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## Three Novel Components of the Human Exosome\*

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The yeast exosome is a complex of 3' → 5' exoribonucleases. Sequence analysis identified putative human homologues for exosome components, although several were found only as expressed sequence tags. Here we report the cloning of full-length cDNAs, which encode putative human homologues of the Rrp40p, Rrp41p, and Rrp46p components of the exosome. Recombinant proteins were expressed and used to raise rabbit antisera. In Western blotting experiments, these decorated HeLa cell proteins of the predicted sizes. All three human proteins were enriched in the HeLa cells nucleus and nucleolus, but were also clearly detected in the cytoplasm. Size exclusion chromatography revealed that hRrp40p, hRrp41p, and hRrp46p were present in a large complex. This cofractionated with the human homologues of other exosome components, hRrp4p and PM/Scl-100. Anti-PM/Scl-positive patient sera coimmunoprecipitated hRrp40p, hRrp41p, and hRrp46p demonstrating their physical association. The immunoprecipitated complex exhibited 3' → 5' exoribonuclease activity *in vitro*. hRrp41p was expressed in yeast and shown to suppress the lethality of genetic depletion of yeast Rrp41p. We conclude that hRrp40p, hRrp41p, and hRrp46p represent novel components of the human exosome complex.

In both bacteria and eukaryotes, the processing and degradation of many RNA species involves multiprotein complexes (reviewed in Refs. 1–4). The *Escherichia coli* degradosome includes the endoribonuclease E (RNase E), the 3' → 5' exonuclease polynucleotide phosphorylase, the DEAD box RNA helicase RhlB, and several additional proteins whose role is unclear (5–7). Related complexes are implicated in RNA processing in chloroplasts and mitochondria (8–10). The yeast exosome contains at least 11 components, which are known or predicted to be 3' → 5' exoribonucleases (11, 12). Ten of these (Rrp4p, Rrp40–46p, Mtr3p, and Csl4p) have been demonstrated to be encoded by essential genes. These 10 components were found in both cytoplasmic and nuclear complexes,

whereas the nonessential *RRP6* gene product was detected only in the nucleus (11, 13).

The 3' processing of many RNAs is affected by the absence or mutation of exosome components. The nuclear exosome is implicated in the processing of ribosomal RNA (rRNA), spliceosomal small nuclear RNAs, and small nucleolar RNAs, as well as the degradation of pre-rRNA spacers and unspliced pre-mRNAs (12–22). The cytoplasmic exosome complex is involved in the 3' → 5' pathway of mRNA degradation (22). The activity of the exosome complex may be regulated by cofactors including, for example, the putative ATP-dependent DEVH box RNA helicases Dob1p and Ski2p (23, 24).

Human cells also contain a multiprotein complex that is related to the yeast exosome (11). This complex, initially designated as the polymyositis/scleroderma (PM/Scl)<sup>1</sup> overlap syndrome particle and herein referred to as the human exosome, was reported to contain 11 (25) to 16 (26) subunits ranging from 20 to 110 kDa. Two proteins of this complex were identified as autoantigens, which are targeted by autoantibodies present in the serum of patients suffering from myositis, scleroderma, or PM/Scl overlap syndrome (27). All tested anti-PM/Scl-positive sera recognize a nuclear protein, known as PM/Scl-100, while some also recognize a protein migrating at about 75 kDa (PM/Scl-75) in SDS-polyacrylamide gel electrophoresis (28–30). PM/Scl-100 and -75 are the human homologues of yeast Rrp6p and Rrp45p, respectively (13, 31). The cloning of five additional human homologues of yeast exosome components has been reported, while ESTs were identified for three further homologues (11, 12, 32–34). However, detailed characterization of human exosome components is limited. The functional conservation has been reported for hRrp4p, hRrp44p/hDis3p, and hCsl4p, and direct evidence for complex formation has been described for hRrp4p and the PM/Scl autoantigens (11).

Here we report the cloning of the human homologues of yeast Rrp40p, Rrp41p (also designated Ski6p), and Rrp46p. Subcellular distribution, coimmunoprecipitation, and *in vivo* as well as *in vitro* activity assays show that these three proteins are components of the human exosome.

### EXPERIMENTAL PROCEDURES

*Isolation of hRrp40p, hRrp41p, and hRrp46p cDNAs*—Data base homology searches revealed human ESTs, which could be assembled into contigs with apparent homology to yeast exosome components (11). ESTs homologous to yeast Rrp40p (accession numbers AA747303, AA282142, H25417, and AA057832), Rrp41p/Ski6p (accession numbers U46288 and AA129848) and Rrp46p (accession numbers AA428915,

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF281132–AF281134.

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<sup>1</sup> The abbreviations used are: PM/Scl, polymyositis/scleroderma; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; VSV, vesicular stomatitis virus G epitope; EST, expressed sequence tag; GST, glutathione S-transferase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; contig, group of overlapping clones.

TABLE I  
Sequences of gene-specific oligonucleotides used in the cloning of hRrp40p, hRrp41p, and hRrp46p

Underlined sequences encode introduced restriction sites to enable cloning into the appropriate expression vectors. Start and stop codons are indicated in bold.

hRrp40-a	5'-CAACCACAACTGGCCATAGATG-3'
hRrp40-b	5'-GTCCACCCAGTAAACACCGCCGCG-3'
hRrp40-c	5'-CTGCCACTGCCGGGCTCCTTGTGAC-3'
hRrp40-5S	5'-CAGAATTCACATATGGCCGAACCTGCGTCTGTC-3'
hRrp40-3AS	5'-CTGGATCCGTCGACTCATCTAGAGGTACCCTTTCTGCCAATCTGGAGAAG-3'
hRrp41-b	5'-ATATTGACAGTTCCTAGGGCC-3'
hRrp41-c	5'-CTCGTGGCGGGCCGTAGACCACAGCC-3'
hRrp41-5S	5'-CTGAATTCACATATGGCGGGGCTGGAGCTTTG-3'
hRrp41-3AS	5'-GTGGATCCGTCGACTCATCTAGAGGTACCCTCCCCAGCAAGATAGAGGC-3'
hRrp46-a	5'-CAATCTTCGGCCTCAGGATCAC-3'
hRrp46-b	5'-GCGCAGGCGACCCACAGAAGAGAG-3'
hRrp46-c	5'-CCGCATGGGCACACCTGCATCCACC-3'
hRrp46-5S	5'-CTGAATTCACATATGGAGGAGGAGACGCATACT-3'
hRrp46-3AS	5'-CTGAATTCGTCGACTCATCTAGAGGTACCAGAGCTGCAGGCTCAAGGAG-3'

