Published online 30 January 2007 *Nucleic Acids Research, 2007, Vol. 35, No. 5* ***1391–1401***
doi:10.1093/nar/gkl1144

Proteomic analysis of the U1 snRNP of *Schizosaccharomyces pombe* reveals three essential organism-specific proteins

Alain N. S. Newo[1], Martin Lützelberger[1], Claudia A. Bottner[1], Jürgen Wehland[2], Josef Wissing[2], Lothar Jänsch[2] and Norbert F. Käufer[1,*]

[1]Institute of Genetics, Technical University of Braunschweig, Spielmannstr. 7, 38106 Braunschweig, Germany and [2]Helmholtz Center for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany

Received May 21, 2006; Revised and Accepted December 15, 2006

ABSTRACT

Characterization of spliceosomal complexes in the fission yeast *Schizosaccharomyces pombe* revealed particles sedimenting in the range of 30–60S, exclusively containing U1 snRNA. Here, we report the tandem affinity purification (TAP) of U1-specific protein complexes. The components of the complexes were identified using (LC-MS/MS) mass spectrometry. The fission yeast U1 snRNP contains 16 proteins, including the 7 Sm snRNP core proteins. In both fission and budding yeast, the U1 snRNP contains 9 and 10 U1 specific proteins, respectively, whereas the U1 particle found in mammalian cells contains only 3. Among the U1-specific proteins in *S. pombe*, three are homolog to the mammalian and six to the budding yeast *Saccharomyces cerevisiae* U1-specific proteins, whereas three, called U1H, U1J and U1L, are proteins specific to *S. pombe*. Furthermore, we demonstrate that the homolog of U1-70K and the three proteins specific to *S. pombe* are essential for growth. We will discuss the differences between the U1 snRNPs with respect to the organism-specific proteins found in the two yeasts and the resulting effect it has on pre-mRNA splicing.

INTRODUCTION

Introns are removed by a highly dynamic multicomponent ribonucleoprotein complex, called the spliceosome. A splicing-competent spliceosome consists of the five snRNPs U1, U2, U5, basepaired U4/6 and many associated proteins, which when activated undergoes conformational changes to catalyze the two transesterification reactions in an snRNP particle consisting of U2, U5 and U6 (1–3). Several studies indicate that pre-mRNA splicing *in vivo* is coupled to transcription. This requires that the assembly of competent spliceosomes be coordinated with transcription (4–6). It is still under debate whether spliceosome assembly *in vivo* proceeds in a strictly ordered stepwise manner by recruiting individual splicing components on to the nascent pre-mRNAs, as suggested for spliceosome assembly *in vitro*, or whether pre-assembled splicing-competent spliceosomes are recruited to introns (1,7). In *Saccharomyces cerevisiae*, the U1 snRNP contacts introns first *in vitro* and *in vivo*, thus playing an important role in intron recognition (4,5,8).

There are very few examples of regulated splicing in *S. cerevisiae* and *S. pombe*, and the size of introns is considerably smaller than in mammalian genes. The removal of introns in the two yeasts appears to be mainly constitutive (9). However, with respect to splicing regulation, major differences exist between these organisms. In *S. pombe*, the phosphorylation of the spliceosomal protein Prp1p by Prp4p kinase is involved in the activation of spliceosomes *in vivo*, whereas for *S. cerevisiae*, which does not contain a counterpart of Prp4p kinase, it is suggested that a spliceosome undergoes the ordered series of interactions and allosteric conformational changes as soon as a splicing-competent spliceosome is fully assembled on an intron (9–12). Interestingly, almost all eukaryotic organisms whose genome is known contain a counterpart of Prp4 kinase, with the exception of the hemiascomycetes to which *S. cerevisiae* belongs (9,13,14).

By employing tandem affinity purification (TAP), a large complex was purified from *S. pombe* containing Prp1p, Prp31p and other spliceosomal proteins, indicating that a tetra-snRNP particle containing U2, U4, U5 and U6 may exist *in vivo* (15). These large complexes associated with the spliceosomal proteins Prp1p and Prp31 seem to be a mixture of spliceosomal particles containing U2, U5 and basepaired U4/U6

*To whom correspondence should be addressed. Tel: +49 531 391 5774; Fax: +49 531 391 5765; Email: n.kaeufer@tu-bs.de

(tetra-snRNP) and splicing-competent spliceosomes containing U2, U5, U4/U6 and U1 (penta-snRNP, 10). Another *in-vivo*-derived complex from *S. pombe* was purified and analyzed using proteomics. This complex lacks Prp1p and Prp31p, but contains 27 splicing factors and the snRNAs U2, U5 and U6, indicating that these complexes might be a mixture of activated and post-catalytic spliceosomes (16).

The U1 snRNP of *S. cerevisiae* has been extensively characterized since it differs in many ways from that found in mammals. The U1 snRNA of *S. cerevisiae* is 568 nucleotides long, whereas human snRNA U1 counts 164 nucleotides (22). Ten proteins associated with the U1 snRNA are highly conserved from yeast to man, including the heteroheptameric Sm proteins binding to the Sm site, and three U1 specific proteins called U1-70K, U1A and U1C (18,19). The U1 snRNP of *Trypanosoma brucei* contains a U1-70K and U1C related protein, but instead of U1A a protein called U1-24K was identified (17). The U1 particle of *S. cerevisiae* is associated with seven additional proteins, which are essential for function in pre-mRNA splicing (20,21). As mentioned before, the U1 particle plays a major role in intron recognition. It has been suggested that one reason for the greater complexity of the budding yeast U1 particle might be the unique architecture of the introns in *S. cerevisiae*. *S. cerevisiae* contains only 256 introns in 5% of its genes, which are between 35 and 600 bp in size (mean size 264), and their splicing consensus sequences are highly conserved. In contrast, mammalian introns show an enormous variety in architecture and vary in size from 35 bp to thousands of kilobases. The splicing consensus sequences in mammalian introns are less highly conserved, and more than 70% of the genes are alternatively spliced (23). This implicates that in mammalia a much more versatile set of proteins might be recruited and transiently interacting with the basic U1 particle early in the splicing process, depending on features of the intron exon context that needs to be recognized (21).

The *S. pombe* snRNA U1 is 148 nucleotides long, and RNA folding analysis suggest that its secondary structure is similar to the human U1 snRNA (24,25). *S. pombe* contains 4730 introns, and 43% of its genes display introns. The introns are very short (mean size 81 bp), and the splicing consensus sequences are much less conserved than in *S. cerevisiae* (9,26). A comparison of the splicing factors of *S. pombe* with *S. cerevisiae* and mammals revealed that the *S. pombe* genome contains homologs of U1-70K, U1A, U1C and of seven Sm proteins. In addition, homologs of three U1-specific proteins of *S. cerevisiae*, called Prp39p, Prp40p and Luc7p, have been identified. Homologs of the other four *S. cerevisiae*-specific U1 proteins Snu71p, Prp42p, Snu56p and Nam8p have not been identified, however (27).

In order to further characterize spliceosomal complexes existing *in vivo* in *S. pombe*, we fused the U1-specific protein U1-70K with TAP-tag and HA-tag, respectively, and analysed the molecular complexes identified with this approach. Much to our surprise, we discovered particles sedimenting in the range of 30–60S, containing exclusively U1 snRNA.

Using the TAP method and mass spectrometric analyses, we identified in these complexes the homologs of U1-70K, U1A, U1C and the seven Sm homologs. We also identified the homologs of the *S. cerevisiae* U1-specific proteins Prp39p, Prp40p and Luc7p. In addition, we found three *S. pombe*-specific proteins U1H (Usp107p), UIJ (Usp108p) and U1L (Usp109p) associated with U1 snRNP. We show that the genes encoding U1-70K and the three *S. pombe*-specific proteins are essential for growth.

MATERIALS AND METHODS

Strains, media, gene disruption, *in vivo* tagging

Standard genetic and molecular techniques were used as described previously (10). Strains were crossed and grown in media described by Gutz *et al.* (28). Genes were disrupted by flanking the *ura4*$^+$ gene with 300 bp both upstream and downstream of the ORFs. These constructs were transformed into diploid *S. pombe* cells lacking the *ura4*$^+$ gene and stable uracil prototrophic colonies were selected (29). Prototrophic transformants were checked for gene disruption by PCR. Heterozygous diploids were sporulated at 25°C, and tetrads were dissected using a Singer MSM micromanipulator (Singer Instruments, UK). Strains expressing epitop tagged versions of Usp101p (U1-70K), Usp107p (U1H), Usp108 (U1J) and Usp109p (U1L) were constructed by introducing the *HA3-Kan*^{r} or *TAP-Kan*^{r} cassette by homologous recombination at the 3′ end of the endogenous locus.

