

SPF30 Is an Essential Human Splicing Factor Required for Assembly of the U4/U5/U6 Tri-small Nuclear Ribonucleoprotein into the Spliceosome*

Received for publication, April 23, 2001
Published, JBC Papers in Press, April 30, 2001, DOI 10.1074/jbc.M103620200

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Spliceosome assembly involves the sequential recruitment of small nuclear ribonucleoproteins (snRNPs) onto a pre-mRNA substrate. Although several non-snRNP proteins function during the binding of U1 and U2 snRNPs, little is known about the subsequent binding of the U4/U5/U6 tri-snRNP. A recent proteomic analysis of the human spliceosome identified SPF30 (Neubauer, G., King, A., Rappsilber, J., Calvio, C., Watson, M., Ajuh, P., Sleeman, J., Lamond, A., and Mann, M. (1998) *Nat. Genet.* 20, 46–50), a homolog of the survival of motor neurons (SMN) protein, as a spliceosome factor. We show here that SPF30 is a nuclear protein that associates with both U4/U5/U6 and U2 snRNP components. In the absence of SPF30, the preformed tri-snRNP fails to assemble into the spliceosome. Mass spectrometric analysis shows that a recombinant glutathione S-transferase-SPF30 fusion protein associates with complexes containing core Sm and U4/U5/U6 tri-snRNP proteins when added to HeLa nuclear extract, most strongly to U4/U6–90. The data indicate that SPF30 is an essential human splicing factor that may act to dock the U4/U5/U6 tri-snRNP to the A complex during spliceosome assembly or, alternatively, may act as a late assembly factor in both the tri-snRNP and the A-complex.

The splicing of nuclear mRNA precursors, pre-mRNAs, is an essential step for the expression of all intron-containing eukaryotic genes. Splicing takes place in the nucleus, in most cases directly on nascent gene transcripts. The splicing mechanism removes introns in a two-step mechanism involving sequential transesterification reactions and is catalyzed by a dedicated machinery termed the spliceosome (reviewed by Ref. 2). The spliceosome complex is composed of small nuclear ribonucleoprotein (snRNP)¹ subunits; specifically, the U1, U2, U4, U5, and U6 snRNPs. The U4/U5/U6 snRNPs preassemble into

a tri-snRNP subunit before joining the spliceosome. In addition to the snRNPs, a group of additional protein splicing factors is also present in spliceosomes.

In vitro studies in both mammalian and yeast systems have shown that spliceosomes assemble on pre-mRNAs in a stepwise pathway involving the sequential binding of the U1, U2, and U4/U5/U6 snRNP subunits. Thus, assembly of the active spliceosome is preceded by the formation of partial complexes corresponding to assembly intermediates (3–5). The U1 snRNP binds to the 5' splice site of the nuclear pre-mRNA (6) assisted by non-snRNP proteins like ASF/SF2 (7), whereas a protein complex termed U2AF (U2 snRNP auxiliary factor) associates with the 3' splice site (8). The two splice sites are thought to be bridged by members of the SR protein family, proteins with a sequence stretch rich in alternating serine and arginine residues (9, 10). This assembly intermediate is called mammalian E complex (or yeast commitment complex), and it subsequently recruits U2 snRNP to form the A complex (3, 11–13). A number of non-snRNP protein factors are also required for formation of these intermediate complexes. For example, protein factors in the E-complex include SF1 (14) and U2AF65 (15), and in the U2 snRNP, the proteins of the SF3a and SF3b complexes are involved (16–18).

The final stage of spliceosome formation involves the addition of the pre-assembled U4/U5/U6 tri-snRNP onto the A complex. A number of snRNP proteins are required for the preassembly of the tri-snRNP. Genetic and biochemical work in yeast has shown that PRP8 (19), PRP4 (20), and PRP6 (21, 22) gene products are essential. Additionally, a set of tri-snRNP-specific proteins is required for reconstitution of purified and disassembled human tri-snRNP, whereas purified 20 S U5 snRNP together with 10 S U4/U6 snRNP alone failed to bind (23). However, surprisingly little is known about the binding of the tri-snRNP to the A complex. Most observed mutations in yeast that affect the assembly of the spliceosome do so by reducing the levels of snRNPs or the level of the tri-snRNP (24). However, antibodies against either the PRP4 or PRP8 proteins prevent spliceosome formation but apparently do not prevent assembly of the tri-snRNP (19, 20). The temperature-sensitive mutation PRP8–1 prevents spliceosome formation and lies in the region of the antibody epitope. But in this experiment it is not clear whether the effect is direct or due instead to destabilization of the U5 snRNP or tri-snRNP (19). In mammalian extracts, SR domain-containing proteins have been suggested to be involved in docking the tri-snRNP to the pre-spliceosome (25), but the evidence is not conclusive (see "Discussion"). Thus, it is still an open question which proteins are directly involved in this step and whether non-snRNP proteins are required for the binding of the tri-snRNP to the A complex.

The biogenesis of spliceosomal snRNPs is itself a complex

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¹ The abbreviations used are: snRNP, small nuclear ribonucleoprotein particle; SPF30, spliceosomal protein factor 30 kDa; SMN, survival of motor neurons; GST, glutathione S-transferase; GFP, green fluorescent protein; PBS, phosphate-buffered saline; Pipes, 1,4-piperazinediethanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.

pathway. Four of the five snRNAs (U1, U2, U4, and U5) are transcribed by RNA polymerase II, then leave the nucleus to assemble with the set of seven core Sm proteins in the cytosol. After the Sm proteins bind the snRNAs are hypermethylated to form the m3G 5' cap structure and reimported into the nucleus (26). The fifth snRNA, U6, is an RNA polymerase III transcript that has a γ monomethyl cap structure and does not leave the nucleus before its incorporation into the U4/U5/U6 tri-snRNP. Newly assembled snRNPs preferentially localize in subnuclear structures called Cajal bodies (also called coiled bodies (27), which were found to be identical with gems in most cell lines (28)) when they first return to the nucleus during their maturation pathway (29). At later times they accumulate in separate nuclear structures termed speckles, together with other splicing factors. A multi-protein complex containing the proteins SMN1, SIP1, Gemin3, Gemin4, and a number of yet unidentified members has been implicated in snRNP biogenesis as well as in shuttling the snRNPs into the nucleus (30–34). It has also been reported that SMN1 plays a role in recycling snRNPs from spliceosomes after splicing has taken place. There is much interest in SMN1 because mutations in this gene cause spinal muscular atrophy, the most common inherited cause of childhood mortality (35). The link between the disease and the biochemical function of the protein has not been elucidated.

Recently, we reported a large scale proteomic analysis of purified human spliceosomes in which a number of novel proteins were identified by mass spectrometry and linked to splicing (1). One of these proteins, denoted splicing factor 30 kDa (SPF30), exhibited a sequence similarity to SMN1. Here we show that SPF30 is an essential protein-splicing factor. Nuclear extracts lacking SPF30 are not capable of *in vitro* splicing, and spliceosome assembly is halted at the A complex. We find that SPF30 interacts with both U2 snRNP and tri-snRNP components and propose that it may act either as a bridging factor between the A complex and the tri-snRNP during spliceosome assembly or have a chaperon-like function in the A complex and the tri-snRNP, allowing their fusion.

EXPERIMENTAL PROCEDURES

Recombinant Protein, Antibody Production, and Extract Preparation—The cDNA for SPF30 was cloned into pGEX4T-3, resulting in an N-terminal GST tag with a thrombin cleavage site separating the tag from the protein. After expression in *Escherichia coli* the protein was purified by fast protein liquid chromatography on a glutathione column. For antibody production in rabbits the fusion protein was cleaved with thrombin and SPF30-purified on DEAE Sepharose. For affinity purification of anti-SPF30, the fusion protein was immobilized on a *N*-hydroxysuccinimide-activated HiTrap column (Amersham Pharmacia Biotech).