AA461395, and AA426493) were selected. Nucleotide sequences of five independent clones of each EST were determined by the dideoxynucleotide termination method. Complete (*hRRP41*) or partial (*hRRP40* and *hRRP46*) open reading frames (ORFs) were identified. To isolate additional cDNAs corresponding to sequences required to complete the ORFs, human teratocarcinoma and placenta cDNA libraries were screened by PCR using gene-specific primers for *hRRP40* and *hRRP46* (hRrp40-a or hRrp46-a, respectively; see Table I) in combination with  $\lambda$ LacZ library primers. The largest PCR products were identified by Southern blotting and cloned into the pCRII-TOPO vector (Invitrogen). Sequence analysis revealed putative start codons for both *hRRP40* and *hRRP46*. The cDNAs isolated from the teratocarcinoma cDNA library were ligated to the coding sequence present in the EST clones. To further investigate the 5' ends of the corresponding mRNAs, a 5' RACE analysis was performed using total human placenta RNA (Smart RACE cDNA amplification kit, CLONTECH). Gene specific primers used for the 5' RACE analysis were hRrp40-b, hRrp40-c, hRrp41-b, hRrp41-c, hRrp46-b, and hRrp46-c. Finally, primers (-5S and -3AS) were designed to introduce restriction sites at the termini of the isolated cDNAs to allow cloning into appropriate expression vectors (see below). The sequences of the oligonucleotides used in the cloning of *hRRP40*, *hRRP41*, and *hRRP46* are listed in Table I.

**Complementation Experiments**—To test for complementation of the yeast conditional alleles *GAL::rrp40*, *GAL::rrp41*, and *GAL::rrp46*, strains P147, P118, and YCA21 were transformed with plasmids pRRP40, pRRP41, and pRRP46, respectively (Table II). Complementation of the conditional alleles was assayed by growth on repressive YPD medium at 30 °C and compared with the growth of the isogenic wild-type strain (YDL401). Plasmids were constructed by inserting the cDNA of hRRP40, hRRP41, and hRRP46 into plasmid pNOPATA1L (CEN, LEU2) (generously provided by E. Hurt) under the control of a NOP1 promoter, at restriction sites *NdeI*-*Bam*HI for hRRP40 and hRRP41, and *NdeI*-*SaI*I for hRRP46.

**Expression of Recombinant Proteins**—For prokaryotic expression, the hRRP40, hRRP41, and hRRP46 cDNAs were cloned into pET116b and/or pGEX2T<sup>+</sup>G resulting in N-terminally His-tagged or glutathione S-transferase (GST)-tagged recombinant proteins, respectively. His-tagged proteins were purified by Ni<sup>2+</sup>-NTA-agarose beads (Qiagen) essentially according to the manufacturer's protocol. GST-tagged proteins were purified as described previously (35). In addition, recombinant N-terminally His-tagged hRrp40p and hRrp46p were expressed in and purified from SF-9 cells using the Bac-to-Bac baculovirus expression system according to the manufacturer's protocol (Life Technologies, Inc.).

**Generation of Rabbit Antisera**—Polyclonal antisera were raised in rabbits by immunization with 100–200  $\mu$ g of purified recombinant protein according to standard procedures (36). Serum H70 (anti-hRrp40p), H71 (anti-hRrp41p), and H73 (anti-hRrp46p) were generated using His-tagged hRrp40p expressed in *E. coli*, His-, and GST-tagged hRrp41p expressed in *E. coli* and His-tagged hRrp46p expressed in the baculovirus system, respectively.

**Western Blot Analysis**—For Western blot analysis, autoimmune patient and rabbit antisera were diluted 5000- and 500-fold, respectively, in blocking buffer (3% nonfat milk, phosphate-buffered saline (PBS), 0.1% Tween 20). As secondary antibody, horseradish peroxidase-conjugated rabbit anti-human IgG or swine anti-rabbit IgG (Dako Immunoglobulins) were used at a 2500-fold dilution in blocking buffer. Visualization was performed by chemiluminescence.

**Transient Transfection of HEP-2 Cells and Indirect Immunofluorescence**—For transfection, hRRP40, hRRP41, and hRRP46 cDNAs were cloned into the pCI-Neo vector (Promega), which contained a sequence element encoding the vesicular stomatitis virus G epitope (VSV) to allow expression of either N-terminally or C-terminally VSV-tagged proteins. HEP-2 cells were grown to 80% confluence by standard tissue culture techniques in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal calf serum. Approximately  $2 \times 10^6$  cells were transfected with 10–20  $\mu$ g of DNA in 800  $\mu$ l of Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum by electroporation, which was performed at 270 V and 950 microfarads using a Gene Pulser II (Bio-Rad). After transfection, cells were seeded onto coverslips and cultured for 24 h. Cells were washed twice with PBS, fixed with methanol for 5 min at -20 °C, briefly rinsed in acetone, air-dried, and stored at -20 °C until use.

For indirect immunofluorescence, fixed cells were subsequently incubated for 1 h at room temperature with an affinity-purified mouse anti-VSV tag monoclonal antibody (Roche Molecular Biochemicals) and subsequently with fluorescein isothiocyanate-conjugated rabbit anti-mouse Ig (Dako Immunoglobulins) and fluorescein isothiocyanate-conjugated swine anti-rabbit Ig (Dako Immunoglobulins). All antibodies were diluted 50-fold in PBS. Cells were mounted with PBS/glycerol containing Mowiol, and bound antibodies were visualized by fluorescence microscopy.

**Preparation of HeLa Cell Extracts**—Cytoplasmic and nuclear HeLa cell extracts were prepared according to a modification of the Dignam procedure essentially as described by Wahle *et al.* (37).

**Immunoprecipitation**—Immunoprecipitations were essentially performed as described previously (38). Per immunoprecipitation, 10  $\mu$ l of patient serum or 20  $\mu$ l of rabbit serum was coupled to 10  $\mu$ l of protein A-agarose (Biozym) and extract of  $2.5 \times 10^6$  HeLa cells was used. For Western blotting, the immunoprecipitates were heated for 5 min in SDS sample buffer and fractionated by SDS-polyacrylamide gel electrophoresis (39).