To construct a Myc-tagged version of *usp101*, we joined in a pUC18 vector three PCR fragments comprising the promoter region of *usp101*, a cassette comprising three Myc-epitopes and a fragment containing the ORF and 3′ UTR. We also inserted the complete *leu1*$^+$ gene in this vector. For integration via homologous recombination into the *leu1* locus, the plasmid was linearized in the *leu1* gene and transformed into a strain auxotroph for leucine containing the *leu1-32* allele and expressing HA-U170K from its authentic locus. Proper integration into the *leu1* locus was checked by PCR. Expression of epitope-tagged proteins were confirmed by immunoblotting using the appropriate antibody (30).

Protein extracts

Proteins were extracted as described by Bottner *et al.* (10). Briefly, cells were harvested, washed in STOP buffer (150 mM NaCl; 50 mM NaF; 10 mM EGTA; 1 mM NaN_3 [pH 8.0]) resuspended in HB buffer (25 mM MOPS [pH 7.2]; 15 mM EGTA; 15 mM $MgCl_2$; 50 mM NaCl; 60 mM β-glycerolphosphat; 1 mM DTT; 0.1 mM $NaVO_3$; 0.0125 g/l proteolysis inhibitor [BIOMOL]) and broken with glass beads. After centrifugation (13 800×g at 4°C for 15 min), the supernatant was frozen in liquid nitrogen and stored at −80°C until use.

Glycerol gradient centrifugation

Five milligrams of total protein extract was loaded onto an 11 ml 10–30% glycerol gradient in HB buffer, and spun

at 24 500 rpm for 15 h at 4°C in an SW41 rotor (Beckman). Nineteen 500 µl fractions were collected manually from top to bottom. Ten microliters of each fraction was subjected to solution hybridisation for snRNA analysis. 30S and 50S ribosomal subunits from *E. coli* were used to calibrate the gradients.

Solution Hybridisation of snRNA

Spliceosomal snRNA was detected by a solution hybridization developed by Li and Brow (31). The samples were incubated in 50 ml SHS buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 0.1% SDS, 1 mg/ml proteinase K) for 30 min (37°C) and extracted with phenol/chloroform/isoamyl alcohol (50:48:2). The RNA was hybridized with 0.1 pmol ^{32}P labeled oligonucleotides complementary (c) to U1, U2, U4, U5 and U6 for 30 min (37°C). After adding of 6× loading buffer (50% glycerol, 10 mM EDTA [pH8.0], 0.25% bromophenol blue, 0.25% xylene cyanol), RNA was separated on a native polyacrylamide gel (9%; 30:1) in 0.5 × TBE by 5 W and 4°C.

Oligonucleotides used: cU1 (1–26) GCTGCAGAAAC TCATGCCAGGTAAGT; cU2(35–55) GAACAGATAC TACACTTGATC; cU4 (71–92) GTTGGAGCGGTCA GGGTAATAG; cU5 (77–107) GATTACAAAAACTAT ACAGTCAAATTAGCAC; cU6 (18–40) CTCTGTAT CGTTTCAATTTGACC.

Northern analysis of snRNA

For Northern analysis, RNA was separated on a denaturing polyacrylamid gel (16%;19:1; 8 M urea) in 0.5 × TBE. After electrophoresis, the gel was stained with ethidium bromide. After staining, the RNA was transferred on to nitrocellulose. Random priming was used for labeling the probes with ^{32}P. *snu1* (U1), geneDBno. SPSNRNA.01; *snu2* (U2) SPSNRNA.02; *snu4* (U4) SPSNRNA.04; *snu5* (U5), SPSNRNA.05; *snu6* (U6), SPSNRNA.06 were subcloned into pUC19 after amplifying the complete genes by PCR using chromosomal DNA as template.

Immunoprecipitation

For immunoprecipitation assays, 5 µg of monoclonal antibodies (HA.11; BAbCO) bound to proteinA sepharose (Sigma-Aldrich) was incubated with 500 µl of each gradient fraction at 4°C for 2–16 h. The immunoprecipitates were washed two times with 1 ml buffer containing 0.15 M NaCl; 0.01 M Tris HCl [pH 7.5]; 0.05% NP40 at 4°C. For immunoblotting, 50% of the immunoprecipitates were separated on an SDS-PAGE and transferred onto Hybond-N membrane (Schleicher und Schuell) by electroblotting, according to the manufacturer's instructions. After blocking with 10% nonfat milk powder in TBS, the membranes were incubated with HA-antibody (mouse; BAbCO) and, after washing two times with TBS, the membranes were incubated with the HRP-labeled anti-mouse antibody (Amersham Life-science). Protein bands were visualized with the ECL kit (Amersham Life-science) according to the manufacturer's instructions. To detect snRNA, 50% of the immunoprecipitates were incubated in 50 µl SHS buffer, as described above.

Purification of Usp101p, Usp107p, Usp108, Usp109 complexes by TAP

We used, with some modification, the protocol for the TAP-method described by Gould *et al.* (32). 2 or 8 l cultures of strains containing *usp101-TAP*, *usp107-TAP, usp108-TAP* or *usp109-TAP* were grown at 25°C and harvested in mid-log phase (1.2×10^7–1.5×10^7 cells/ml, $OD_{600} = 0.6$–0.8). The cell pellet was frozen in liquid nitrogen and stored at −80°C until use. As a control, a wild type strain (L972) was cultivated under the same conditions.

The cells were lysed in a bead beater (BioSpec Products) in 100 ml NP-40 buffer (6 mM Na_2HPO_4, 4 mM NaH_2PO_4 H_2O, 1% NONIDET P-40, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 4 µg/ml leupeptin, 0.1 mM $NaVO_3$) containing EDTA-free protease inhibitors (Boehringer Mannheim), 1.3 mM of benzamidin and 1 mM PMSF. The glass beads were extracted with an additional 50 ml of NP-40 buffer containing protease inhibitors. The lysate was cleared by centrifugation at 16 000 rpm and 4°C for 40 min, frozen in liquid nitrogen and stored at −80°C until use.

The protein extract was added to 400 µl bed volume of IgG-sepharose beads (Pharmacia) equilibrated in NP-40 buffer and incubated by rotation at 4°C for at least 4 h. The beads were washed four times with 10 ml of IPP150 (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP-40) and three times with 1 ml of TEV cleavage buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP-40, 0.5 mM EDTA, 1.0 mM DTT). After the last wash, the beads were resuspended in 300 µl of TEV cleavage buffer. Then 50 to 100 units TEV protease (Invitrogen) were added and incubated by rotation at 16°C for 2 to 3 h or at 4°C overnight. The TEV protease eluate was collected. The beads were washed three times in 300 µl calmodulin binding buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM magnesiumacetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.1% NP-40, 10 mM β-mercaptoethanol). The supernatants were added to the TEV protease eluate. $CaCl_2$ (4.8 µl; 1 M) was added to quench the EDTA of the cleavage buffer. Then, 300 µl bed volume of calmodulin affinity resin (Stratagene) equilibrated in calmodulin binding buffer were added and incubated by rotation at 4°C for 2–4 h. The resin was collected and washed six times with 1 ml of calmodulin binding buffer. The resin bound complexes were eluted either under denaturing conditions by resuspending the resin in 100–200 µl × 2 SDS-PAGE sample buffer (100 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 200 mM β-mercaptoethanol, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerin), or under native conditions by resuspending the resin in 200 µl of calmodulin elution buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM magnesiumacetate, 1 mM imidazole, 2 mM $CaCl_2$, 0.02% NP-40, 20 mM EGTA, 10 mM β-mercaptoethanol) and incubating the suspension by rotation for 15 min at room temperature. The elution procedure was repeated 4× using 200 µl elution buffer each time. The protein complex eluted was separated by SDS-PAGE, and proteins were visualized. For mass spectrometry, the protein complex was separated by Tricine-SDS-PAGE as

described by Schägger and von Jagow (33), and proteins were stained with the fluorescent dye ruthenium (II) tris-bathophenanthrolin disulphonate (RuBPS) according to Rabilloud (34) or coomassie blue. The protein complex eluted under native conditions was precipitated with TCA and washed twice with cold (−20°C) acetone.

