 ORF were expressed as GST fusion proteins in *E.coli* and purified as described (Smith and Johnson, 1988). 100–300 μg of purified fusion protein were used for each rabbit immunization, following standard protocols (Harlow and Lane, 1988). To obtain specific anti-hPop1 antibodies, an affinity column was prepared by immobilizing 4.3 mg of the GST–hPop1(508–835) fusion polypeptide used for immunization of rabbit II, on 1 ml of Affigel 10 slurry (Biorad) in 0.1 M MES, pH 6.5, following the manufacturer's instructions. Similarly, 8 mg of GST, expressed and purified from *E.coli*, were immobilized on 1 ml Affigel 10 slurry. Serum from rabbit II was first precleared from antibodies recognizing GST by incubation with the GST affinity column for 1 h at 4°C. The unbound fraction was incubated with the GST–hPop1(508–835) column for 2 h at 4°C. After washing the column with 10 mM Tris–Cl, pH 7.5, followed by 500 mM NaCl, 10 mM Tris–Cl, pH 7.5, bound antibodies were eluted with 100 mM glycine, pH 2.5.

Immunoprecipitations and assay of RNase P enzymatic activity

For immunoprecipitations with anti-hPop1 antibodies, 100 μl serum or affinity-purified anti-hPop1 antibodies were coupled to 100 μl of a 50% suspension of Protein A–Sepharose beads (Sigma) in IPP500 (500 mM NaCl, 10 mM Tris–Cl, pH 8, 0.1% NP40) by incubating for 1 h at room temperature. Beads were washed three times with IPP500 and once with IPP150 (150 mM NaCl, 10 mM Tris–Cl, pH 8, 0.1% NP40, 0.5 mM PMSF, 2 mM benzamidin, 17 μg/ml aprotinin). 100 μl of HeLa cell nuclear extract (Dignam *et al.*, 1983) were precleared by incubating under rotation with Protein A–Sepharose coupled pre-immune serum, for 30 min at 4°C, in IPP150 supplemented with RNasin to 0.1 U/μl. The unbound fraction was incubated with the corresponding Protein A–Sepharose-coupled serum (100 μl of a 50% suspension), for 2 h at 4°C. Beads were subsequently washed four times with IPP150.

To analyse co-precipitating RNAs, proteins were removed from immunoprecipitates, immune-supernatants and total HeLa cell nuclear extract by digestion with 80 μg Proteinase K in 1×PK buffer (100 mM Tris–Cl, pH 7.5, 12.5 mM EDTA, pH 8.0, 150 mM NaCl, 1% SDS) in the presence of 10 μg *E.coli* tRNA for 45 min at 50°C. RNAs were extracted by two phenol/chloroform/*iso*-amyl alcohol extractions, precipitated with ethanol and analysed on a 6% denaturing acrylamide gel. Northern hybridization with antisense riboprobes specific for the human RNase P, MRP, U1 and U3 snRNAs was as described (Cheng and Abelson, 1987).

To assay for RNase P enzymatic activity in the immunoprecipitates, an internally labelled pre-tRNA substrate (*S.pombe* $tRNA^{Ser}$ SupS1; Krupp *et al.*, 1986) was transcribed *in vitro* to a specific activity of 10^3 c.p.m./fmol and gel-purified. This 110 nt-long substrate contains an extension of 28 nt at the 5′ end of the mature tRNA. An aliquot of each immunoprecipitate, corresponding to 4 μl of HeLa cell nuclear extract, was incubated with 5 fmol of the substrate, in assay buffer (20 mM Tris–Cl, pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 50 mM KCl, 50 mg/ml BSA, 60 U/ml RNasin), for 30 min at 37°C, under constant shaking. RNA was extracted with phenol/chloroform/*iso*-amyl alcohol and precipitated with ethanol in the presence of 10 μg *E.coli* tRNA as carrier. RNAs were resolved on an 8% polyacrylamide–urea gel and visualized by autoradiography.

Immunolocalization and Western blots

Indirect immunocytochemistry on fixed and permeabilized HeLa cell monolayers was performed as described (Carmo-Fonseca *et al.*, 1992). The affinity-purified anti-hPop1 antibodies and the corresponding pre-immune serum were used in a 1:100 dilution. The monoclonal anti-fibrillarin antibody has been previously described (Reimer *et al.*, 1987), while the monoclonal anti-coilin antibody was a gift of M.Carmo-Fonseca. For Western blot analysis, the affinity-purified antibody was used in 1:1000 dilution and detection was performed using the enhanced chemiluminescence Western Blotting Detection System Kit (Amersham) following the manufacturer's instructions. Fluorescently labelled secondary antibodies were from Vector and Dianova, while horseradish peroxidase-conjugated anti-rabbit IgGs were from Amersham.

In vitro transcription and translation

In vitro transcription was performed using T7 RNA polymerase and hPop1 cDNA (full-length and a fragment encoding amino acids 214–512 cloned in vector pT7-7TT) essentially as described by Scherly *et al.* (1989). *In vitro* translation of full-length hPop1 was performed with [^{35}S]methionine (ICN) in rabbit reticulocyte lysate. For *in vitro* translation of hPop1 aa214–512, the corresponding T7-mRNA was incubated in wheat germ extract in the presence of [^{35}S]methionine.

Immunoprecipitation of hPop1 protein with patient sera

Patient sera were obtained from the University Hospital (St Radboud) of Nijmegen and selected on their ability to immunoprecipitate RNase MRP RNA. For immunoprecipitations with patient sera, 5 μl serum were coupled to 20 μl of a 50% suspension of Protein A–agarose beads (Biozym) in IPP500 by incubating for 1 h at room temperature or 16 h at 4°C. Beads were washed twice with IPP500 and once with IPP200 (200 mM NaCl, 10 mM Tris–Cl, pH 8, 0.05% NP40, 0.5 mM PMSF). *In vitro*-translated ^{35}S-labelled hPop1 protein (full-length or amino acids 214–512) in IPP200 was added, beads were incubated for 3 h at 4°C and washed three times with IPP200. Beads were resuspended in SDS sample buffer and precipitated proteins were analysed by 8 or 13% SDS–PAGE.

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