**Size Exclusion Chromatography**—Nuclear and cytoplasmic extract of  $75 \times 10^6$  HeLa cells was fractionated by automated liquid chromatography (BioLogic, Bio-Rad) using the Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech) in a buffer containing 10 mM Hepes-KOH, pH7.9, and 100 mM KCl at 0.5 ml/min. Thirty samples of ~330  $\mu$ l were collected, and proteins were immediately precipitated by the addition of four volumes of acetone. After overnight incubation at -70 °C, pellets were collected by centrifugation at  $13,000 \times g$  for 30 min. Air-dried pellets were solubilized in SDS sample buffer, and proteins were analyzed by Western blotting (20% of each fraction). To estimate complex sizes, gel filtration standards (Bio-Rad), including thyroglobin (670 kDa), bovine IgG (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa), and vitamin B<sub>12</sub> (1.35 kDa), were analyzed as described above.

**In Vitro Exonuclease Assay**—Immunoprecipitations were performed as described above. After removal of nonbinding material, immune complexes bound to the protein A-agarose beads were washed twice with buffer A (10 mM Tris-HCl, pH 7.5, 50 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM Na<sub>2</sub>HPO<sub>4</sub>). Substrate solution (<sup>32</sup>P-labeled substrate in buffer A) was added to the protein A beads, and the suspension was incubated at 37 °C with gentle agitation. Formamide loading buffer was added to samples taken at regular intervals and immediately frozen until analysis. Samples were analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. Substrates, transcribed *in vitro*



TABLE II  
Yeast strains used in this study

YDL401	<i>MATa his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108</i>	(40)
P147	As YDL401 but <i>GAL10::prot.A-RRP40</i>	(11)
P118	As YDL401 but <i>GAL10::prot.A-RRP41</i>	(40)
YCA21	As YDL401 but <i>GAL10::prot.A-RRP46</i>	(11)

by T3 RNA polymerase from *Xba*I-linearized pBS(-) template (Stratagene), were labeled randomly using [<sup>32</sup>P]UTP (Amersham Pharmacia Biotech) or at the 3' end using [<sup>32</sup>P]pCp (Amersham Pharmacia Biotech), respectively (38).

## RESULTS

**Cloning of Human Homologues of Yeast *Rrp40p*, *Rrp41p*/*Ski6p*, and *Rrp46p***—Full length cDNAs encoding human homologues of the *S. cerevisiae* *Rrp40p*, *Rrp41p*/*Ski6p*, and *Rrp46p* (open reading frames YOL142w, YGR195w, and YGR095c, respectively) were generated based upon EST clones, cDNA-specific PCR fragments isolated from a teratocarcinoma cDNA library, and 5' RACE products obtained from human placental RNA (Fig. 1). These sequence data have been submitted to the DDBJ/EMBL/GenBank<sup>®</sup> data bases under accession numbers AF281132, AF281133, and AF281134. From the cDNAs, hRrp40p, hRrp41p, and hRrp46p have predicted molecular masses of 31 kDa (275 amino acids), 27 kDa (245 amino acids), and 26 kDa (235 amino acids), respectively. The data in Table III show that the predicted protein sequences of hRrp40p, hRrp41p, and hRrp46p are relatively well conserved to the *S. cerevisiae* (Sc) homologues, and clear homologues are also present in *M. musculus* (Mm), *C. elegans* (Ce), and *S. pombe* (Sp). hRrp41p and hRrp46p are both homologous to *E. coli* RNase PH (11, 12, 31) and therefore show homology to each other, whereas hRrp40p is not significantly homologous to hRrp41p or hRrp46p.

Two genomic sequences for hRRP46 are present in the data base; a complete sequence on chromosome 19 and a partial sequence (accession no. L08634) corresponding to nucleotides -23 to +150 of the isolated hRRP46 cDNA clone. A T/C polymorphism is evident in these sequences, resulting in an amino acid substitution (methionine/threonine) at position 5 of hRrp46p. Both types of cDNA were obtained from the 5' RACE analyses, and ESTs exist with each nucleotide. In the studies described below, the cDNA clone encoding threonine at position 5 was used. Polymorphism was also evident in the region 5' to the ORF, with an in frame upstream stop codon present in L08634, but not in the chromosome 19 sequence. In the latter, the ORF could potentially extend 33 amino acids further 5'. However, no cDNA that extends beyond 23 nucleotides of the 5'-untranslated region was isolated and the extended ORF sequence would be in poorer agreement with the molecular weight predicted from the observed gel migration of the protein from HeLa cell lysates, compared with that synthesized in *E. coli*. Therefore, we believe that the cDNA used encodes the full-length protein. An EST with an internal truncation in the hRrp46p ORF was also found, presumably as a consequence of alternative splicing, that leads to a frameshift at Gly-85 and truncation of the polypeptide.

Two EST clones encoding a possible splicing variant of hRrp40p (accession nos. AA282142 and H25417) were identified, and the same sequence is listed as apoptosis-related protein PNAS-3 mRNA (accession no. AF229833). These lack an internal region of the ORF leading to frameshift at Gln-158 and truncation of the polypeptide.

***hRrp41p* Encodes a Functional Homologue of Yeast *Rrp41p*/*Ski6p***—To test whether hRrp40p, hRrp41p, or hRrp46p can complement mutations in the corresponding yeast genes, the full-length cDNAs were cloned into yeast expression vectors under the control of the strong constitutive *NOP1* promoter

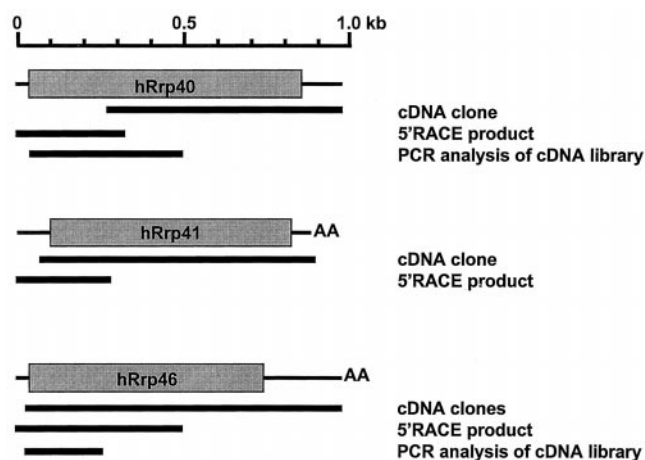


FIG. 1. Schematic representation of the cloning strategy used to generate the cDNAs encoding hRrp40p, hRrp41p, and hRrp46p. Sequencing of human EST clones, which were selected based upon homology with yeast *Rrp40p*, *Rrp41p*/*Ski6p*, and *Rrp46p* proteins, resulted in sequence information represented by the cDNA clones. Additional 5' RACE analyses and PCR analyses on cDNA libraries complemented the cDNA clones with additional 5' end sequences.