Characterization of Usp101p, Usp107p and Usp109p complexes by mass spectrometry

All visible bands of the Tricine-SDS-PAGE were sliced into small cubes and washed with Milli-Q water for 5 min followed by a two-step dehydration in 50 and 100% acetonitrile (ACN), respectively. Subsequently, the gel pieces were rehydrated in 100 mM NH_4HCO_3 (3× volume of rehydrated gel), and the dehydration procedure was repeated. The gel pieces were then completely dried in a SpeedVac concentrator and rehydrated with digestion buffer (20 mg/ml sequencing grade modified porcine trypsin [Promega, Madison, WI, USA], 50 mM NH_4HCO_3, 10% ACN) followed by overnight digestion at 37°C. Peptides were extracted from the gel pieces as previously described in Wehmhöner *et al.* (35). Extracted peptides were purified using ZipTip C18-microcolumns (Millipore, Billerica, MA, USA), following the manufacturer's instructions. Precipitated protein complex was directly digested by adding 50 ml of the same digestion buffer. All extracts and digests were lyophilised in a SpeedVac concentrator and resolubilized in 10 ml 0.1% TFA. The RP-HPLC separation of the peptide samples was performed using a bioinert Ultimate nano-HPLC system (Dionex, Sunnyvale, CA, USA). 10 µl of each sample as injected, and peptides were purified and concentrated on a C18-PepMap precolumn (0.3 mm i.d. × 5 mm, 100 Å pore size, 3 mm particle size, Dionex) at a flowrate of 30 ml min^{-1} 0.1% TFA. Subsequently, peptides were separated on an analytical 75 mm i.d. × 150 mm C18-PepMap column (Dionex, 100 Å pore size, 3 mm particle size) at a column flowrate of 250 nl min^{-1}. The acetonitrile gradient (Solution A: 0.1% formic acid, 5% ACN; Solution B: 0.1% formic acid, 80% ACN) started at 5% and ended at 60% B. A gradient length of 30 min was chosen for the characterization of individual bands from the Tricine-SDS-PAGE, whereas precipitated protein complexes were analyzed by applying a 120-min gradient.

MS and MS/MS data were acquired using a Micromass Q-TOF mass spectrometer (micro Q-TOF™ Waters, Milford, MA, USA). Doubly and triply charged peptide-ions were automatically chosen data-dependent by the MassLynx software (Waters) and fragmented for a maximum of 18 s for each component. MSdata were automatically processed, and peak lists for database searches were generated by the MassLynx software (MassLynx 4.0, Mass Measure All, 2× Savitzky Golay Smooth Window 5, minimum peak width at half height 5). Database searches were carried out with an in-house MASCOT server (Matrix Science) using an *S. pombe* protein database. For assessment of predicted protein identifications, the MASCOT default significance criteria were chosen. Only peptides with a MASCOT rank of 1 were considered as significant and used for the combined peptide score.

RESULTS

Spliceosomal particles in fission yeast

To characterize native spliceosomal complexes and their snRNA composition in *S. pombe*, initially we optimized a solution hybridization assay that preserved basepairing of U4 and U6 (31). Total RNA was hybridized to 5′ labeled oligonucleotides complementary with the spliceosomal snRNAs U1, U2, U4, U5 and U6. The hybridization solution containing single or all five complementary oligonucleotides was separated on a non-denaturing gel and visualized by autoradiography. Notably, extracts of fission yeast do not contain appreciable amounts of individual U4 snRNA molecules. Almost all U4 appears to be basepaired with U6 snRNA (Figure 1A, lane 4, asterisk and arrow). However, U6 snRNA exists in particles basepaired with and free of U4 snRNA (Figure 1A, lane 6).

In order to sort out spliceosomal snRNP complexes existing in fission yeast, cell extract was separated on a 10–30% glycerol gradient containing 150 mM sodium chloride and fractionated. RNA was isolated from each fraction and hybridized against the labeled oligonucleotides complementary to U1, U2, U5 and U6. The hybridization solution of each fraction was separated on a non-denaturing gel. The spliceosomal snRNA profile of the cell extract reflects the steady state concentration of individual spliceosomal snRNPs and multiple higher-order complexes of different sizes in their native state. It is noteworthy that most U6 and U5 snRNPs are found sedimenting in complexes larger than 30S, and that U4/U6 snRNP molecules are also found in higher-order complexes sedimenting in regions >30S. The profile of the U1 and U2 snRNPs shows particles sedimenting in a wide range from 12 to 60S (Figure 1B).

snRNP U1 particles associated with U1-70K sediment in the range of 30–60S

To identify snRNP particles associated with U1-70K (referred to as Usp101p in fission yeast, for U1-specific protein), we used a strain in which the genomic copy of *usp101* was fused with the HA-epitope and separated cell extract on a glycerol gradient. Each fraction was immunoprecipitated with anti-HA antibody and immunoblotted. Most of U1-70K-HA (Usp101p) sediments in the range of 30–60S (Figure 2, panel U1-70K-HA). From aliquots of the immunoprecipitates, RNA was isolated by phenol extraction. The snRNA composition co-precipitating with HA-U1-70K was analyzed with the solution hybridization assay by hybridizing the mixture of labeled oligonucleotides complementary to U1, U2, U5 and U6. The hybridization solution was then separated on a non-denaturing gel. Unexpectedly, the particles containing U1-70K-HA sedimenting in the range of 30–60S contain exclusively U1 snRNA (Figure 2). However, U1 snRNPs are also detected sedimenting below 30S (Figure 1B). The mammalian U1 snRNP sediments at

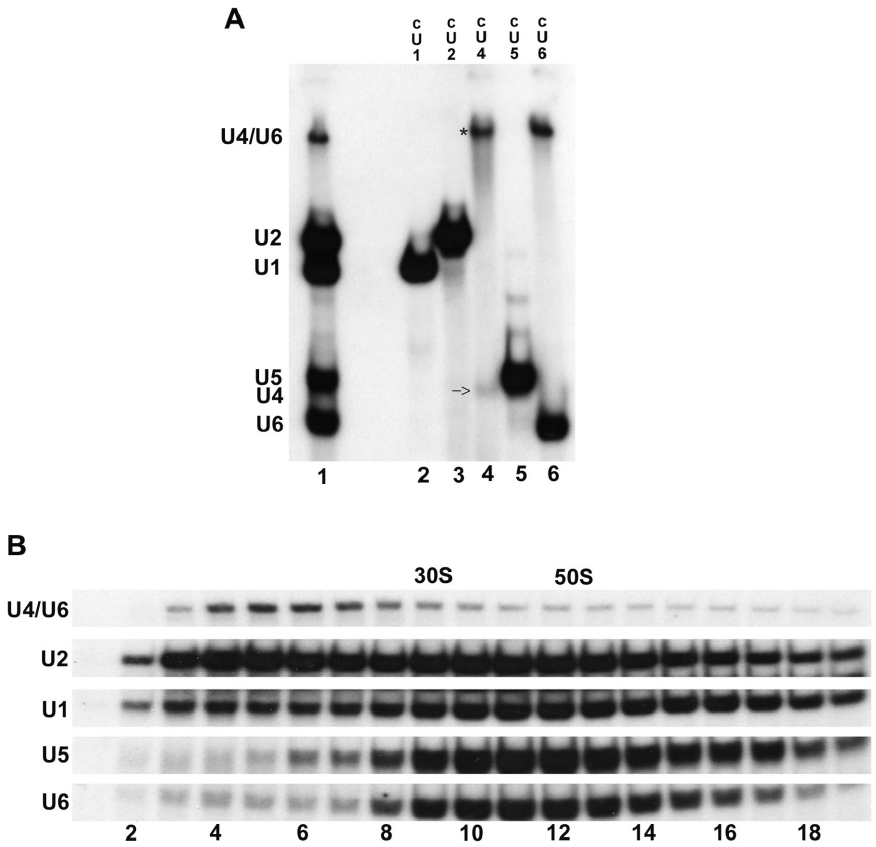

Figure 1. Determination of U snRNPs in native extract. (**A**) Native extract was used to isolate total RNA. ^{32}P-labeled oligonucleotides complementary (c) with the snRNAs U1, U2, U4, U5 and U6 were hybridized with the RNA. After hybridization, the RNA was separated by non-denaturing polyacrylamide gel electophoresis and visualized by autoradiography. It may be noted that this procedure allows to detect basepaired U4/U6. Lane 1, hybridization with all five cUs; lanes 2–6, hybridization with individual cU as indicated. *, position of basepaired snRNA U4/U6; arrow: position of individual snRNA U4. (**B**) Native extract was separated on a 10–30% glyerol gradient (top fraction 1-bottom fraction 19). RNA was isolated from each fraction and hybridized with the 5′- labeled oligonucleotides cU1, cU2, cU5 and cU6. After hybridization, each fraction was separated by non-denaturing PAGE and visualized by autoradiography. The positions of the different U snRNAs are indicated on the left. The gradient was calibrated with small (30S) and large (50S) ribosomal subunits from *Escherichia coli*.