HeLa nuclear extracts (Computer Cell Culture Center) were depleted using anti-SPF30 serum and pre-immune serum for the mock control. The serum (2 ml) was incubated with 500 μ l of settled protein A-Sepharose for 2 h at 4 °C, and the beads were washed with PBS and buffer D (20 mM HEPES-KOH (pH 7.9), 100 mM KCl, 20% glycerol). The extract (1 ml) was incubated twice with 250 μ l of protein A-Sepharose-bound antibodies supplemented with 100 μ l of fresh protein A-Sepharose for 1 h at room temperature.

Cell Culture and Transfection Assays—Human HeLa or MCF7 cells were grown on coverslips in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum and 100 units/ml penicillin and streptomycin (Life Technologies, Inc.). Transfections were performed after cells had reached ~60–70% confluency using Fugene 6 (Roche Molecular Biochemicals), and transfection conditions were as recommended by the manufacturer. Cells were fixed 18–24 h after transfection.

Cell Staining and Immunofluorescence—Cells were washed in PBS and fixed for 5 min with 3.7% w/v paraformaldehyde in CSK buffer (10 mM Pipes (pH 6.8), 10 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 2 mM EDTA) at room temperature. Permeabilization was performed with 1% Triton X-100 in PBS for 15 min at room temperature. Samples were blocked with 1% v/v normal goat serum (Sigma). Incubation with primary antibodies diluted in 1% v/v normal goat serum was carried out

for 40 min at room temperature. Coverslips were then washed 3 \times 10 min with PBS. Incubation with secondary antibodies (Texas Red-conjugated anti-rabbit and Texas Red-conjugated anti-mouse, Jackson ImmunoResearch) was carried out for 40 min at room temperature. After an additional 3 \times 10 min washes with PBS, coverslips were mounted in Mowiol (Sigma). All fluorescence microscopy work was carried out using a Zeiss LSM 410 confocal laser-scanning microscope. Excitation wavelengths were at 488 nm (for GFP-SPF30) and 543 nm for Texas Red. The following primary antibodies were used: rabbit anti-p80-coilin polyclonal serum 204/5 (dilution 1:350 (36)), antibody Y12 (dilution 1:1000 (37)), and anti-SMN monoclonal MANSMA1 (dilution 1:10 (38)).

In Vitro Splicing Reactions—Splicing reactions (30 μ l final volume) contained 11 μ l of nuclear extract. Reaction conditions were as described (39). Proteins were digested by the addition of 6.75 μ l of buffer K (3 μ l of a stock solution containing 6%(w/v) SDS, 250 mM EDTA, 250 mM Tris-HCl (pH 8.0), and 3.75 μ l of a stock solution containing 16 mg/ml proteinase K and 2 mg/ml tRNA) and incubation at 65 °C for 20 min. The RNA was purified by binding to RNATack Resin (Biotec), eluted in formamide loading buffer (pH 8.0), and analyzed by gel electrophoresis (10% gels) and autoradiography. For the analysis of splicing complexes, reactions were terminated by the addition of heparin to a final concentration of 5 mg/ml. The reactions were loaded onto polyacrylamide/agarose composite gels (4) and run for 5 h at 25 mA.

Affinity Purification—The snRNPs were precipitated from 250 μ l of nuclear extract using a 20- μ l emulsion of anti-2,2,7-trimethylguanosine agarose conjugate (Calbiochem) for 2 h at 4 °C. After extensive washing with buffer D, proteins were eluted with 2% (w/v) SDS and analyzed by gel electrophoresis (12%) and Western blotting (1:3000 dilution anti-SPF30, 1:8000 dilution anti-P99 (40), 1:500 dilution 4G3 (41), 1:500 dilution Y12 (37)).

400 μ g of affinity-purified anti SPF30 were conjugated to 200 μ l of protein A-Sepharose (Amersham Pharmacia Biotech) using dimethylpiperimidate (Sigma) (42). Purified IgG (Sigma) served as mock control. 1 ml of nuclear extract containing 0.1% Triton X-100 was pre-cleared with 500 μ l of IgG-Sepharose and 170 μ l of protein A-Sepharose each for 1 h at 4 °C. The snRNPs were co-precipitated using 12 μ l of the anti-SPF30-Sepharose conjugate for 1 h at 4 °C. The beads were washed with buffer D. Bound material was eluted using 100 mM glycine (pH 2.5) and analyzed by Northern blotting.

The extracts for the precipitated using U snRNA-specific antisense 2'-OCH₃ RNA oligonucleotides carrying terminal biotin residues were prepared as published (43) with the following alterations in the final concentration: 100 mM KCl, 0.1% Nonidet P-40, and 50 μ g of yeast tRNA. 150 pmol of the following 2'-OCH₃ RNA oligonucleotides were added to 200- μ l extracts in the respective reaction: U1 (1–13) ICCAIIUAAIUUAU-C*³C*³C*³ (44), U2 (31–40) IAUACUACACC*³C*³C*³ (45), U4 (53–72) C*³C*³C*³AAAIUUUCAUUAICAAUA (46), U5 (31–45) IAACAGAUACUACACC*³C*³C*³ (44), U6 (57–74) AUCCUICICAIIIIICAC*³C*³ (46), IntB C*³C*³C*³AUUAUCAUAIUUAU (45) (C*³ is biotinylated 2'-deoxycytidine and I is 2'-OCH₃ inosine in place of a guanosine residue). Mixtures were incubated at 30 °C for 1 h. Streptavidine-agarose beads (Sigma) were added (50 μ l) for 30 min at 4 °C, isolated, and washed (20 mM Hepes (pH 7.9), 100 mM KCl, 0.01% Nonidet P-40). Bound proteins were eluted with loading buffer for subsequent SDS-PAGE analysis and Western blotting. One set of samples was used to purify the precipitated U snRNAs by proteinase K digest and ethanol precipitation and investigated by hybridization analysis.

Proteins interacting with recombinant GST-SPF30 were purified at room temperature starting from 15 ml of HeLa nuclear extract containing 0.1% Triton X-100. The extract was pre-cleared with 5 ml of glutathione-Sepharose for 2 h and split, and 80 μ g of GST-SPF30 or GST, respectively, were added. After incubation for 1 h, 100 μ l of glutathione-Sepharose were added for another hour. The beads were collected in a spin column (MoBiCol) and washed with buffer D. Bound material was eluted stepwise with 2 times 80 μ l each of 20 mM HEPES-KOH (pH 7.9) containing 200, 400, or 600 mM KCl, respectively, and finely with 100 mM glycine (pH 2.5). For RNA analysis, 10 μ l of each fraction was kept. The remaining material was pooled and loaded onto a 5–20% gradient SDS-PAGE gel for protein analysis. Silver staining was done as described previously (47).

Mass Spectrometry, Data Base Searches, and Multiple Alignment—Protein bands from gel electrophoresis were excised and processed as described (48, 49). An aliquot of the sample was used for matrix-assisted laser desorption ionization mass spectrometry (50) on a Reflex III mass spectrometer (Bruker-Franzen) equipped with delayed extraction. Spectra were analyzed in LaserOne software (Mortensen and Mann) and a non-redundant data base (European Bioinformatics Institute (EBI), Hinxton, Cambridge, UK) was searched with PeptideSearch

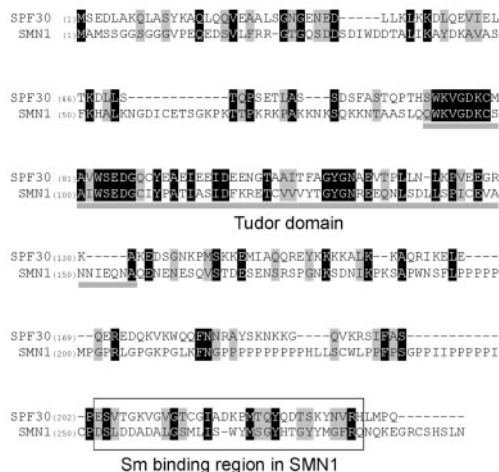


FIG. 1. **SPF30 is related to SMN.** The figure shows a multiple sequence alignment of the human SPF30 and SMN1 proteins. The sequences were aligned using the ClustalW program. Identical and similar amino acids are indicated in *black* and *light shading*, respectively. The sequence to which RNA binding was mapped in SMN1, the positions of the Tudor domain in SMN1 and SPF30, and the position of Sm protein binding to SMN1 are indicated with *gray bars* and a *box*, respectively. The charged amino acids, which are on the same side of a predicted amphipathic α -helix, are marked by an *asterisk*.