(see "Experimental Procedures"). The constructs were transformed into yeast strains in which the endogenous genes were subject to *GAL* regulation; strains *GAL::rrp40*, *GAL::rrp41*, and *GAL::rrp46*. Western blotting (data not shown) confirmed that each of the human proteins was well expressed. In plate assays, the growth inhibition of the *GAL::rrp41* strain on glucose medium was efficiently suppressed by expression of hRrp41p (Fig. 2), showing it to be the functional homologue of *Rrp41p*/*Ski6p*. The expression of hRrp40p or hRrp46p did not support the growth of *GAL::rrp40* or *GAL::rrp46* strains, respectively, indicating that the human proteins are unable to perform all of the essential functions of their yeast homologues.

**Detection of hRrp40p, hRrp41p, and hRrp46p in Human Cells**—The cloned cDNAs were expressed as His-tagged and/or GST-tagged polypeptides using the bacterial and baculovirus expression systems. In each case this resulted in the synthesis of proteins with gel mobilities close to those expected for the predicted molecular weights. The recombinant proteins were purified by Ni<sup>2+</sup> or glutathione affinity chromatography and used to raise rabbit antisera, designated H70 (anti-hRrp40p), H71 (anti-hRrp41p), and H73 (anti-hRrp46p). All sera recognized the corresponding recombinant His-tagged protein on Western blots (Fig. 3, A–C, lanes Ag). The reactivity of all antisera with all three recombinant proteins was analyzed by Western blotting. Purification of the baculovirus-expressed hRrp46p might, in principle, have led to the copurification of the endogenous insect exosome complex; moreover, hRrp46p and hRrp41p are homologous (see Table III). However, none of the anti-sera showed detectable cross-reactivity.

To demonstrate that hRrp40p, hRrp41p, and hRrp46p are expressed in HeLa cells, Western blots containing total HeLa cell extract were probed with the rabbit antisera (Fig. 3, A–C, lanes T). Although the sera recognized more than one protein in the total cell extract, prominent species (indicated by arrowheads) were decorated in each case that migrated somewhat faster than the corresponding recombinant His-tagged proteins

TABLE III

	Mm <sup>a</sup>	Ce <sup>b</sup>	Sp <sup>c</sup>	Sc <sup>d</sup>	hRrp40p	hRrp41p	hRrp46p
hRrp40p	88/91 <sup>e</sup>	34/56	31/50	30/46	100/100	12/26	18/28
hRrp41p	96/97	44/62	42/57	35/54	12/26	100/100	28/43
hRrp46p	89/93	31/46	29/46	26/42	18/28	28/43	100/100

<sup>a</sup> Polypeptide encoded by contig assembled with mouse (*M. musculus*) ESTs.

<sup>b</sup> *Caenorhabditis elegans* protein entries in data base (ceRrp40p: CAB01875, ceRrp40p: Q17533, ceRrp46p: CAA90109).

<sup>c</sup> *Schizosaccharomyces pombe* protein entries in data base (spRrp40p: CAB16582, spRrp41p: BAA13803, spRrp46p: CAA17913).

<sup>d</sup> *Saccharomyces cerevisiae* protein entries in data base (scRrp40p: NP\_01499, scRrp41p: NP\_011711, scRrp46p: NP\_011609).

<sup>e</sup> Values are percentages of identical and similar amino acid residues, respectively.

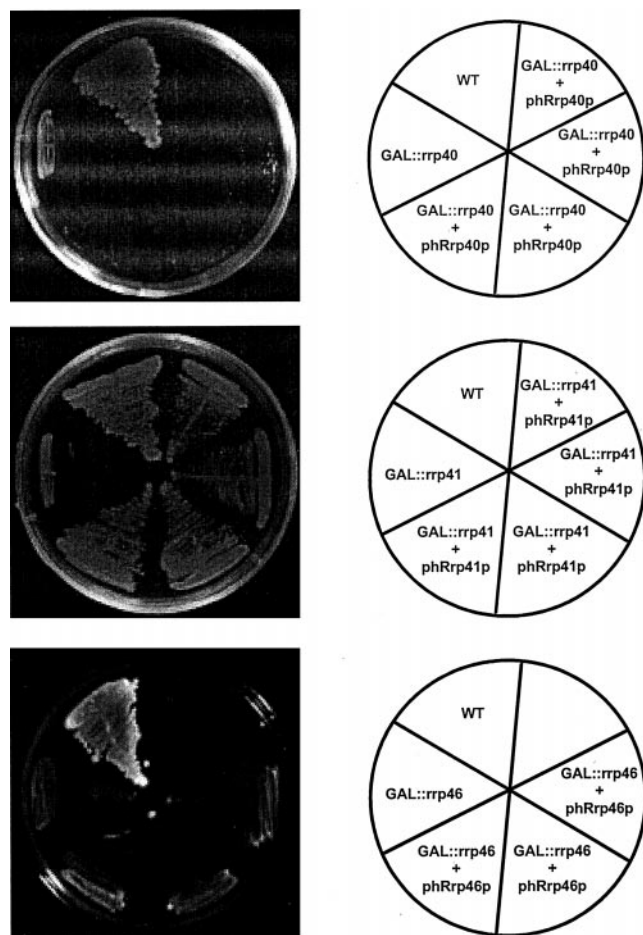


FIG. 2. Complementation of growth defects in yeast strain depleted of Rrp40p, Rrp41p/Ski6p, or Rrp46p by the human homologues. Figure shows plate assay for growth of GAL::Rrp40, GAL::Rrp41, and GAL::Rrp46 transformed with phRrp40p, phRrp41p, and phRrp46p, respectively, on repressive YPD medium. WT, wild-type.

(Fig. 3, A–C, compare lanes Ag and T). Immunoprecipitation experiments (see below) confirmed that these correspond to the hRrp40p, hRrp41p, and hRrp46p.

We conclude that hRrp40p, hRrp41p, and hRrp46p are present in HeLa cell lysates and show gel mobilities close to those expected for their predicted molecular weights.