12S and the U1 snRNP of *S. cerevisiae* at 18S (21). Therefore, our result suggests that in *S. pombe* the U1 particles sedimenting in the range of 30–60S contain U1-70K, whereas the U1 particles sedimenting lower than 30S do not.

Isolation of U1-70K complexes by TAP purification

To determine the protein composition of the U1 particles, we chose to purify the complex with the tandem affinity purification method (TAP). For this purpose, we used a strain expressing a U1-70K-TAP (Usp101p-TAP). This strain grows with the same viability as a wild-type strain and shows no morphological abnormalities. The two affinity steps using immunoglobulin G (IgG)-Sepharose and calmodulin resin, respectively, were carried out as described by Gould *et al.* (32). The U1-70K-TAP complex was separated by Tricine SDS-PAGE and stained with a fluorescent dye (Figure 3). The fluorescent bands were individually subjected to an in-gel-digestion procedure, and the eluted peptides were sequenced and identified by liquid chromatography coupled tandem mass spectrometry (LC-MS/MS). Proteins present in a mock TAP purification performed with extract from a wild-type strain that does not express a TAP-tag-fused protein were assumed to be nonspecific contaminants. In addition, to identify the polypeptide composition of the U1-70K-TAP complex, the eluate from the second affinity step was precipitated with TCA, subjected to tryptic digestion and analyzed by LC-MS/MS.

With these approaches, we identified, in addition to U1-70K (Usp101p), the seven Sm protein family members and the homologs of U1A and U1C, which we now call Usp102p and Usp103p, respectively (Table 1 and Figure 3). The homologs of the *S. cerevisiae* U1-specific proteins Prp40p, Prp39p and Luc7p were identified and named Usp104p, Usp105p and Usp106p. Three *S. pombe*-specific proteins displaying conserved RNA binding motifs were also detected and named U1H (Usp107p), U1J (Usp108p) and U1L (Usp109p) (Table 1 and Figure 3). According to this analysis, the snRNP U1 in *S. pombe* consists at least of nine U1-specific proteins. The fluorescent bands labeled with asterisk contained ribosomal proteins of the large and small subunits and other proteins (Figure 3).

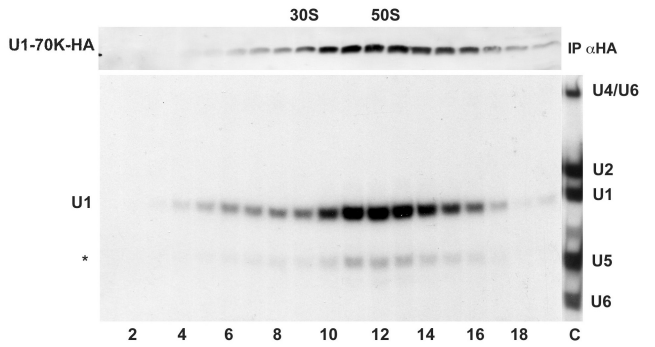

Figure 2. U1-70K (Usp101p) is associated with a U1 snRNP complex sedimenting at 45S. Native extract of growing cells expressing epitope-tagged U1-70K-HA were separated on a 10–30% glycerol gradient. The gradient fractions (1–19) were immunoprecipitated (IP) with HA-antibody and separated by SDS-PAGE, immunoblotted and probed with HA antibody (αHA) to determine the distribution of U1-70K-HA as indicated. Aliquots of the HA immunoprecipitates (IP) were used to isolate RNA, hybridized with the 5′ labeled oligonucleotides cU1, cU2, cU5 and cU6 and after electrophoresis on a non-denaturing polyacrylamide gel visualized as in Figure 1. The positions of the U snRNAs are indicated on the right (lane C). *Although this signal is weak, it is specific for U1 snRNA (see Figure 5). The gradient was calibrated with small (30S) and large (50S) ribosomal subunits from *Escherichia coli*.

For validation of these results, we fused the TAP tag with the *S. pombe*-specific proteins U1H (Usp107p), U1J (Usp108p) and U1L (Usp109p). Proteomic analysis of the U1H-TAP-, U1J-TAP- and U1L-TAP- purified complexes confirmed the protein composition of the U1-70K-TAP complex, revealing nine U1-specific proteins and seven Sm proteins (Figure 3 and Figure 1S A, B supplement).

Next, we wanted to confirm that the *S. pombe*-specific proteins U1H, U1L and U1K are exclusively associated with U1 snRNA. Therefore, we constructed strains expressing U1H-TAP and U1-70K-HA, U1J-TAP and U1-70K-HA, U1L-TAP and U1-70K-HA, respectively. Protein extract of each strain was purified by the TAP method. The material eluted from the calmodulin resin was separated on SDS-PAGE, immunoblotted and probed with antibody against the calmodulin binding peptide (CBP, called αTAP) in the TAP domain to detect U1H, U1J and U1L, respectively, and probed with HA-antibodies to detect U1-70K-HA. All three *S. pombe*-specific proteins U1H, UIJ and U1L are associated with a complex containing U1-70K and reveal the size expected after removal of the protein A domains (Figure 4).

The material eluted from the second affinity step was also used to isolate RNA. The RNA was separated on a denaturing gel and stained with ethidium bromide (Figure 5C). Then the RNA was transferred to nitrocellulose and hybridized with a ^{32}P-labeled probe comprising the complete *snu1* (U1 snRNA) gene and visualized by autoradiography. Noteworthy, the *snu1* probe detects a second major and several minor degradation products. The major degradation product is approximately 10 nucleotides smaller than the 148 nt U1 snRNA and can also be stained with ethidium bromide

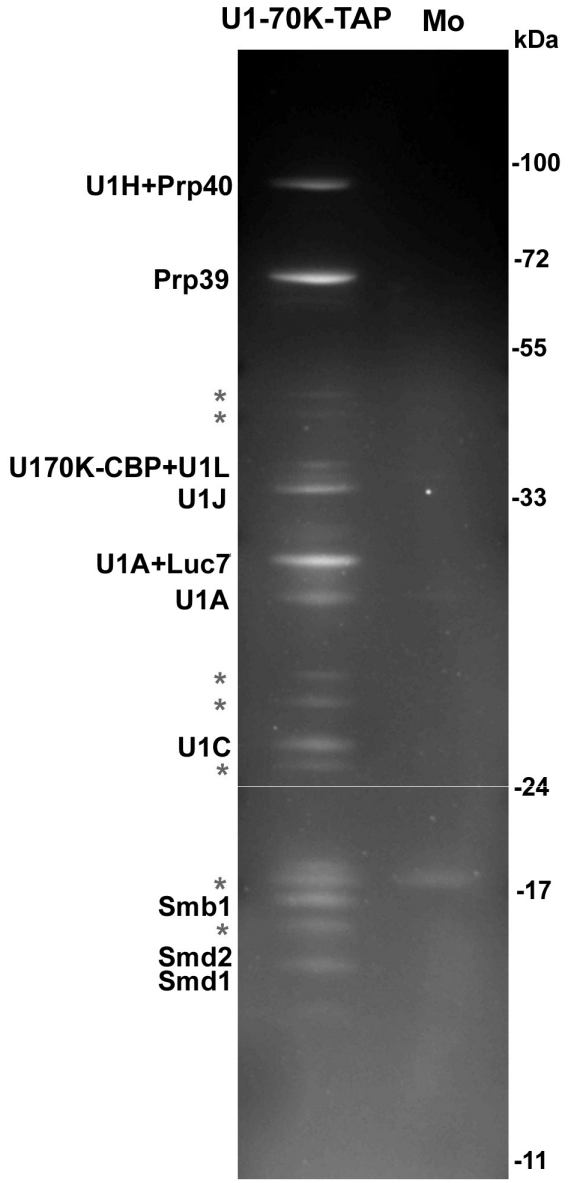

Figure 3. Purification of U1-70K-TAP. Protein extract was made from 21 of the *usp101-TAP* strain. The eluate from the calmodulin resin after the second affinity step was separated with Tricine-PAGE and stained with the fluorescent stain ruthenium (II) tris-bathophenanthrolin disulphonate. Bands were labeled with the names of proteins identified by (LC-MS/MS) mass spectrometry. U1-70K-CBP (calmodulin binding peptide). U1A appears in two bands, the smaller is presumably a degradation product. Bands labeled with asterisk (from top down) contained peptides from translation elongation factor EF-1 alpha, ribosomal proteins rpl301, rpl702, rpl1002, rps5, rpl801, rps1201 of the small (rps) and large (rpl) subunit. Mo, eluate after the second affinity step using extract from a wildtype strain; Marker proteins in kilodalton (kDa).