(51). Tandem mass spectrometry spectra were recorded on a prototype QSTAR (PE-Sciex) and analyzed. The sequence tag approach (52) was used to search the mass spectrometric data against the non-redundant (47), EST (1), and genomic (74) data bases in the Protein and Peptide Software Suite (Protana A/S).

Homology searches were done using the BLAST algorithm (53), and multiple alignments were constructed using the ClustalW (54) WWW Service at the European Bioinformatics Institute (EBI) and the Jalview program from M. Clamp as well as Vector NTI suite (InforMax).

In Vitro Transcription and Antisense Probes—The Adeno pre-mRNA was transcribed from *Sau3AI*-digested plasmid pBSAd1 (11).

Antisense probes for Northern blot hybridization were generated using materials and protocols described in Ryder et al. (Ref. 43 and references therein). All transcripts were purified by ethanol precipitation or using RNATack Resin (Biotech) and taken up in TE buffer (pH 8.0).

Sedimentation of snRNPs in Glycerol Gradients—1.5 ml of HeLa nuclear extract depleted in SPF30 or mock-treated were layered onto 10 ml of 10–30% (v/v) glycerol gradients in 20 mM HEPES-NaOH (pH 7.9), 150 mM NaCl, and 1.5 mM MgCl₂. After sedimentation at 30,000 rpm for 20 h in a SW41 Ti rotor (Beckman) at 4 °C, fractions of 550 μ l were harvested from top to bottom. Proteins were precipitated from 250 μ l of each fraction by the addition of 20 μ g of glycogen, 0.1 volume of 5 M ammonium acetate, and 3 volumes of ethanol and incubation for 3 h at room temperature. To 300 μ l of each fraction, 10 μ g of tRNA were added. The RNA was extracted from these aliquots for the SPF30-depleted samples by phenol/chloroform extraction and ethanol precipitation (0.1 volume of 5 M ammonium acetate and 2.5 volumes of ethanol, 30 min at 0 °C). In the case of the mock-treated samples, 65 μ l of buffer K was added, and the mix was incubated at 65 °C for 40 min before ethanol precipitation.

RESULTS

SPF30 Is Similar to SMN1—We previously identified SPF30 by mass spectrometric analysis of proteins associated with purified human spliceosomes (1). SPF30 was also cloned independently due to the sequence similarity of its central domain to SMN1 (55). The alignment of SPF30 to SMN1 reveals colinearity of the two proteins (Fig. 1). One region of strong similarity is composed of the Tudor domain, which has been found in a number of proteins with putative RNA binding activity (56). However, RNA binding activity of SMN1 could not be mapped to this domain but was found in the region of amino acids 28–91 (57) and 1–76 (58). Exon 2a of SMN1 (position 28–51) is predicted to form an amphipathic α -helix which mediates the main part of the RNA interaction (58). The conserved structure of the amphipathic α -helix in SPF30 suggests that

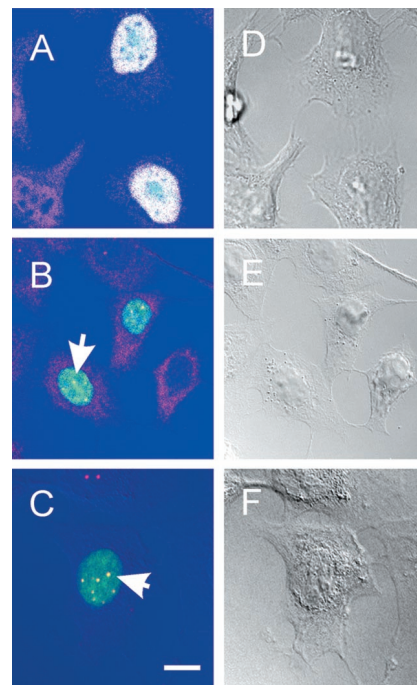


FIG. 2. **Nuclear localization of SPF30.** Micrographs show co-localization of GFP-SPF30 fusion protein with Sm proteins (A), SMN1 (B), and coilin (C) and the corresponding Nomarski images (D, E, F). The signal of GFP-SPF30 (*green*) is confined to the nucleus and displays a speckled pattern that is identical to the snRNP containing speckles (*cf. white label in panel A with green label in panels B and C*). SPF30 additionally localizes to Cajal bodies (*arrows*) together with SMN1 (B) and coilin (C). Bar, 10 μ m.

SPF30 may also bind to RNA. Another region of high similarity is near the C termini of the two proteins. Interestingly, the Sm protein binding part of SMN1 is located in this sequence stretch (30), suggesting that SPF30 may also bind to Sm proteins. Both proteins also show differences in their C-terminal regions. SPF30 has a putative bipartite nuclear localization signal (position 142–159), and SMN1 has a proline-rich region (position 195–248) that is absent from SPF30.

Homology searches were performed in protein, EST, and genomic data bases using the amino acid and the nucleotide sequence of SPF30. Potential orthologs with complete sequence were found in *Arabidopsis thaliana* (GenBank™ accession number AAC18929), *Caenorhabditis elegans* (complete coding sequence can be assembled from EST GenBank™ accession numbers AV202411, C71011, and AV189731), *Drosophila melanogaster* (GenBank™ accession number AAF45352), *Schizosaccharomyces pombe* (GenBank™ accession number CAA22823.1), and *Plasmodium falciparum* (GenBank™ accession number CAB39051). However, no homolog could be detected in *Saccharomyces cerevisiae*.

Nuclear Localization of SPF30—The cellular localization of SPF30 was analyzed by fusing the SPF30 cDNA to the green fluorescent protein (GFP) in a plasmid vector that allows efficient expression of the GFP-SPF30 fusion protein in mammalian cells (pGFP-SPF30). The pGFP-SPF30 plasmid was transiently transfected into HeLa cells, and then the cells were fixed, and the expression pattern of GFP-SPF30 was analyzed by confocal fluorescence microscopy (Fig. 2). The GFP-SPF30 fusion protein was specifically localized in the nucleus, where it showed a speckled pattern characteristic of known pre-mRNA splicing factors (Fig. 2A). Immunolabeling with an anti-Sm monoclonal antibody showed that the GFP-SPF30 protein colocalized with endogenous snRNPs. The *in vivo* colocalization of SPF30 with snRNPs in HeLa cell nuclei is consistent with our