**Subcellular Localization of hRrp40p, hRrp41p, and hRrp46p**—In yeast, 10 out of 11 known components are associated with both the cytoplasmic and the nuclear forms of the exosome, while Rrp6p is detected only in the nucleus (11). Western blotting experiments showed that hRrp4p and PM/Scl-75 are components of both nuclear and cytoplasmic complexes in human cells, while PM/Scl-100 (the homologue of Rrp6p) appears to be solely nuclear (11).

To determine the subcellular distribution of hRrp40p, hRrp41p, and hRrp46p, HeLa cells were separated into three fractions: cytoplasmic extract (C), nuclear extract (N), and the

nuclear material retained after salt extraction (R). The material of an equal number of cells ( $5 \times 10^5$  cells) from each fraction was used for Western blotting. As controls for the fractionation, we analyzed the largely cytoplasmic histidyl-tRNA synthetase (Jo-1, Fig. 3F), the nuclear DNA topoisomerase I (DNA Topo I, Fig. 3G), and the nuclear matrix-associated protein lamin B (Fig. 3H). The positions of migration are indicated by arrowheads. These results confirmed that the cells were fractionated as expected.

Western blots were probed with the rabbit sera H70 (Fig. 3A), H71 (Fig. 3B), and H73 (Fig. 3C). hRrp40p, hRrp41p, and hRrp46p were each detected in both the nuclear (N) and cytoplasmic (C) fraction, with lower yields in the cytoplasm (C). Weaker signals were seen in residual nuclear material after salt extraction (R). A similar distribution was found for hRrp4p (Fig. 3D). In contrast, PM/Scl-100 was recovered almost entirely in the nuclear fraction (Fig. 3E).

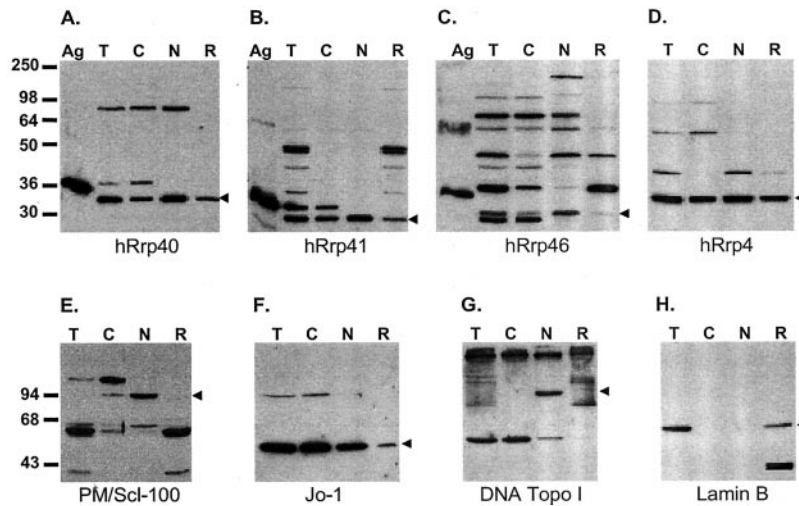
To confirm the subcellular localization of hRrp40p, hRrp41p, and hRrp46p, polypeptides carrying N-terminal and C-terminal VSV tags were expressed in HEP-2 cells. Indirect immunofluorescence of cells transfected with these constructs showed that all of these VSV-tagged proteins were strongly enriched in the nucleoli (Fig. 4). In addition, a weaker diffuse staining was widely distributed in the nucleoplasm and cytoplasm. Indirect immunofluorescence using the rabbit antisera H70 and H73 also resulted in prominent staining of the nucleoli of HEP-2 cells. Rabbit antiserum H71 did not give signals in indirect immunofluorescence (data not shown).

We conclude that hRrp40p, hRrp41p, and hRrp46p are present in both the cytoplasm and nucleus, with the highest concentration in the nucleolus.

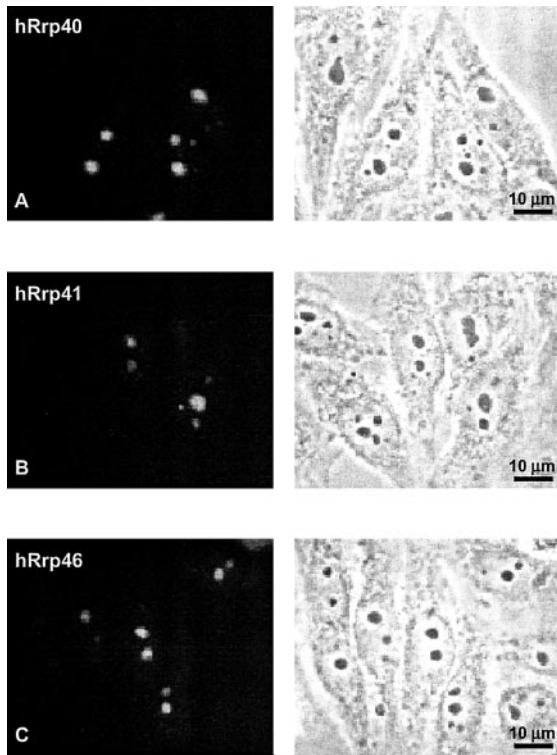
**hRrp40p, hRrp41p, and hRrp46p Are Part of the PM/Scl Complex**—The previously identified components of the human exosome (hRrp4p, PM/Scl-75, and PM/Scl-100) cosedimented in a large complex on glycerol gradient centrifugation of HeLa cell lysates (11). To determine whether hRrp40p, hRrp41p, and hRrp46p are associated with complexes of similar size, HeLa cell extracts were fractionated by size exclusion chromatography.

Cytoplasmic and nuclear extracts were prepared from  $75 \times 10^6$  cells and separately fractionated by chromatography on a Superdex 200 column. For each extract, 30 fractions were collected and analyzed by Western blotting with rabbit antisera H70, H71, H73, anti-hRrp4p, and anti-PM/Scl-100 (Fig. 5). These analyses revealed that hRrp40p, hRrp41p, and hRrp46p are associated with relatively large complexes in the cytoplasmic (Fig. 5A) and nuclear (Fig. 5B) extracts. All three proteins cofractionated with PM/Scl-100 and hRrp4p in both the cytoplasmic and nuclear extracts.

To estimate the size of the complexes, gel filtration standards were fractionated using the same conditions. In the cytoplasmic extract, the exosome components peaked in fractions three and four corresponding to a molecular mass of  $\sim 700$  kDa. In the nuclear extract, all exosome proteins analyzed except PM/Scl-100 showed a broader distribution over fractions 3–11, corresponding to estimated molecular masses of  $\sim 250$ –700 kDa.