(Figure 5A and C). The same nitrocellulose was re-probed with a mixture of ^{32}P-labeled DNA probes comprising the complete *snu2*, *snu4*, *snu5* and *snu6* genes. These probes do not detect additional U snRNAs, suggesting that the complexes U1H, U1L, U1J and U1-70K isolated by the TAP-purification method contain exclusively snRNA U1 (Figure 5A and B).

Table 1. U1-specific proteins and Sm proteins identified by LC-Ms/Ms mass spectrometry

No.	*S. pombe*protein	Mol. wt kDa	Accession no. GeneDB *S.pombe*	Motifs	*S. cerevisiae* homolog	*H. sapiens* homolog
1	Usp101p	30	SPAC19A8.13	1 RRM	Snp1p	U1-70K
2	Usp102p	28	SPBC4B4.07c	2 RRM	Mud1p	U1A
3	Usp103p	20	SPBP35G2.09	zn finger	Yhc1p	U1C
4	Usp104p	82	SPAC4D7.13	2 WW, 5 FF	Prp40p	
5	Usp105p	71	SPBC4B4.09	7 HAT	Prp39p	–
6	Usp106p	31	SPCC16A11.13	–	Luc7p	–
7	Usp107p (U1H)	−81	SPBC839.1	1 PW1	–	–
8	Usp108p (U1J)	42	SPAC23D3.08	1 KH	–	–
9	Usp109p (U1L)	40	SPBC1289.12	2 RRM	–	–
	–	71		1 PW1	Snu71p	–
	–	65		TPRs	Prp42p	–
	–	56		–	Snu56p	–
	–	56	–	3 RRM	Nam8p	–
10	Smb1p	15	SPAC26A3.08	Sm	Smb1p	SmB1
11	Smd1p	13	SPAC27D7.07c	Sm	Smd1p	SmD1
12	Smd2p	13	SPAC2C4.03c	Sm	Smd2p	SmD2
13	Smd3p	11	SPBC19C2.14	Sm	Smd3p	SmD3
14	Sme1p	10	SPBC11G11.06c	Sm	Sme1p	SmE1
15	Smf1p	9	SPBC4F6.01	Sm	Smf1p	SmF1
16	Smg1p	9	SPBC4B4.05	Sm	Smg1p	SmG1

Usp1, U1-specific protein; –, homolog not apparent; for U1H, U1J and U1L, no homologs were apparent in all databases; RRM, RNA recognition motif; KH, RNA recognition motif; PWI, putative RNA, DNA recognition motif; WW, FF, HAT, TPR protein–protein interaction motifs, zn finger, Zink finger motif; Mol. wt kDa, molecular weight in kilodalton. For detailed information about the human and budding yeast snRNP U1 proteins, see references 18, 20 and 21.

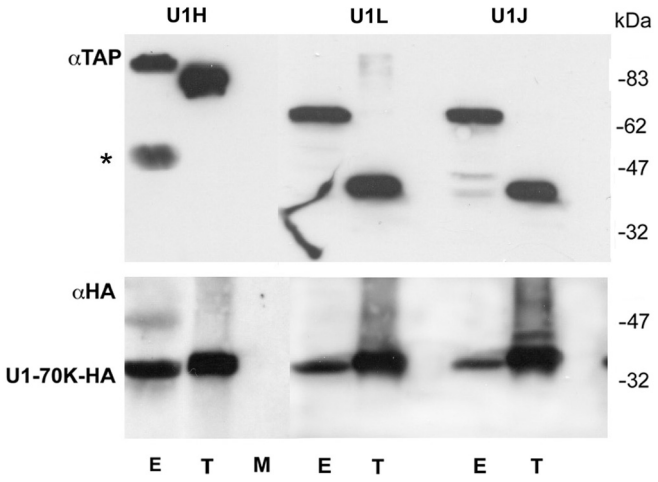

Figure 4. Purification of U1H-TAP, U1L-TAP and U1J-TAP. Protein extracts of 21 cultures were made from strains expressing U1H-TAP, U1L-TAP and U1J-TAP. Each strain also expresses U1-70K-HA. Protein extract (E, 0.02% of total) and the eluate from the calmodulin resin after the second affinity step (T, 10% of the eluate) was separated with SDS-PAGE, immunoblotted and probed with anti-TAP (αTAP) and anti-HA (αHA) antibodies as indicated. *, degradation product of U1H. M, eluate after the second affinity step using extract from a wild-type strain; Marker proteins in kilodalton (kDa). See also supplementary data (Figure S1 A/B).

To investigate whether U1H, U1L and U1J sediment with particles of the same size range as U1-70K, protein extract was separated on a glycerol gradient. The gradient fractions were separated on SDS-PAGE, immunoblotted and probed with the appropriate antibodies. U1H-TAP is associated with particles sedimenting in the range of 30–60S as observed for U1-70K-TAP (Figure 6). The sedimentation profile of U1J-TAP and U1L-TAP, however, is different. Both proteins sediment in a wide range between 11 and 60S (Figure 6A).

In order to examine whether U1L-TAP sedimenting below 30S is associated with snRNA U1 and U1-70K-HA, the gradient fractions 3–8 and 10–15 were pooled and IgG-Sepharose was added to bind U1L-TAP complexes. The IgG-Sepharose-bound material was separated on SDS-PAGE, immunoblotted and probed with IgG and HA antibodies to detect U1L-TAP and U1-70K-HA, respectively. RNA was also isolated from the IgG-bound material and hybridized with a U1 probe. Both pooled gradient fractions contain snRNA U1, indicating that U1L sedimenting below 30S is associated with U1 snRNA (Figure 6A, B). Furthermore, in the gradient fractions 10–15, significantly more U1-70K-HA was found associated with U1L than in the gradient fractions 3–8 (Figure 6B). This is consistent with the sedimentation profile of U1-70K, indicating that most of U1-70K is associated with U1 particles sedimenting in the range of 30–60S (Figures 6A and 2). Based on the sedimentation profile of U1H (Figure 6A), we would predict that the U1 particles sedimenting below 30S (Figure 1B) also do not contain U1H. Noteworthy here, U1-70K and U1H remain stably associated with the particles sedimenting in the range of 30–60S at salt concentrations less than or equal to 500 mM sodium chloride (results not shown).