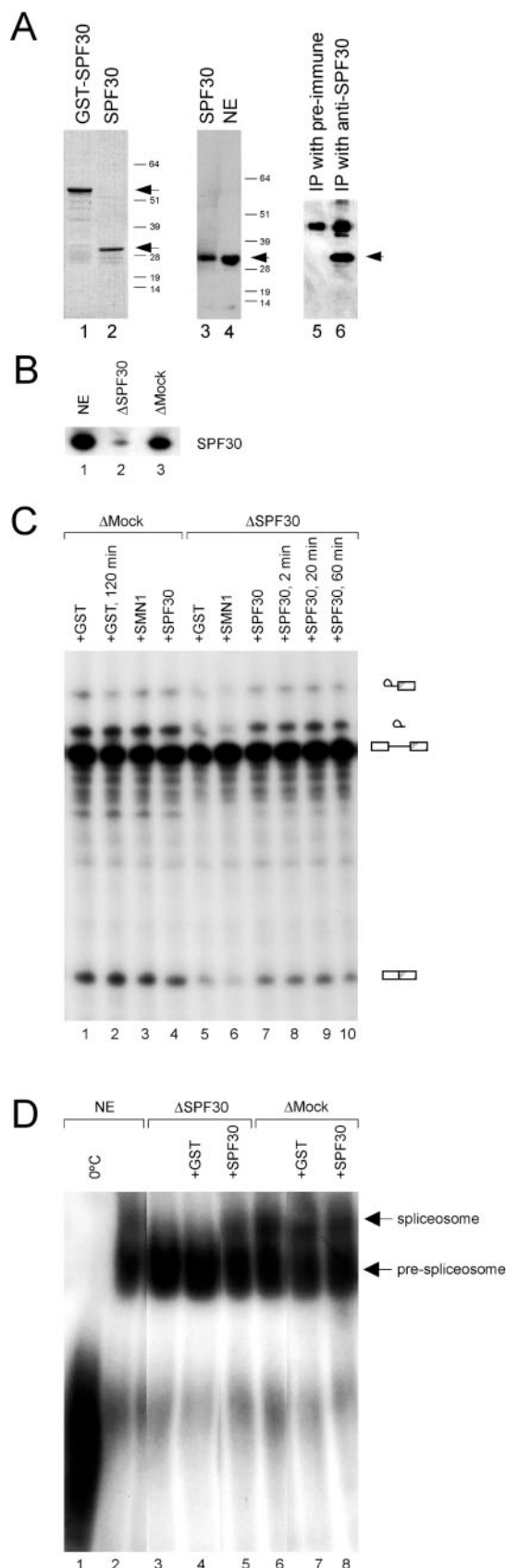


FIG. 3. Influence of SPF30 in spliceosome assembly and splicing *in vitro*. A, Coomassie-stained SDS-PAGE gel showing the purity of the GST-SPF30 fusion protein used for complementation of the Δ SPF30 extracts and as a bait in fishing experiments (lane 1) and the proteolytically released SPF30 protein used for antibody production (lane 2). Protein blot of recombinant SPF30 (lane 3) and nuclear extract (lane 4) showing that recombinant and endogenous SPF30 migrate at

previous finding that SPF30 copurified *in vitro* with spliceosomes formed in HeLa cell nuclear extracts (1). Additional immunofluorescence experiments were performed in which cells transiently expressing GFP-SPF30 were fixed and labeled with antibodies specific for either SMN1 (Fig. 2B) or p80 coilin (Fig. 2C). This revealed that SPF30 is also present in Cajal bodies (also known as coiled bodies (27)), a nuclear structure implicated in snRNP maturation and sometimes located at the site of snRNA gene loci (reviewed by (Matera (28) and Lamond and Earnshaw (59)). We note that the SMN protein is selectively localized within the nucleus to Cajal bodies and is not detected in the additional speckled structures that contain SPF30 and other spliceosome components (Fig. 2, cf. panels A and B). This is consistent with previous observations (Ref. 60; see also Ref. 28). Taken together, the localization data are consistent with an *in vivo* role for SPF30 involving its interaction with splicing complexes and/or snRNPs.

SPF30 Is an Essential Splicing Factor—To characterize the SPF30 protein in greater detail, a rabbit polyclonal antiserum was raised against the recombinantly expressed SPF30 protein after removal of the GST tag by proteolytic cleavage. Fig. 3A shows SDS-PAGE analysis of the purified GST-SPF30 fusion protein (lane 1) and recombinant SPF30 protein purified after proteolytic cleavage of the linker (lane 2). Probing with the rabbit anti-SPF30 antiserum detected a specific band in HeLa nuclear extract of ~ 30 kDa (lane 4) that comigrated with the *E. coli*-expressed, purified SPF30 protein (lane 3). The endogenous SPF30 protein in HeLa nuclear extract was specifically immunoprecipitated by the anti-SPF30 antiserum (lanes 6).

A role for SPF30 in pre-mRNA splicing was investigated by using the anti-SPF30 antiserum to deplete SPF30 from HeLa nuclear extract. Approximately 90% of the SPF30 protein was depleted from HeLa nuclear extract by this procedure (Fig. 3B). Splicing assays performed using SPF30-depleted (Δ SPF30) extracts showed a reduced level of splicing relative to undepleted or mock-depleted control HeLa extracts (Fig. 3C). Splicing is blocked either at or prior to the first transesterification reaction, as evidenced by the reduction in both splicing intermediates and products (lane 5). This suggests an essential role for SPF30 either in the first catalytic step of the splicing reaction or in the prior assembly of the active spliceosome or both.

the same apparent molecular weight. Anti-SPF30 serum but not pre-immune serum immunoprecipitates SPF30. Anti-SPF30 (lane 6) and preimmune (lane 5) sera were used to immunoprecipitate SPF30 from nuclear extract. The SPF30 protein was detected by transferring the immunoprecipitated (IP) proteins to nitrocellulose and probing with anti-SPF30 serum. B, a protein blot showing HeLa nuclear extract probed with anti-SPF30 antibodies. The level of SPF30 is reduced by $\sim 90\%$ in Δ SPF30 extracts (lane 2) as compared with levels of SPF30 in nuclear extract (NE) (lane 1) and mock-depleted nuclear extract (Δ Mock extract) (lane 3). C, *in vitro* splicing assay using Δ Mock (lanes 1–4) and Δ SPF30 extracts (lanes 5–10) complemented with recombinant protein as denoted and analyzed on 10% urea-PAGE. The splicing defect of Δ SPF30 extracts can only be compensated by the addition of SPF30 (lane 7) but not by GST (lane 5) or SMN1 (lane 6), whereas none of these proteins exhibits a notable effect on the splicing activity in Δ Mock extracts (lanes 1–4). SPF30 was added 2, 20, or 60 min (lanes 8–10) after the shift of the splicing mixtures to 30 °C. After the addition of SPF30, the splicing mixtures were left for another 90 min at 30 °C, resulting in resumed splicing for all time points. The length of incubation and the addition of GST alone did not affect the splicing reaction (lanes 1 and 2). D, complexes containing radioactive pre-mRNA separated on non-denaturing agarose-acrylamide composite gels. A complexes (pre-spliceosome) can form in Δ SPF30 extracts (lanes 3–5), whereas formation of B complexes (spliceosome) requires the addition of SPF30 (lane 5). The negative and positive controls for the formation of the splicing-related complexes are shown in lanes 1 and 2, whereas lanes 6–8 show that complex formation in the mock-depleted extract is not affected by the depletion procedure nor by the addition of GST or SPF30.

The suppression of splicing in Δ SPF30 extracts could be caused either by the reduction of the SPF30 concentration or by the co-depletion of one or more essential splicing factor(s) that binds to SPF30. To test this, a complementation experiment was performed by adding the purified, *E. coli*-expressed GST-SPF30 fusion protein to the depleted extract (Fig. 3C). This showed that splicing activity was stimulated by adding recombinant GST-SPF30 alone (lane 7). Therefore, we conclude that SPF30 is an essential splicing factor and that no other essential factor is co-depleted from the extract by the anti-SPF30 antiserum to a level where it is rate-limiting for splicing. The addition of recombinant SMN1 to Δ SPF30 extracts did not restore splicing, indicating that SMN cannot replace the function of SPF30 (lane 6). The *E. coli*-expressed SPF30 protein enhanced splicing regardless of whether it was added to the Δ SPF30 extract at 0 °C, *i.e.* before the spliceosome can assemble (lane 7), or added back at different time points after the shift to 30 °C, which allows spliceosome assembly (lanes 8–10). Thus, whatever spliceosome assembly step or function is blocked by the absence of SPF30, it can always be rescued by the subsequent addition of the SPF30 alone. In undepleted, control HeLa extracts, the concentration of SPF30 does not appear to be rate-limiting for splicing because the addition of excess SPF30 neither increases nor decreases splicing activity (lane 4). In summary, we conclude that SPF30 is an essential splicing factor.