**FIG. 3. Western blot analyses of fractionated HeLa cells to determine the subcellular distribution of exosome components.** HeLa cells were lysed by hypotonic Dounce homogenizer, nuclei were extracted with 0.3 M NaCl, and the retained nuclear material was recovered after sonification of the salt-extracted nuclei. Western blots containing total (T), cytoplasmic (C), nuclear (N), and retained nuclear (R) HeLa cell fractions, each obtained from an equal number of cells, were probed with rabbit antisera reactive with hRrp40p (panel A), hRrp41p (panel B), hRrp46p (panel C), hRrp4p (panel D), or PM/Sc1-100 (panel E). Western blots were also decorated with autoimmune patient sera reactive with histidyl-tRNA synthetase (Jo-1, panel F), with DNA topoisomerase I (panel G), or with monoclonal antibodies reactive with lamin B (panel H). The relevant proteins are indicated by arrowheads. Western blots in panels A–C also contain the corresponding recombinant His-tagged proteins (Ag lanes).



**FIG. 4. Nucleolar accumulation of the VSV-tagged hRrp40p, hRrp41p, and hRrp46p in HEp-2 cells.** HEp-2 cells were transfected with VSV-tagged constructs by electroporation and cultured for 24 h at 37 °C. The localization of C-terminally VSV-tagged hRrp40p (A), hRrp41p (B), and hRrp46p (C) was determined by indirect immunofluorescence using a monoclonal anti-VSV tag antibody (left panels). The corresponding phase-contrast images are shown on the right.

The peak of PM/Sc1-100 was limited to fractions 3 and 4, suggesting association with only the higher molecular weight complexes. Similar results were obtained following fractionation of nuclear extracts by glycerol gradient centrifugation, which showed cosedimentation of hRrp40p and hRrp41p with hRrp4p and the PM/Sc1 autoantigens (data not shown).

Coimmunoprecipitation experiments were performed to con-

firm the physical association of hRrp40p, hRrp41p, and hRrp46p with the known human exosome components. Five anti-PM/Sc1-positive patient sera were used to immunoprecipitate the exosome complex from a HeLa cell nuclear extract; three anti-PM/Sc1-negative sera served as controls (Fig. 6A). The immunoprecipitates were analyzed by Western blotting using the rabbit antisera H70, H71, and H73. hRrp40, hRrp41, and hRrp46 were each immunoprecipitated by the anti-PM/Sc1-positive sera, but not by the control sera. Although the rabbit antisera showed reactivity with several proteins present in the total nuclear extract (Fig. 6A, lanes i), each rabbit antiserum consistently stained only one protein in the immunoprecipitates. In each case the gel mobility corresponded with the predicted molecular weight, confirming that these represent the cognate proteins.

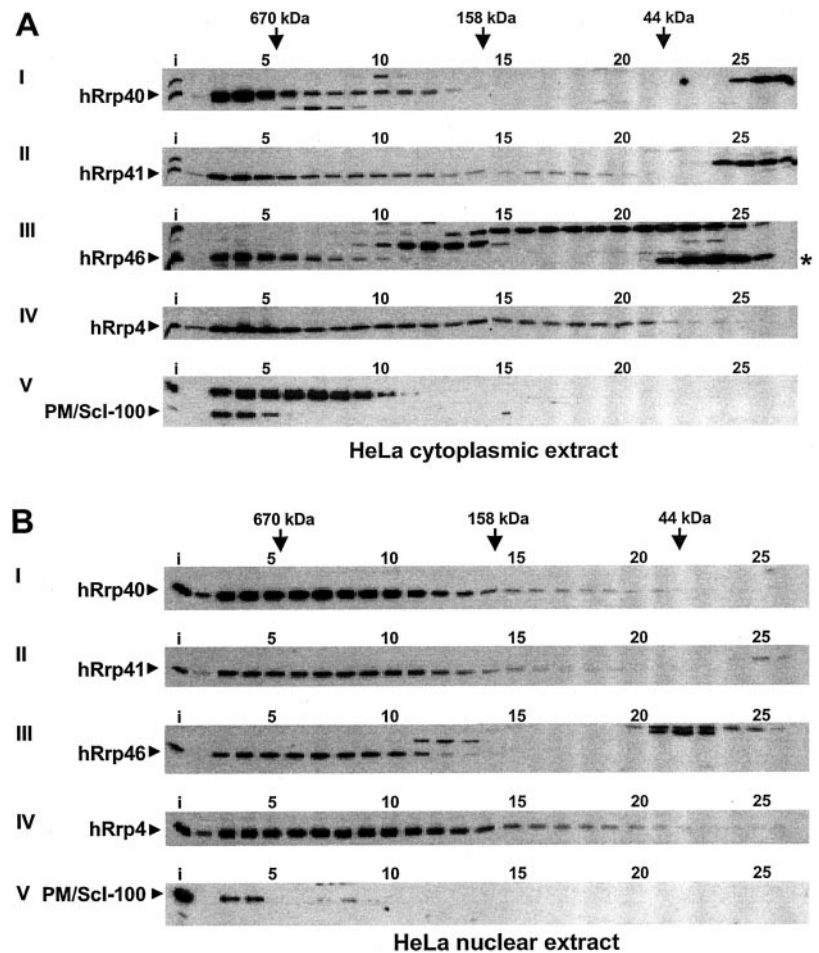
In the converse experiments, sera H70, H71, and H73 were used for immunoprecipitation and the coprecipitation of PM/Sc1 autoantigens was detected with two anti-PM/Sc1 patient sera, Lun7 and Lun36. Sera H70 and H73 each coimmunoprecipitated PM/Sc1-100 from a nuclear HeLa cell extract (Fig. 6B, right panel). In addition, both patient sera stained several smaller proteins that were immunoprecipitated by H70 and H73 from the nuclear extract, which may represent other components of the PM/Sc1 particle. Neither pre-immune sera (PI lanes) precipitated the PM/Sc1-100 autoantigen or any of the smaller proteins. PM/Sc1-100 was also coimmunoprecipitated at low levels from a cytoplasmic extract by sera H70 and H73 (Fig. 6B, left panels). Serum H71 failed to precipitate PM/Sc1-100 (Fig. 6B) and also failed to decorate the nucleoli of HEp-2 cells in immunofluorescence microscopy (data not shown). This may be due either to inaccessibility of hRrp41p in the complex or to the inability of the antibodies to recognize native hRrp41p.