In order to compare the sedimentation profile of U1-70K-TAP complexes observed with whole cell extract (Figure 7, panel U1-70K-C) with the sedimentation profile of U1-70K-TAP complexes after TAP-purification, the material eluted from the calmodulin resin was separated on a glycerol gradient containing 150 mM sodium chloride. The gradient fractions were then separated by SDS-PAGE, immunoblotted and probed

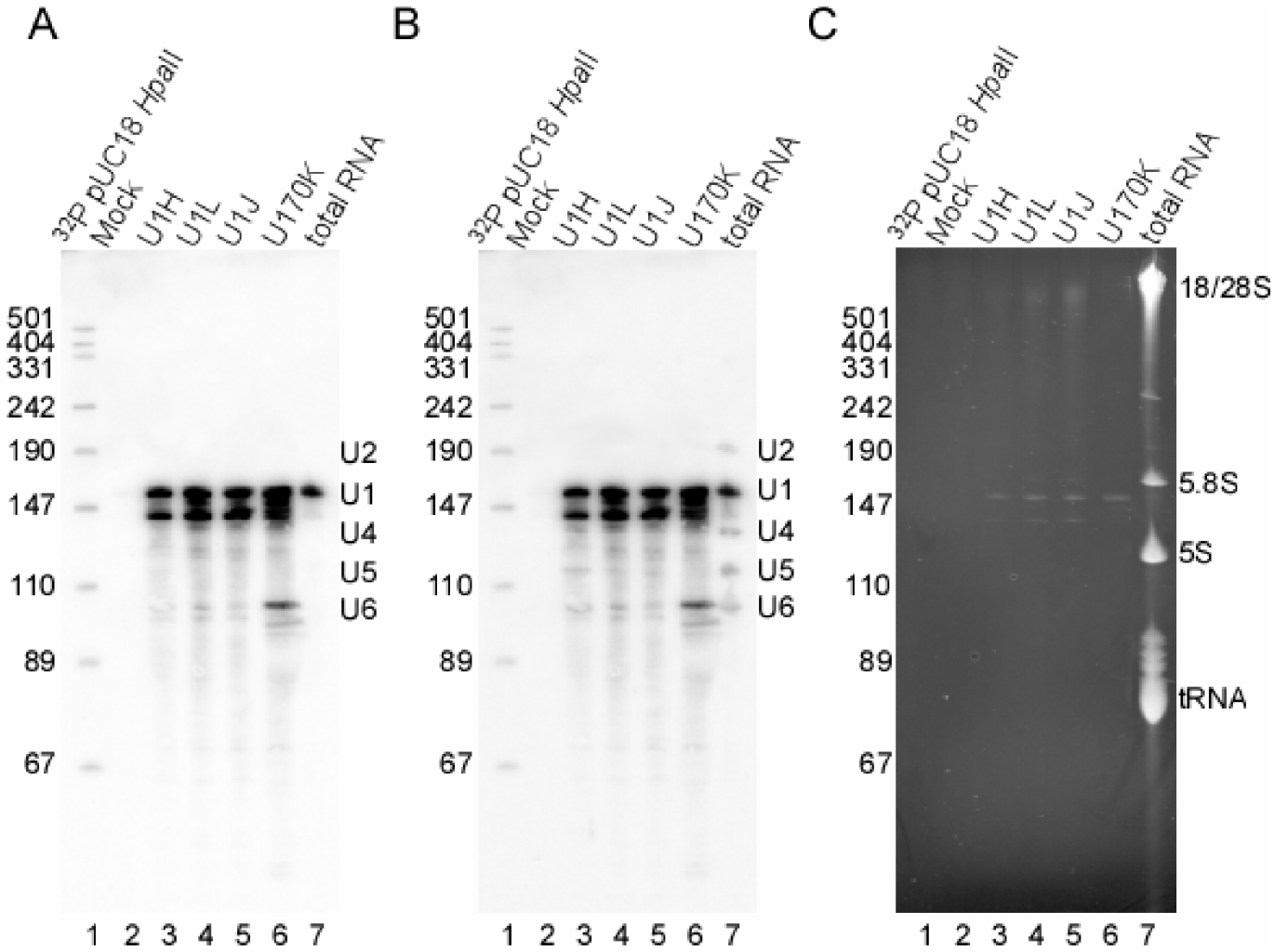

Figure 5. Northern analysis of purified U1H, U1L, U1J and U1-70K complexes. RNA was isolated from 20% of the eluate of the calmodulin resin and separated on a denaturing (16%, 8 M urea) polyacrylamide gel. (**A**) RNA was transferred on to nitrocellulose and hybridized with a ^{32}P-labeled U1 probe comprising the complete gene. (**B**) The nitrocellulose was re-hybridized with a mixture of ^{32}P-labeled U1, U2, U4, U5 and U6 probes. (**C**) Polyacrylamide gel stained with ethidium bromide. Lane 1, ^{32}P-labeled DNA fragments of *H*paII digested pUC18 plasmid as marker. Numbers indicate fragment length in basepairs; lane 2, Mock, RNA isolated from an eluate with protein extract from a wild-type strain; lane 3, RNA isolated from U1H complex; lane 4, RNA isolated from U1L complex; lane 5, RNA isolated from U1J complex; lane 6, RNA isolated from U1-70K complex; lane 7, total RNA isolated from a wild-type strain. 18/28S, ribosomal RNA; 5.8S, ribosomal RNA; 5S, ribosomal RNA; tRNA, transfer RNA.

with the appropriate antibodies. Strikingly, the purified U1-70K-TAP complexes sediment in a wide range of 11–60S (Figure 7, panel U1-70K-T). Northern analysis of the RNA isolated from the gradient fractions and probing with the complete *snu1* gene revealed that the TAP-purified U1-70K complexes sedimenting below 30S also contain U1 snRNA (Figure 7, panel snRNA). This result suggests that in whole-cell extract the U1 snRNP particles may exist as aggregates of more than one U1 particle (30–60S), partially falling apart to monomeric U1 snRNP particles sedimenting in the region of 20S. We do not know when the particles fall apart during the TAP-procedure.

In a first approach to investigate whether the U1 particles sedimenting in the range of 30–60S might be at least dimeric, we constructed a strain expressing U1-70K fused with HA epitopes at the C-terminus (U1-70K-HA) and also expressing U1-70K fused with Myc-epitopes at the N-terminus (U1-70K-Myc). Whole cell extract was immunoprecipitated with anti-HA and anti-Myc antibodies, respectively. The immunoprecipitates were separated by SDS-PAGE, immunoblotted and probed with the appropriate antibodies. The results show that U1-70K-HA co-precipitates U1-70K-Myc and vice verca, indicating that U1-particles sedimenting in the region of 30–60S, indeed, may be at least dimeric (Figure 2S).

In order to determine if the newly identified U1-specific proteins are essential, we disrupted the gene encoding Usp101p (U1-70K) and the three *S. pombe*-specific genes encoding Usp107p (U1H), Usp108p (U1J) and Usp109 (U1L) in diploid cells. Tetrad analyses indicated that all four genes are essential for growth (Figure 3S).