Spliceosome Assembly Halts at the Pre-spliceosome A Complex in the Absence of SPF30—The absence of SPF30 may inhibit splicing either by preventing spliceosome assembly or by blocking catalysis of the splicing reaction. To distinguish between these alternative possibilities, we next analyzed the formation of splicing complexes in Δ SPF30 and control HeLa nuclear extracts by native gel electrophoresis (Fig. 3D). This shows that the pre-spliceosome A complex, but not the mature spliceosome, forms in Δ SPF30 extracts (lane 3). Formation of the spliceosome in depleted extract is restored by the addition of recombinant SPF30 alone (lane 5). Taking into account that spliceosome assembly is restored to the depleted extract even when SPF30 is added at late time points (see above), we infer that the A complex formed in the Δ SPF30 extract is potentially functional. Although this finding does not exclude an additional role for SPF30 at a later point in spliceosome assembly or in the splicing reaction itself, it demonstrates that SPF30 is essential for the formation of the spliceosome. The fact that assembly is halted at the A complex indicates that SPF30 is required before the addition of the U4/U5/U6 snRNP to the spliceosome. This could be due to an essential role of SPF30 either in the formation of the tri-snRNP or in some function required for the docking of the assembled tri-snRNP to the A complex.

SPF30 Is Not Essential for Assembly of the Tri-snRNP—To address whether the suppression of splicing in Δ SPF30 extracts results from a failure to assemble the tri-snRNP or, instead, results from a defect in docking of the tri-snRNP to the A complex, we analyzed snRNP complexes in a gradient centrifugation experiment. The U4, U5, and U6 snRNPs from Δ SPF30 extracts migrate in a similar fashion to mock-treated, control HeLa extracts that are active for spliceosome assembly and splicing (Fig. 4, A and B). This shows that in Δ SPF30 extracts, the tri-snRNP is still formed. We conclude that blocking of the splicing reaction in Δ SPF30 extract is not specifically due to a visible failure of tri-snRNP assembly but rather results from failure of the tri-snRNP to either become competent or to add to the A complex.

SPF30 Interacts with the Tri-snRNP and with the U2 snRNP—As tri-snRNP binding to the A complex is dependent upon SPF30, we performed next a series of experiments to examine if SPF30 directly interacts with snRNPs.

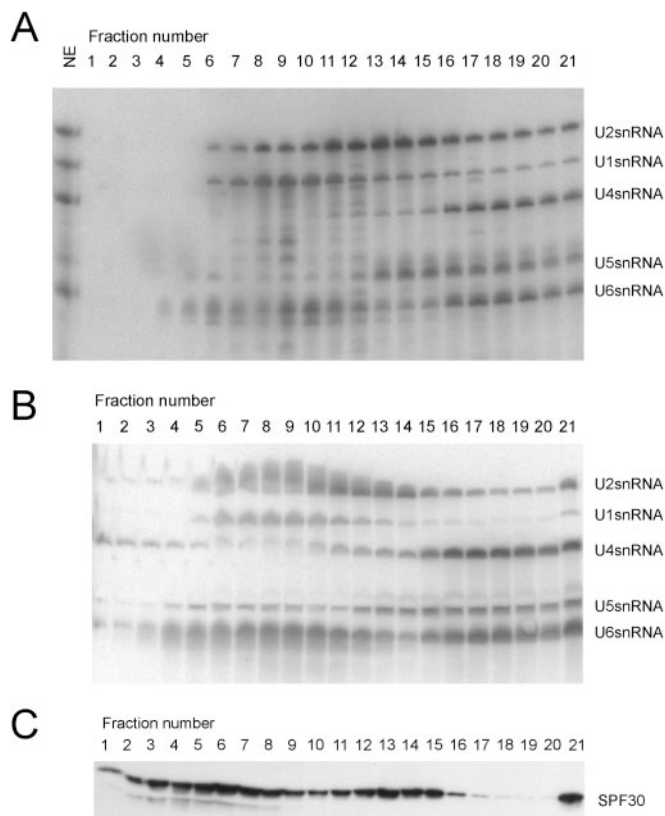


FIG. 4. Gradient centrifugation of nuclear extract. Shown is fractionation of Δ SPF30 and Δ Mock extracts by 5–20% glycerol gradient centrifugation. The fractions were taken from top to bottom and split to analyze the position of SPF30 and of the U snRNAs. A and B, the U snRNA content of the fractions was analyzed by urea-PAGE and RNA hybridization. The U snRNAs show the same migration behavior in Δ SPF30 (A) and Δ Mock (B) extracts, revealing that SPF30 is not required for tri-snRNP stability. C, a protein blot of the SDS-PAGE-separated fractions shows that SPF30 migrates in two populations, one peaking in fraction 6 and the other in fraction 13. Fraction 21 contains pelleted SPF30.

First, SPF30 migrated in the glycerol centrifugation in two populations peaking in fractions 6 and 13 (Fig. 4C). The population at higher glycerol density co-migrated with U2 snRNA, which also peaked in fraction 13 (Fig. 4B). Therefore, SPF30 may interact with U2 snRNP.

A second experiment directly demonstrated the interaction of SPF30s with complexes containing snRNPs. For this snRNPs were isolated by immunoprecipitation using an antibody that specifically recognizes the snRNA trimethylated cap structure (anti-m³G cap). The immunoprecipitated proteins were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the anti-SPF30 antiserum (Fig. 5A). This revealed the presence of SPF30 in the precipitate (lane 2). In contrast, the nuclear protein p99, which is not associated with snRNPs, was not detected in the m³G cap precipitate (lane 2). However, SPF30 does not behave like a *bona fide* snRNP protein because a smaller fraction of SPF30 is immunoprecipitated relative to the core snRNP protein Sm B (Fig. 5A, *cf.* lane 1 and lane 2). Therefore, SPF30 either binds directly to snRNPs or else is a component of larger complexes that also contain snRNPs.

In a third experiment, HeLa nuclear extracts were depleted using either anti-SPF30 or control antibodies, and the presence of U snRNAs in the immunoprecipitates was investigated by hybridization analysis (Fig. 5B). Anti-SPF30 but not the con-