We conclude that hRrp40p, hRrp41p, and hRrp46p are present in a complex containing the known human exosome components, PM/Sc1-100 and hRrp4p.

**Exoribonuclease Activity of the Complexes Containing hRrp40p, hRrp41p, and hRrp46p**—The human exosome complex was immunoprecipitated from a HeLa cell extract using rabbit antisera H70, H71, and H73 or anti-PM/Sc1-positive patient serum R212. The pre-immune rabbit sera and a pool of



FIG. 5. **hRrp40p, hRrp41p, and hRrp46p are associated with high molecular weight complexes in HeLa cells.** A, cytoplasmic extract. B, salt-extractable nuclear extract. Lysates (from an equal number of cells) were fractionated by size exclusion chromatography and fractions 2–27 were analyzed by Western blotting using rabbit antisera specific for hRrp40p (I), hRrp41p (II), hRrp46p (III), hRrp4p (IV), and PM/Scl-100 (V). In the left lanes (i), total cytoplasmic or nuclear extract was loaded. Note that serum H73 is also reactive with a cytoplasmic polypeptide that migrates slightly faster than hRrp46p (see also Fig. 3C), but fractionated only in the lower molecular weight fractions (panel A, III, indicated by \*).



10 normal human sera served as negative controls. Associated exoribonuclease activity was assayed *in vitro* with an internally labeled 37-nucleotide single-stranded RNA substrate. The immunoprecipitates obtained with rabbit antisera H70 and H73 and patient serum R212 exhibited ribonuclease activity, with progressive disappearance of the RNA substrate and the accumulation of the labeled end-product (Fig. 7, left panel, lanes 2–4 and 8–13). Analysis of the reaction products by thin layer chromatography revealed that the accumulating end products are nucleoside monophosphates consistent with exonucleolytic degradation of the substrate (data not shown). Omission of phosphate from the buffer used in this assay, which has been shown to affect the activity of yeast Rrp41p/Ski6p (12), did not significantly inhibit the reaction (data not shown). Immunoprecipitates obtained with rabbit antiserum H71 (Fig. 7, left panel, lanes 5–7) and the control sera (Fig. 7, right panel) did not show nucleolytic activities. The similar patterns of reaction products seen with H70 and H73 indicate that the complexes immunoprecipitated by each serum are related.

When the substrate was 3'-labeled with  $^{32}\text{P}$ , incubation with the immunoprecipitates obtained with sera H70, H73, or R212 resulted in the disappearance of the RNA substrate without detectable intermediate products. We conclude that the immunoprecipitated complexes exhibit 3'  $\rightarrow$  5' exonuclease activity. Removal of the 3' phosphate group, inherently introduced by the [ $^{32}\text{P}$ ]pCp labeling procedure, resulted in a slightly enhanced reaction (data not shown) indicating a preference for a 3'-hydroxyl residue, as seen for the yeast exosome (12).

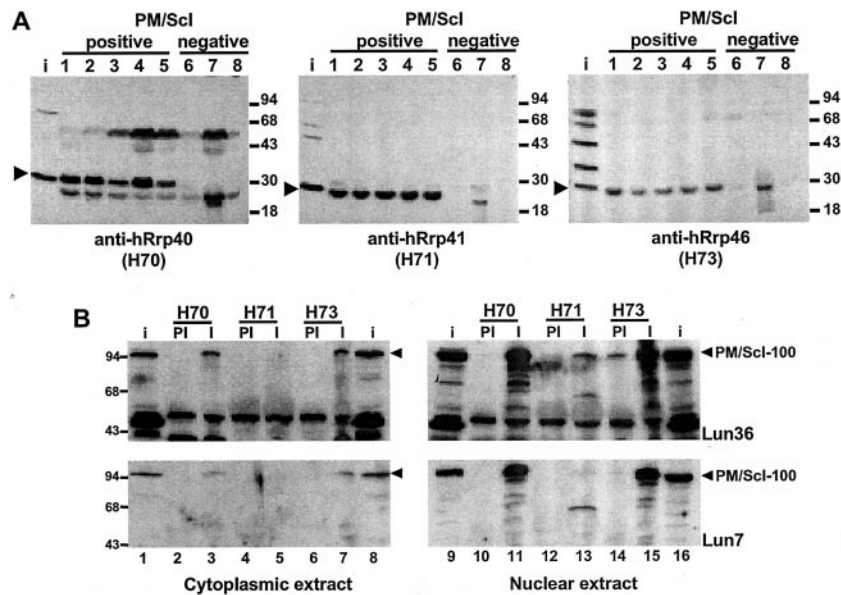
These results show that hRrp40 and hRrp46 are associated with a complex displaying 3'  $\rightarrow$  5' exoribonuclease activity *in*

*vitro*. We conclude that these proteins are indeed novel human exosome components.

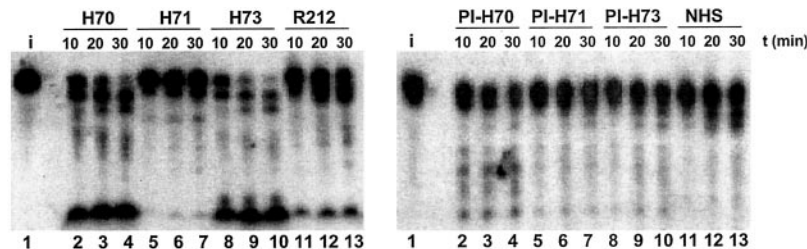
#### DISCUSSION

We have characterized three novel human polypeptides, hRrp40p, hRrp41p, and hRrp46p, encoded by cDNAs that were isolated on the basis of homology to the yeast exosome components Rrp40p, Rrp41p/Ski6p, and Rrp46p, respectively (11). Western blotting experiments using rabbit antisera raised against each of the recombinant proteins showed that HeLa cells express proteins of the predicted molecular weights. Consistent with data previously obtained for human exosome components, all three proteins are present in both the cytoplasm and nucleus, with nucleolar enrichment. The novel proteins were shown to be part of a large complex cofractionating with hRrp4p and PM/Scl-100. The physical association with the PM/Scl autoantigens was confirmed by coimmunoprecipitation. Functional assays demonstrated that the complexes containing hRrp40p and hRrp46p display 3'  $\rightarrow$  5' exoribonuclease activity *in vitro*. Moreover, expression of hRrp41p in yeast is able to support the growth of cells depleted of yeast Rrp41p/Ski6p. We conclude that hRrp40p, hRrp41p, and hRrp46p are novel components of the human exosome complex.