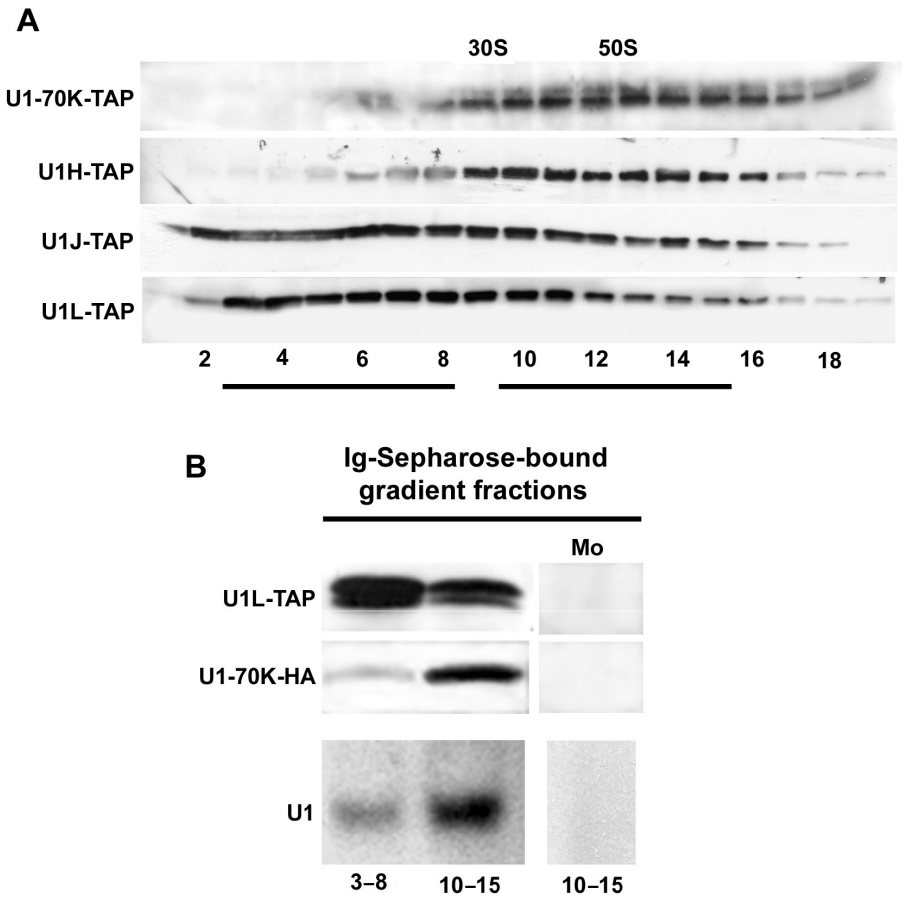

Figure 6. Sedimentation profiles of U1-70K (Usp101p), U1H (Usp107p), U1J (Usp108) and U1L (Usp109p) complexes. (**A**) Protein extract (5 mg) of cells expressing U1-70K-TAP, U1H-TAP and U170-HA or U1J-TAP and U1-70K-HA or U1L-TAP and U1-10K-HA were separated on a 10–30% glycerol gradient. The gradient fractions (1–19) were precipitated with IgG antibodies and separated by SDS-PAGE, immunoblotted and probed with αTAP antibodies to determine the distribution of U1-70K-TAP, U1H-TAP, U1J-TAP and U1L-TAP, as indicated. (**B**) The gradient fractions 3–8 and 10–15 of a gradient-containing U1L-TAP and U1-70K-HA were pooled bound to IgG-Sepharose. The bound material was separated on SDS-PAGE, immunoblotted and probed with IgG and HA antibodies. An aliquot (50%) of the bound material was used to isolate RNA and hybridized with a labeled U1 probe to visualize U1 snRNA as indicated. Mo, pooled gradient fractions 10–15 of a protein extract from a wild-type strain. The pooled gradient fractions 3–8 from this gradient also showed no signals (not shown). The gradient was calibrated with small (30S) and large (50S) ribosomal subunits from *Escherichia coli*.

DISCUSSION

In this study, we used the TAP-method to purify complexes associated with the *S. pombe* homolog of the U1-specific protein U1-70K and identified its proteins with LC-MS/MS tandem mass spectrometry. This approach revealed the existence of complexes containing at least 16 proteins. Three of them are the homologs of U1-70K, U1A and U1C, which are conserved throughout eukaryotes. The proteins Usp104p (Prp40p), Usp105p (Prp39p) and Usp106p (Luc7p) appear to be conserved in fungi (Table 1). U1H (Usp107p), U1J (Usp108p) and U1L (Usp109p) had no sequences with significant similarity and, therefore, these three proteins appear to be fission yeast specific. The seven Sm proteins are conserved throughout eukaryotes and are not only associated with the snRNP U1, but are also found associated with the snRNPs U2, U4 and U5 (19). We have confirmed these protein interactions by purifying and analyzing U1H-TAP, U1J-TAP and U1L-TAP complexes. The density gradient analyses revealed that U1-70K and U1H are associated with U1 particles sedimenting in the range of 30–60S, whereas U1J and U1L appear to be associated with U1 particles sedimenting in wider range (11–60S). Based on these observations, we suggest that the U1 particles sedimenting at 30–60S and containing at least 16 proteins represent the pool of functional U1 particles ready to engage in pre-mRNA splicing. The U1 snRNPs sedimenting below 30S obviously do not contain a full set of the nine U1-specific proteins (Figure 6A and B). As mentioned before, we were surprised that the particle containing 16 proteins and the 148 nt U1 snRNA component sediments in the region of 30–60S. In contrast, the U1 particle of *S. cerevisiae* associated with 17 proteins and a 568 nt U1 snRNA component sediments at 18S (21). We are aware that the association of multimeric U1 snRNPs might be an artifact due to cell lysis. However, based on our analyses, it is also reasonable to suggest that the U1 snRNPs ready for splicing might be stored as higher-order structures in the nucleus and that the U1

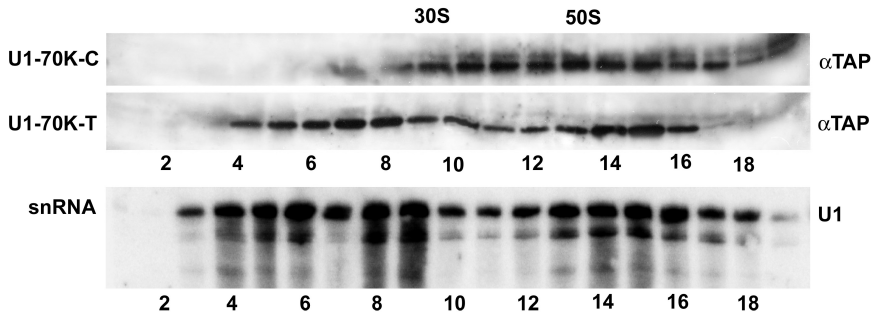

Figure 7. Sedimentation profile of U1-70K complexes after TAP purification. Extract from 8 L culture was prepared. The eluate from the calmodulin resin after the second affinity step was separated on a 10–30% glycerol gradient containing 150 mM sodium chloride. The gradient fractions (70% of each fraction) were separated on SDS-PAGE, immunoblotted and probed with αTAP antibody (U1-70K-T). From the remaining 30%, RNA was isolated, separated on a denaturing polyacrylamide gel, transferred on to nitrocellulose and hybridized with a ^{32}P-labeled U1 probe (snRNA U1). For comparison, U1-70K-C shows the sedimentation profile of U1-70K complexes when cell extract was loaded on the gradient (see Figure 6, panel U1-70K-TAP).

particles sedimenting below 30S might be U1 particles just released from activated spliceosomes to be recycled for the splicing process.

The U1-specific proteins Snu71p, Prp42p, Snu56p and Nam8p of *S. cerevisiae* have no apparent homolog in *S. pombe* and have not yet been found in any other eukaryote (36).

The same is true for U1H, U 1J and U1L; we did not find homologues in any other eukaryote.

However, Snu71p, for example, could be the functional counterpart of U1H. The two proteins are similar in size and display a PWI motif in the C-terminus of the protein (Table 1). The motifs found in U1H, U1J and U1L of *S. pombe*, as well as in Snu71p, Prp42p, Snu56p and Nam8p of *S. cerevisiae*, predict that these proteins are putative RNA-binding proteins, suggesting that they may be involved in intron recognition. The strong divergence of these proteins is consistent with the observation that introns in *S. pombe* and *S. cerevisiae* clearly show organism-specific features, as discussed in the introduction (9,13).

Based on the results presented here and those published previously, we hypothesize that the proteins U1H, U1J and U1L associated with the U1 snRNP in *S. pombe* are specific proteins involved in intron recognition. Once an intron is defined by a U1 particle, a tetra-snRNP particle consisting of U2, U5 and U4/U6 is recruited to the pre-mRNA, and a splicing-competent spliceosome (penta snRNP) is formed. The formation and activation of a splicing-competent spliceosome involves the phosphorylation of the spliceosomal component Prp1p by Prp4p kinase (10,11).

We have suggested that *S. pombe* represents the archetype of the pre-mRNA splicing machinery in eukaryotes (27). The present study supports this prediction. Here, we extend this prediction to the mechanism of spliceosome activation. All eukaryotic genomes sequenced to date, with the exception of the hemiascomycetes, contain a Prp4p kinase counterpart. Therefore, further analysis using *S. pombe* as a genetically and biochemically amenable organism will help provide insight into the complex mechanisms of pre-mRNA splicing regulation.

ACKNOWLEDGEMENTS

We thank Dr. Judy Potashkin (Rosalind Franklin University of Medicine and Science, IL, USA) for careful reading of the manuscript, and Susanne Zock-Emmenthal and Kirsten Minkart for their valuable technical assistance. Thanks to Henning Schmidt (TU Braunschweig) for his permanent support. We thank Charles C. Query (Albert Einstein College of Medicine, NY, USA) for tagged U1-70K *S. pombe* strains. Dr. Alain N.S. Newo thanks the DAAD for a fellowship in the programme: 'Research Grants for Young Academics and Scientists' and the Helmholtz Center for Infection Research for financial support for further 6 months. Funding to pay the Open Access publication charge was provided by Technical University of Braunschweig.

Conflict of interest statement. None declared.