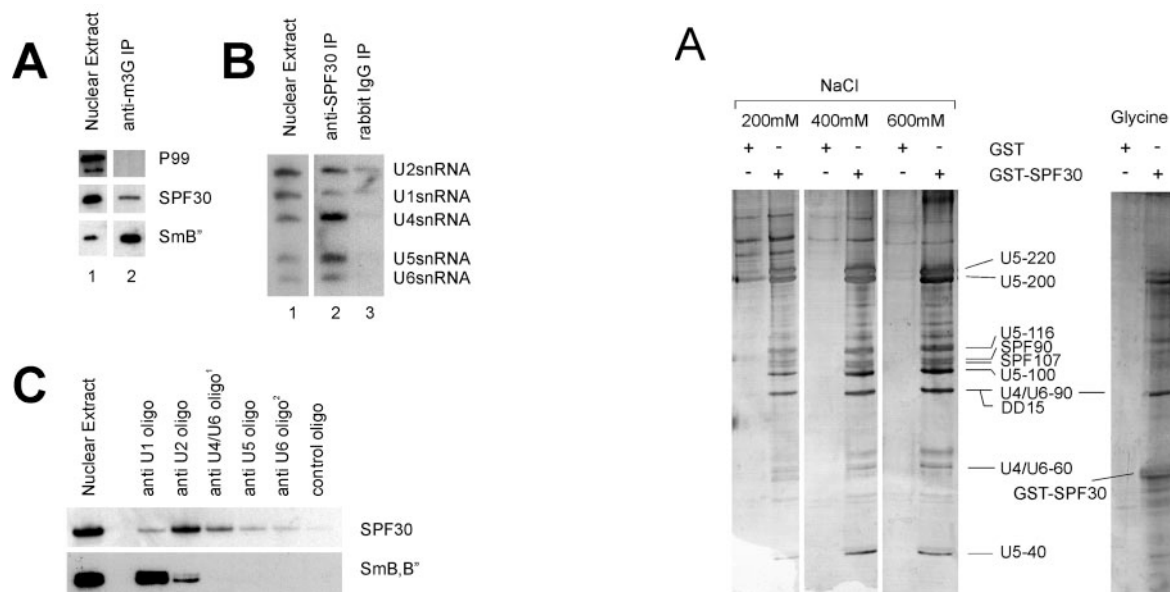


FIG. 5. Interaction of endogenous and recombinant SPF30 with snRNPs. A, SPF30 is co-immunoprecipitated using m3G-cap-specific antibodies against snRNPs, as shown by probing protein blots with the anti-SPF30 antibody. A small fraction of the total SPF30 is isolated in the anti-m3G cap immunoprecipitate as compared with the efficient precipitation of Sm B, one of the core snRNP proteins. P99, a nuclear protein unrelated to splicing, is not detected in the immunoprecipitate, and serves as a negative control. *Lane 1*, shows total HeLa nuclear extract proteins; *lane 2* shows proteins in the m3G immunoprecipitate (*IP*). B, U snRNAs co-immunoprecipitated with the anti-SPF30 antibody were analyzed on a 10% urea-PAGE gel followed by transfer to a nylon membrane and hybridization with anti-snRNA probes. *Lane 1* shows total snRNAs in HeLa nuclear extract, *lane 2* shows snRNAs isolated with the anti-SPF30 antibody, and *lane 3* shows the negative control selection with nonspecific antibodies. Anti-SPF30 specifically binds U2, U4, U5, and U6 snRNAs, whereas U1 snRNA is not enriched relative to the control (*lane 3*). C, U snRNPs were precipitated using U snRNA-specific antisense 2'-OCH₃ RNA oligonucleotides carrying terminal biotin residues. The protein content was analyzed by SDS-PAGE and Western blotting using anti SPF30 antiserum and the Sm-specific antibody Y12. ¹, the U4-specific oligonucleotide precipitates the U4/U6 snRNP. ², the U6-specific oligonucleotide precipitates approximately equal amounts of U4/U6 snRNP and free U6 snRNP.

control antibodies resulted in co-precipitation of U4, U5, and U6 snRNAs (Fig. 5B, cf. lanes 2 and 3). In addition, U2 snRNA is specifically precipitated by anti-SPF30 antibodies as compared with the control, whereas a small amount of U1 bound nonspecifically to both the anti-SPF30 and the control antibodies (cf. lanes 2 and 3). These data are consistent with the binding of SPF30 to complexes containing U2 snRNP, as previously suggested by their co-migration in glycerol gradients and the U4/U5/U6 tri-snRNP.

In a fourth approach to analyze the interaction of SPF30 with snRNP components, recombinant GST-SPF30 protein was used to affinity-select interaction partners that bind in HeLa nuclear extracts. The material bound to GST-SPF30 was eluted, split, and separated by respective PAGE to investigate for their RNA (data not shown) and protein content (Fig. 6A). Hybridization analysis showed that the U4, U5, and U6 snRNA bound to recombinant GST-SPF30. In addition, traces of U2 snRNA could be isolated. No U1 snRNA could be detected, serving as an internal control. Additional control experiments using the GST protein tag alone as a bait showed that it failed to select any interacting U snRNAs, confirming the specificity of the GST-SPF30 data (data not shown).

To specify in which U snRNP complexes SPF30 can be found, we used in a fifth approach a series of U snRNA-specific antisense 2'-OCH₃ RNA oligonucleotides carrying terminal biotin

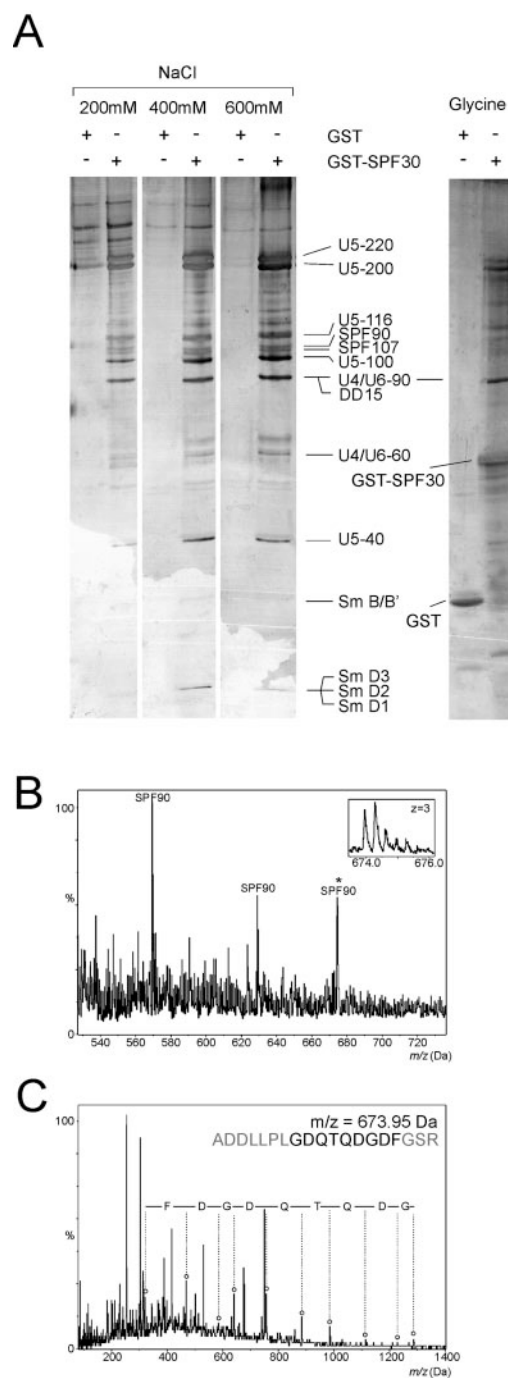


FIG. 6. SPF30 interacts with snRNP proteins. A, analysis of proteins bound to GST-SPF30 and eluted in the same experiment described in Fig. 5C. Proteins were separated by 10–20% gradient SDS-PAGE and visualized by silver-staining. Bands were excised, digested with trypsin, and analyzed by mass spectrometry to identify the components. hSad1p, DD15, SPF107, and SPF90 are the human homologs to *S. cerevisiae*-splicing proteins Sad1p, PRP43, PRP6, and Snu66, respectively. B and C, mass spectra leading to the identification of SPF90/SART1₈₀₀. B, nano-electrospray mass spectrum of the mixture of tryptic peptides obtained by in-gel digest of a protein band. The inset shows the peptide signal selected for fragmentation (marked by an asterisk). The peaks arise from the natural occurrence of ¹²C and ¹³C and are spaced 0.33-Da intervals, indicating that the peptide is triply charged. C, the spectrum shows a series of fragments obtained by collision of the selected peptide with N₂ in the mass spectrometer. The fragments differ in length by a single amino acid, which allows assignment of part of the peptide sequence. The mass of the peptide together with the fragment masses and the sequence TL is sufficient to uniquely identify SPF90/SART1₈₀₀ by the peptide sequence tag algorithm in a nonredundant data base containing nearly 500,000 entries. The rest of the sequence was used to confirm the result.