**Subcellular Localization of hRrp40p, hRrp41p and hRrp46p**—Consistent with previous data using anti-PM/Scl positive patient sera, hRrp40p, hRrp41p, and hRrp46p were shown by indirect immunofluorescence to be enriched in the nucleolus. However, subcellular fractionation showed that hRrp40p, hRrp41p, and hRrp46p are present in both nuclear and cytoplasmic fractions, as is hRrp4p (11). Salt extraction released most of the nuclear hRrp40p, hRrp41p, and hRrp46p,



**FIG. 6. hRrp40p, hRrp41p, and hRrp46p are physically associated with the human exosome.** *A*, coimmunoprecipitation experiments were performed using five anti-PM/Scl-positive patient sera (lanes 1–5) and three anti-PM/Scl-negative patient sera (lanes 6–8). Immunoprecipitated material was analyzed by Western blotting using the rabbit antisera raised against hRrp40p (H70), hRrp41p (H71), or hRrp46p (H73) to detect coimmunoprecipitation of these proteins. In lanes *i*, total nuclear extract (30%) used for immunoprecipitation was loaded. *B*, Western blot analyses of the converse experiment in which the preimmune (PI, lanes 2, 4, 6, 10, 12, and 14) and immune (I, lanes 3, 5, 7, 11, 13, and 15) rabbit antisera were used for the immunoprecipitations and two anti-PM/Scl-positive patient sera, Lun7 (lower panels) and Lun36 (upper panels), were used for detection. Cytoplasmic (lanes 1–8) and salt-extractable nuclear (lanes 9–16) HeLa cell extracts were used for immunoprecipitations. Total cytoplasmic and nuclear extract was loaded in lanes *i* (lanes 1 and 8 (cytoplasmic) and lanes 9 and 16 (nuclear)). Arrowheads indicate the relevant proteins.



**FIG. 7. hRrp40p- and hRrp46p-containing complexes display ribonuclease activity *in vitro*.** Immunoprecipitated hRrp40p, hRrp41p, hRrp46p, and PM/Scl antigens containing complexes from HeLa cells were assayed for exoribonuclease activity *in vitro*. A uniformly  $^{32}\text{P}$ -labeled RNA substrate was incubated with the immunoprecipitates of anti-hRrp40p (H70), anti-hRrp41p (H71), and anti-hRrp46p (H73) rabbit sera or an anti-PM/Scl-positive patient serum (R212). Samples taken after 10, 20, and 30 min of incubation at 37 °C were analyzed by 10% denaturing polyacrylamide gel electrophoresis followed by autoradiography (left panel). Control experiments (right panel) were performed using the immunoprecipitates of the corresponding preimmune rabbit antisera (PI-H70, PI-H71, and PI-H73) or a pool of ten normal human sera (NHS). In the left lanes (*i*), input RNA substrate was loaded.

but a substantial amount of each protein was retained. The release of DNA topoisomerase I indicated that the high salt extraction was efficient. We speculate that the extracted and retained fractions represent nucleoplasmic and nucleolar pools of the exosome, respectively. Diffusely distributed nucleoplasmic and cytoplasmic populations are presumably less visible in immunofluorescence than is the nucleolar population.

The distribution of the human exosome components is similar to that previously seen for yeast Rrp4p and Rrp43p (11, 40). The yeast exosome is implicated in RNA processing reactions in the nucleolus (pre-rRNA processing and spacer degradation), nucleoplasm (pre-small nucleolar RNA and pre-small nuclear RNA processing and pre-mRNA degradation) and cytoplasm (mRNA degradation) (12–23), and the distribution observed for exosome components presumably reflects these functions. The similarities in the distribution patterns, and the complementation of yeast mutants by the human proteins, make it likely that the human exosome will carry out many or all of the same functions.

The yeast exosome is implicated in the degradation of unspliced pre-mRNAs (18, 22) and might therefore influence the

outcome of alternative splicing events. The identification of cDNAs that apparently result from alternative splicing of *hRRP40* and *hRRP46* therefore raises interesting possibilities for autogenous regulation.

**Characterization of the Complex Containing hRrp40p, hRrp41p, and hRrp46p**—Immunoprecipitation with anti-PM/Scl-positive patient sera, using metabolically labeled human cell extracts, indicated that the PM/Scl complex consists of at least 11 proteins (25, 26). The estimated molecular masses of the proteins found in these studies were 110, 90, 80, 39, 37, 33, 30, 27, 26, 22, and 20 kDa. Comparison of these molecular masses with those of the proteins characterized in the present study suggested that hRrp40, hRrp41 and hRrp46 correspond to the 30-, 27-, and 26-kDa proteins, respectively.

Size exclusion chromatography of the exosome complex in a HeLa cell extract, indicated that the cytoplasmic complex has a molecular mass of ~700 kDa. The nuclear complex gave a broad distribution between 250 and 700 kDa. These complex sizes were estimated based upon the separation of gel filtration standards. However, this fractionation may not be dependent solely on the mass of the complex, but may also reflect its



structure relative to the protein size markers. It is also unclear whether the size distribution of the nuclear exosome reflects the existence of multiple, heterogeneous complexes or whether it is due to instability of the complex. The size of the human exosome complex estimated by size exclusion chromatography was larger than previously estimated from glycerol gradient centrifugation (12), presumably reflecting differences in the physical basis of the separation techniques.

The complexes immunoprecipitated with anti-hRrp40p (H70) or anti-hRrp46p (H73) showed very similar *in vitro* activities. In each case, both processive and distributive activities are suggested by the data. A distributive exonuclease activity removes one (or a few) nucleotide(s) before dissociating from the substrate. In consequence, its activity is "distributed" over the entire substrate population, which is therefore progressively shortened in approximate synchrony. Such an activity would be consistent with the shortening of the RNA population near the top of the gel in the H70 and, particularly, H73 lanes in Fig. 7 (lanes 2–4 and 8–10, respectively). In contrast, binding of a processive exonuclease to the a molecule of substrate results in its rapid degradation to a short residual fragment, at which point the RNA is too short for the enzyme to bind. In consequence, a fraction of the substrate is rapidly shortened, while most of the substrate is untouched. Such an activity may be seen in the early time points of using the H70 and H73 immunoprecipitates shown in Fig. 7.

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### **Three Novel Components of the Human Exosome**

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