REFERENCES

1. Jurica,M.S. and Moore,M.J. (2003) pre-mRNA splicing: awash in a sea of proteins. *Mol. Cell*, **12**, 5–14.
2. Burge,C.B., Tuschl,T. and Sharp,P.A. (1999). Splicing of precursors to mRNAs by the spliceosomes. In Gesteland,R.F., Cech,T.R. and Atkins,J.F. (eds), RNA World II. Cold Spring Harbor Laboratory Press, pp. 525–560.
3. Collins,C.A. and Guthrie,C. (2000) The question remains: is the spliceosome a ribozyme? *Nat. Struct. Biol.*, **7**, 850–854.
4. Görnemann,J., Kotvic,K.M., Hujer,K. and Neugebauer,K.M. (2005) Cotranscriptional spliceosome assembly occurs in a stepwise fashion and requires the cap binding complex. *Mol. Cell*, **19**, 53–64.
5. Lacadie,S.A. and Rosbash,M. (2005) Cotranscriptional spliceosomeassembly dynamics and the role of U1 snRNA: 5′ss base pairing in yeast. *Mol. Cell*, **19**, 65–75.
6. Kornblihtt,A.R., De La Mata,M., Fededa,J.P., Munoz,M.J. and Nogues,G. (2004) Multiple links between transcription and splicing. *RNA*, **10**, 1489–1498.
7. Nilsen,TW (2005) Spliceosome assembly in yeast: one Chip at a time? *Nature Struc. & Mol. Biol.*, **12**, 571–573.
8. Du,H. and Rosbash,M. (2002) The U1 snRNP protein U1C recognizes the 5′ splice site in the absence of base pairing. *Nature*, **419**, 86–90.

9. Kuhn,A.N. and Käufer,N.F. (2003) Pre-mRNA splicing in *Schizosaccharomyces pombe*; Regulatory role of a kinase conserved from fission yeast to mammals. *Curr. Genet.*, **42**, 241–251.
10. Bottner,C.A., Schmidt,H., Vogel,S., Michele,M. and Käufer,N.F. (2005) Multiple genetic and biochemical interactions of Brr2, prp8, Prp31, Prp1p and Prp4 kinas suggest a function in the control of the activation of spliceosomes in *Schizosaccharomyces pombe*. *Curr. Genet.*, **48**, 151–161.
11. Schwelnus,W., Richert,K., Opitz,F., Groß,T., Habara,Y., Tani,T. and Käufer,N.F. (2001) Fission yeast Prp4p kinase regulates pre-mRNA splicing by phosphorylating a non-SR-splicing factor. *EMBO Rep.*, **2**, 35–41.
12. Brow,D.A. (2002) Allosteric cascade of spliceosome activation. *Annu. Rev. Genet.*, **36**, 333–360.
13. Bon,E., Casaregola,S., Blandin,G., Llorente,B., Neuveglise,C., Munsterkotter,M., Guldener,U., Mewes,H.W., Van Helden,J. *et al.* (2003) Molecular evolution of eukaryotic genomes: hemiascomycetous yeast spliceosomal introns. *Nucleic Acids Res.*, **31**, 1121–1135.
14. Dellaire,G., Makarov,E.M., Cowger,J., Longman,D., Sutherland,H.G.E., Lührmann,R., Torchia,J. and Bickmore,WA. (2002) Mammalian PRP4 kinase copurifies and interacts with components of both the U5 snRNP and the N-CoR deacetylase complexes. *Mol. Cell. Biol.*, **22**, 5141–5156.
15. Carnahan,R.H., Feoktistova,A., Ren,L., Niessen,S., Yates,III,J.R. and Gould,K.L. (2005) Dim1p is required for efficient splicing and export of mRNA encoding Lid1p, a component of the fission yeast anaphase-promoting complex. *Eukaryotic Cell*, **4**, 577–587.
16. Ohi,M.D., Link,A.J., Ren,L., Jennings,J.L., McDonald,W.H. and Gould,K.L. (2002) Proteomics analysis reveals stable multiprotein complexes in both fission and budding yeasts containing Myb-related Cdc5p/Cef1p, novel pre-mRNA splicing factors, and snRNAs. *Mol. Cell. Biol.*, **22**, 2011–2024.
17. Palfi,Z., Schimanski,B., Günzl,A., Lücke,S. and Bindereif,A. (2005) U1 small nuclear RNP from Trypanosoma brucei: a minimal U1 snRNA with unusual protein components. *Nucleic Acids Res.*, **33**, 2493–2503.
18. Stark,H., Dube,P., Lührmann,R. and Kastner,B. (2002) Arrangement of RNA and proteins in the spliceosomal U1 small nuclear ribonucleoprotein particle. *Nature*, **409**, 539–542.
19. Will,C.L. and Lührmann,R. (2001) Spliceosomal UsnRNP biogenesis, structure and function. *Curr. Opin. Cell. Biol.*, **13**, 290–301.
20. Rigaut,G., Shevchenko,A., Rutz,B., Wilm,M., Mannn,M. and Seraphin,B (1999) A generic protein purification method for protein complex characterization and proteome exploration. *Nature Biotech.*, **17**, 1030–1032.
21. Gottschalk,A., Tang,J., Puig,O., Salgado,J., Neubauer,G., Colot,H., Mann,M., Seraphin,B., Rosbash,M. *et al.* (1998) A comprehensive biochemical and genetic analysis of the yeast U1 snRNP reveals five novel proteins. *RNA*, **4**, 374–393.
22. Kretzner,L., Krol,A. and Rosbash,M. (1990) *Saccharomyces cerevisiae* U1 small nuclear secondary structure contains both universal and yeast-specific domains. *Proc. Natl. Acad. Sci., USA*, **87**, 851–855.
23. Johnson,J.M., Castle,J., Garrett-Engele,P., Kann,Z., Loerch,P.M., Armour,C.D., Santos,R., Schadt,E.E., Stoughton,R. *et al.* (2003) Genome-wide survey of human alternative pre-mRNA splicing with exon junction microarrays. *Science*, **302**, 2141–2144.
24. Alvarez,C.J., Romfo,C.M., Vanhoy,R.W., Porter,G.L. and Wise,J.A. (1996) Mutational analysis of U1 function in *Schizosacchaomyces pombe*: Pre-mRNAs differ in the extent and nature of their requirements for this snRNA *in vivo*. *RNA*, **2**, 404–418.
25. Porter,G., Brennwald,P. and Wise,J.A. (1990) U1 small nuclear RNA from Schizosaccharomyces pombe has unique and conserved features and is encoded by an essential single-copy gene. *Mol. Cell. Biol.*, **10**, 2874–2881.
26. Wood,V., Gwilliam,R., Rajandream,M.A., Lyne,M., Lyne,R., Stewart,A., Sgouros,J., Peat,N., Hayles,J. *et al.* (2002) The genome sequence of *Schizosaccharomyces pombe*. *Nature*, **415**, 871–880.
27. Käufer,N.F. and Potashkin,J. (2000) Analysis of the splicing machinery in fission yeast: a comparison with budding yeast and mammals. *Nucleic Acids Res.*, **28**, 3003–310.
28. Gutz,H., Heslot,H., Leupold,U. and Loprieno,N. (1974). *Schizosaccharomyces Pombe*, In Handbook of Genetics Vol. 1, King,R.C. (ed.). Plenum Press, New York. pp. 395–446.
29. Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163–168.
30. Moreno,S. and Klar,A. (1991) Molecular and genetic analysis of fission yeast *Schizosaccharomyces pombe*. *Methods Enzymol.*, **194**, 795–823.
31. Li,Z. and Brow,DA (1993) A rapid assay for quantitative detection of specific RNAs. *Nucleic Acids Res.*, **21**, 4645–4646.
32. Gould,K.L., Ren,L., Feoktistova,A.S., Jennings,J.L. and Link,A.J. (2004) Tandem affinity purification and identification of protein complex components. *Methods*, **33**, 239–244.
33. Schägger,H. and von Jago,G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.*, **166**, 368–379.
34. Rabilloud,T., Strub,J.M., Luche,S., van Dorsselaer,A. and Lunardi,J (2001) A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. *Proteomics*, **1**, 699–704.
35. Wehmhöner,D., Dieterich,G., Fischer,E., Baumgartner,M., Wehland,J. and Jänsch,L. (2005) "LaneSpector", a tool for membrane proteome profiling based on sodium dodecyl sulfate-polyacrylamide gel electrophoresis/liquid chromatography-tandem mass spectrometry analysis: application to Listeria monocytogenes membrane proteins. *Electrophoresis*, **26**, 2450–2460.
36. Collins,L. and Penny,D. (2005) Complex spliceosomal organization ancestral to extant eukaryotes. *Mol. Biol. Evol.*, **22**, 1053–1066.