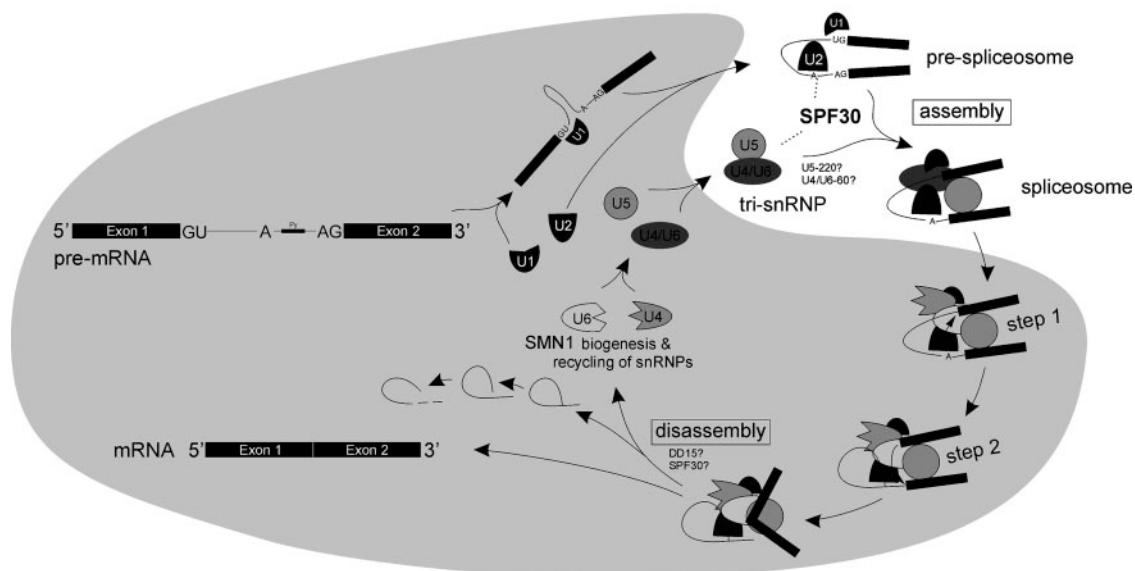


FIG. 7. **SPF30 promotes tri-snRNP assembly onto the A complex.** The schematic shows a representation of the spliceosome assembly cycle, indicating the proposed role for SPF30 in promoting the addition of the U4/U5/U6 tri-snRNP to the A complex.

residues. Binding of the oligonucleotides to the respective U snRNAs allows the specific purification of the U1, U2, U4/U6, U5, and U6 snRNP (44–46). The protein components of the isolated U snRNPs were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and probed with the anti-SPF30 antiserum and the Sm-specific antibody Y12 (Fig. 5C), whereas specificity of the purification was separately controlled by hybridization analysis (data not shown). Following the signal of Sm B, B' shows that large amounts of U1 and U2 snRNP could be purified. In the fractions of the U4/U6, U5, and U6 snRNP, only at long exposure times could a signal be detected, whereas a substantial signal for SPF30 is seen co-precipitating with the U2 snRNP, U4/U6, U5, and U6 snRNP, indicating association of SPF30 to these complexes. In contrast, only very little SPF30 can be isolated with the U1 snRNP considering the large quantities that could be isolated of this complex. The IntB oligonucleotide does not bind any of the U snRNAs and serves as negative control. Taken together, the data indicate that SPF30 associates with, both, complexes containing tri-snRNP components and complexes containing U2 snRNP components.

GST-SFP30 Associates with Complexes Containing Tri-snRNP Proteins—Next, we identified the proteins obtained by elution from the GST-SPF30 beads (see above). Eluates were separated on a one-dimensional SDS-PAGE gel and subsequently stained with silver. Bands were excised and trypsin-digested as described (49) (Fig. 6A). The resulting peptides were subjected to matrix-assisted laser desorption ionization mass fingerprinting and nano electrospray peptide sequencing (48). Of the U5 snRNP, the following proteins were identified: U5–220, U5–200, U5–116, U5–100, and U5–40. The U4/U6 snRNP-specific proteins U4/U6–90 and U4/U6–60 and the snRNP core proteins Sm B/B', D1, D2, and D3 were also identified. Under the conditions of stepwise elution U4/U6–90 is bound most strongly to SPF30, indicating a possible direct interaction. In addition to the known human tri-snRNP proteins, a number of new spliceosomal proteins have been found. SPF107 is a human homolog of *S. cerevisiae* PRP6 and was cloned as a 100-kDa protein (GenBank™ accession number AB006198) recognized by an anti-peptide antibody against an epitope containing the nuclear localization signal of NF- κ B p65 subunit (61). SPF90 is the human homolog to *S. cerevisiae* Snu66, a yeast tri-snRNP protein (62, 63). Two forms of this protein have been described as a carcinoma antigen recognized

by T cells; one is a small cytosolic form, SART1₂₅₉, which is expressed at various levels in different tumors, and the other is a large nuclear form SART1₈₀₀ (GenBank™ accession number AB019219), which is ubiquitously expressed (64). The nuclear localization and the apparent molecular weight made SART1₈₀₀ the likely form of SPF90. This was confirmed by nano electrospray peptide sequencing. Of the five sequenced peptides, two were common to SART1₂₅₉ and SART1₈₀₀, but three were specific to SART1₈₀₀ (Fig. 6B). hSad1p is a homolog of *S. cerevisiae* Sad1p, a protein found to function in U4/U6 snRNP formation (65) and not identified in the yeast tri-snRNP. DD15 is a homolog of *S. cerevisiae* PRP43, a protein containing a helicase domain. PRP43 was, like Sad1p, not identified in the yeast tri-snRNP but has been described as involved in the disassembly of the spliceosome (66, 67). The identification of hSad1p and DD15 here may reflect a difference between the *S. cerevisiae* and *Homo sapiens* tri-snRNP complexes. Alternatively, the direct interaction of hSad1p with SPF30 would indicate a more general role of SPF30 as an snRNP assembly factor. The direct interaction of DD15 with SPF30 may be the equivalent to the binding of SMN1 to the RNA helicase Gemin3 (33). If DD15, like its yeast homolog PRP43, should play a role in spliceosome disassembly, then SPF30 may not only be involved in spliceosome assembly but also in its disassembly.

DISCUSSION

In this work we have analyzed the function of SPF30, a protein that was previously identified in a proteomic screen of the human spliceosome (1). Here we report that SPF30 is an exclusively nuclear protein that colocalizes with Sm proteins in speckled structures known to contain splicing factors. We show that SPF30 is also an essential splicing factor that is required for spliceosome assembly and splicing. Specifically, depletion of SPF30 from HeLa nuclear extracts blocks spliceosome formation at the A complex before the addition of the U4/U5/U6 tri-snRNP. However, the tri-snRNP is still formed in HeLa extracts depleted of SPF30. Both spliceosome formation and splicing are restored to depleted extracts by addition of *E. coli*-expressed SPF30 alone. A combination of immunodepletion and protein interaction experiments indicate that SPF30 associates with complexes containing U2 snRNP components and complexes containing U4/U5/U6 tri-snRNP components.

These data lead us to propose that SPF30 is an essential protein-splicing factor whose function is required for the addition of the tri-snRNP to the A complex during spliceosome assembly (Fig. 7).

SPF30 is the first and so far only reported homologue of SMN1, the gene responsible for spinal muscular atrophy. Both proteins are co-linear and share a central Tudor domain. A second region of strong similarity falls onto the sequence stretch that is involved in the binding of SMN1 to Sm proteins, the core proteins of snRNPs (30). Previous work has implicated SMN1 in the biogenesis as well as in the recycling of snRNPs (31, 32).

We find that SPF30 colocalizes together with SMN1 in Cajal bodies (also called coiled bodies) in HeLa nuclei. These are structures previously implicated in the biogenesis and recycling of snRNPs (27, 29, 32, 68). However, apart from their colocalization in Cajal bodies, the SPF30 and SMN proteins have distinct cellular distributions. Thus, SPF30, but not SMN, is found in nuclear speckles together with other splicing factors and spliceosome proteins, whereas SMN1 is also found in the cytoplasm. These differences in cellular localization may reflect the preferential involvement of SMN1 in snRNP biogenesis, whereas SPF30 is actively involved in the splicing reaction catalyzed by mature snRNPs. Consistent with this view, SPF30, but not SMN1, was shown to associate with purified spliceosomes *in vitro* (1). We also show here that purified SMN1 cannot substitute for the function of SPF30 that is required during spliceosome assembly. Our data indicate that the SPF30 and SMN1 proteins play quite distinct roles connected with splicing. This does not exclude, however, that SMN1 has an important role in the mechanism of splicing *in vivo*, such as in snRNP recycling, as previously suggested (32).

We present a variety of experimental evidence in this study that all points to the conclusion that SPF30 can interact with the spliceosomal snRNPs U2 and U4/U5/U6 as follows. (i) SPF30 is co-immunoprecipitated with anti-m3G cap antibodies; (ii) anti-SPF30 antibodies co-immunoprecipitate U2, U4, U5, and U6 snRNP; (iii) antisense oligonucleotides specific for U2, U4/U6, U5, and U6 snRNP coprecipitate SPF30; (iv) recombinant GST-SPF30 can affinity select U4, U5, and U6 snRNP as well as small amounts of U2 snRNP; and (v) SPF30 co-fractionates with U2 snRNP in glycerol gradients. In contrast, none of the experiments reveal any evidence for interaction of SPF30 with the U1 snRNP. Taken together, these data show that SPF30 either binds directly to U2, U4/U6, and U5 snRNP or else forms complexes with other nuclear factors that include these snRNPs. However, it is not likely that SPF30 is a new snRNP protein. Only a minor fraction of the total SPF30 protein in HeLa nuclear extract can be coprecipitated with snRNPs, and only a fraction co-migrates with the U2 snRNP. In addition, despite extensive analysis of the snRNPs by the laboratories of Lührmann (23, 69, 70) and others, SPF30 has not been reported so far. On the other hand, purifications of the U2 snRNP (23), the U5 snRNP (69), and the tri-snRNP (70) show a substoichiometric band of the approximate size of SPF30. This would be consistent with a loose attachment of SPF30 to these snRNPs or with SPF30 associating only with a subset of these snRNP complexes.

The co-fractionation of endogenous SPF30 with the U2 snRNP and the fact that anti-SPF30 antibody precipitates U2 snRNA whereas antisense oligonucleotides specific for U2 snRNA precipitate SPF30 suggests that SPF30 binds to this complex. In addition, tri-snRNP components can be affinity-purified using recombinant GST-SPF30. Anti-SPF30 also co-immunoprecipitates the U4, U5, and U6 snRNAs that are found in the tri-snRNP, and antisense oligonucleotides specific for U4, U5, and U6 snRNAs precipitate SPF30. Taken together,

these findings also suggest that SPF30 binds to the U4/U6 and U5 snRNP. Although all snRNP complexes may bind to the same site in SPF30, the homology to SMN1 predicts two putative interaction sites in SPF30. SPF30 shares a strong similarity in the C terminus with the Sm protein binding region of SMN1, and both proteins have a Tudor domain. It has been shown that SMN1 binds RNA in its N-terminal region, and the main contribution is thought to come from a predicted amphipathic α -helix (58). This α -helix is conserved in SPF30 and supports the view of direct RNA binding for this protein. Furthermore, recombinant SPF30 is able to bind RNA from total HeLa RNA preparation on its own.²

The fact that SPF30 apparently only co-fractionates with the U2 snRNP in glycerol gradients and not also with the tri-snRNP as could be expected from the other experiments could be explained by a detection problem. The U2 snRNP is much more abundant than the tri-snRNP components. Therefore, the presence of SPF30 in the tri-snRNP may be overlooked in this kind of experiment. A similar effect is seen in the isolation of the individual U snRNPs. Our observation that U2 snRNP is the U snRNP most strongly interacting with SPF30 may be explained by the relative higher amounts of U2 snRNP in nuclear extract compared with the amount of tri-snRNP.

We have shown that SPF30 is an essential factor in splicing, as judged by the reduction in splicing activity in SPF30-depleted extracts that is then enhanced by specific addition of *E. coli*-expressed SPF30 protein alone. The presence of orthologs of SPF30 in model organisms like *C. elegans*, *A. thaliana*, *S. pombe*, and *P. falciparum* is consistent with its having an essential role in splicing. Although we did not detect any SPF30-like protein in budding yeast, we note that some other essential mammalian-splicing factors, including the RS domain proteins, are also not found in *S. cerevisiae*, nor are other Tudor domain proteins like SMN1. SPF30 and SR proteins may be involved in spliceosome assembly mechanisms that are performed differently in this organism.

An analysis of the assembly of the spliceosome in Δ SPF30 extracts reveals that the A complex, which contains U2 snRNP but not the tri-snRNP, can form and accumulates in the absence of SPF30. The addition of recombinant SPF30 at both early and late incubation times restores spliceosome formation and splicing. This indicates that at least some of the A complexes formed in the absence of SPF30 can proceed in the splicing pathway once the lacking component is added. We also found that the tri-snRNP is assembled in Δ SPF30 extracts. These observations suggest that SPF30 is required for the recruitment of the tri-snRNP into the A complex to form the spliceosome. Previous work has been done both in human and *S. cerevisiae*-splicing systems to analyze this assembly step. In *S. cerevisiae*, antibodies against PRP4 (20) and PRP8 (19) prevented the addition of the tri-snRNP into the pre-spliceosome apparently without affecting the stability of the tri-snRNP. Because both proteins are required for tri-snRNP assembly, direct study of tri-snRNP docking is difficult. None of the temperature-sensitive mutants reported so far (19, 71) has been shown to block tri-snRNP docking; thus, it remains unclear whether inactivation of PRP4 and PRP8 blocks only tri-snRNP assembly or also docking to the pre-spliceosome. In HeLa-splicing extracts, SR proteins have been shown to be essential for the incorporation of high salt-precipitated tri-snRNPs into purified pre-spliceosome complexes (25). However, the salt precipitate does not contain the SR proteins, some of which are tri-snRNP-specific and required for its assembly (such as the 27-kDa tri-snRNP-specific protein) (70, 72). Since the tri-

² J. Rappsilber and M. Mann, unpublished data.

snRNP is disassembled after high salt precipitation, it is not clear if the SR proteins were necessary for the docking of the tri-snRNP rather than only for its assembly.

We propose two alternative models that are difficult to distinguish. In the first model SPF30 is a bridging factor between the tri-snRNP on one side and U2 snRNP in the pre-spliceosome on the other side. Given the fact that splicing incompetent Δ SPF30 extracts can be rescued by SPF30 alone, suggesting that no rate-limiting factor is co-depleted, and due to the association of SPF30 to the U2, U4/U6, U5, and possibly U6 snRNP, we suggest that this bridging may be direct (Fig. 7). For example, SPF30 interactions may join the two large complexes to form the spliceosome. In the second model SPF30 has a more general role as an assembly factor. Among the proteins isolated using recombinant SPF30 was hSad1p, the human homolog of *S. cerevisiae* Sad1p, a protein found to function in U4/U6 snRNP formation (65), suggesting a possible role of SPF30 in U4/U6 snRNP formation or an involvement of this protein in later assembly steps. But SPF30 is also found associated with both the U4/U6 snRNP, potentially binding to U4/U6-90, and the U5 snRNP, which must fuse to form the tri-snRNP. Our data also indicate that SPF30 is needed for the docking of the tri-snRNP to the A complex, all supporting the notion of the second model. Furthermore, we have found a possible interaction of SPF30 with DD15, the human homolog of *S. cerevisiae* PRP43, a RNA helicase that has been implicated in spliceosome disassembly. Therefore, SPF30 may be involved not only in the assembly but also in the disassembly of the spliceosome, perhaps as a more general assembly/disassembly factor for higher order snRNP complexes. In this SPF30 may complement the function of SMN1, which is proposed to be involved in snRNP recycling (32). Future studies will be directed toward testing these models and further characterizing the role of SPF30 in the splicing mechanism.

Acknowledgments—We are grateful to Alexander Gottschalk and Maarten Fornerod for discussions and suggestions. We thank Kai te Kaat for help with the expression and purification of the recombinant protein and Ursula Ryder for support during the Northern blot analysis. J. Rappsilber was generously hosted by Matthias Wilm for a prolonged period at EMBL, and we thank the members of the Lamond, Wilm, and Mann laboratories for encouragement and support.

Note Added in Proof—While this manuscript was in print, another report describing SPF30 was published (73).

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