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Essential Role of a Trypanosome U4-Specific Sm Core Protein in Small Nuclear Ribonucleoprotein Assembly and Splicing▽†

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Spliceosomal small nuclear ribonucleoproteins (snRNPs) in trypanosomes contain either the canonical heptameric Sm ring or variant Sm cores with snRNA-specific Sm subunits. Here we show biochemically by a combination of RNase H cleavage and tandem affinity purification that the U4 snRNP contains a variant Sm heteroheptamer core in which only SmD3 is replaced by SSm4. This U4-specific, nuclear-localized Sm core protein is essential for growth and splicing. As shown by RNA interference (RNAi) knockdown, SSm4 is specifically required for the integrity of the U4 snRNA and the U4/U6 di-snRNP in trypanosomes. In addition, we demonstrate by *in vitro* reconstitution of Sm cores that under stringent conditions, the SSm4 protein suffices to specify the assembly of U4 Sm cores. Together, these data indicate that the assembly of the U4-specific Sm core provides an essential step in U4/U6 di-snRNP biogenesis and splicing in trypanosomes.

The excision of intronic sequences from precursor mRNAs is a critical step during eukaryotic gene expression. This reaction is catalyzed by the spliceosome, a macromolecular complex composed of small nuclear ribonucleoproteins (snRNPs) and many additional proteins. Spliceosome assembly and splicing catalysis occur in an ordered multistep process, which includes multiple conformational rearrangements (35). Spliceosomal snRNPs are assembled from snRNAs and protein components, the latter of which fall into two classes: snRNP-specific and common proteins. The common or canonical core proteins are also termed Sm proteins, specifically SmB, SmD1, SmD2, SmD3, SmE, SmF, and SmG (10; reviewed in reference 9), which all share an evolutionarily conserved bipartite sequence motif (Sm1 and Sm2) required for Sm protein interactions and the formation of the heteroheptameric Sm core complex around the Sm sites of the snRNAs (3, 7, 29). Prior to this, the Sm proteins form three heteromeric subcomplexes: SmD3/SmB, SmD1/SmD2, and SmE/SmF/SmG (23; reviewed in reference 34). Individual Sm proteins or Sm subcomplexes cannot stably interact with the snRNA. Instead, a stable subcore forms by an association of the subcomplexes SmD1/SmD2 and SmE/SmF/SmG with the Sm site on the snRNA; the subsequent integration of the SmD3/SmB heterodimer completes Sm core assembly.

In addition to the canonical Sm proteins, other proteins carrying the Sm motif have been identified for many eukaryotes. Those proteins, termed LSm (like Sm) proteins, exist in distinct heptameric complexes that differ in function and localization. For example, a complex composed of LSm1 to LSm7 (LSm1-7) accumulates in cytoplasmic foci and participates in mRNA turnover (4, 8, 31). Another complex, LSm2-8, binds to the 3′ oligo(U) tract of the U6 snRNA in the nucleus (1, 15, 24). Finally, in the U7 snRNP, which is involved in histone mRNA 3′-end processing, the Sm proteins SmD1 and SmD2 are replaced by U7-specific LSm10 and LSm11 proteins, respectively (20, 21; reviewed in reference 28).

This knowledge is based primarily on the mammalian system, where spliceosomal snRNPs are biochemically well characterized (34). In contrast, for trypanosomes, comparatively little is known about the components of the splicing machinery and their assembly and biogenesis. In trypanosomes, the expression of all protein-encoding genes, which are arranged in long polycistronic units, requires *trans* splicing. Only a small number of genes are additionally processed by *cis* splicing (reviewed in reference 11). During *trans* splicing, a short noncoding miniexon, derived from the spliced leader (SL) RNA, is added to each protein-encoding exon. Regarding the trypanosomal splicing machinery, the U2, U4/U6, and U5 snRNPs are considered to be general splicing factors, whereas the U1 and SL snRNPs represent *cis*- and *trans*-splicing-specific components, respectively. In addition to the snRNAs, many protein splicing factors in trypanosomes have been identified based on sequence homology (for example, see references 14 and 19).

Recent studies revealed variations in the Sm core compositions of spliceosomal snRNPs from *Trypanosoma brucei*. Specifically, in the U2 snRNP, two of the canonical Sm proteins, SmD3 and SmB, are replaced by two novel, U2 snRNP-specific proteins, Sm16.5K and Sm15K (33). In this case, an unusual purine nucleotide, interrupting the central uridine stretch of the U2 snRNA Sm site, discriminates between the U2-specific and the canonical Sm cores. A second case of Sm core variation was reported for the U4 snRNP, in which a single protein, SmD3, was suggested to be replaced by the U4-specific LSm

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protein initially called LSm2, and later called SSm4, based on a U4-specific destabilization after SSm4 knockdown (30). A U4-specific Sm core variation was also previously suggested and discussed by Wang et al. (33), based on the inefficient pulldown of U4 snRNA through tagged SmD3 protein. However, neither of these two studies conclusively demonstrated by biochemical criteria that the specific Sm protein resides in the U4 Sm core; a copurification of other snRNPs could not be unequivocally ruled out.

By using a combination of RNase H cleavage, tandem affinity purification, and mass spectrometry, we provide here direct biochemical evidence that in the variant Sm core of the U4 snRNP, only SmD3 is replaced by the U4-specific SSm4. SSm4 is nuclear localized, and the silencing of SSm4 leads to a characteristic phenotype: dramatic growth inhibition, general *trans*- and *cis*-splicing defects, a loss of the integrity of the U4 snRNA, as well as a destabilization of the U4/U6 di-snRNP. Furthermore, *in vitro* reconstitution assays revealed that under stringent conditions, SSm4 is sufficient to specify U4-specific Sm core assembly. In sum, our data establish SSm4 as a specific component of the U4 Sm core and demonstrate its importance in U4/U6 di-snRNP biogenesis, splicing function, and cell viability.

MATERIALS AND METHODS

Cell culture. Cell culture of the procyclic form of *T. brucei* strain 427 and of stably transfected cell lines was done as described previously (5, 25).

RNA interference (RNAi) silencing and reverse transcription (RT)-PCR. The *T. brucei* SSm4 coding region (Tb927.7.6380) from positions 4 to 489 was PCR amplified and cloned as a stem-loop module into the vector pLEW100 (32). For homologous recombination of the pLEW100-SSm4 construct, 10 μg was first linearized with SacII and used for the transfection of *T. brucei* 29-13 cells, followed by cloning in the presence of G418 (15 μg/ml), hygromycin (50 μg/ml), and phleomycin (2.5 μg/ml). For silencing, doxycycline (1 μg/ml) was added. Cells were counted daily and diluted back to 2×10^6 cells per ml.

For semiquantitative and real-time RT-PCR analysis (iCycler with iQ5 software; Bio-Rad), RNA was prepared after 3 days of RNAi induction or from uninduced cells by using TRIzol reagent (Invitrogen) and further purified with the RNeasy minikit (Qiagen). Total RNA was reverse transcribed with the iScript cDNA synthesis kit (Bio-Rad). Amplification products were analyzed by agarose gel electrophoresis. 7SL RNA-specific primers were used in control reactions for semiquantitative RT-PCR as well as for the normalization of the real-time RT-PCR results. Primer sequences are listed in the supplemental material.

Tandem affinity purification and RNase H cleavage of U4 snRNA. Tandem affinity purification of the U4- and U6-specific Sm/LSm proteins was done through PTP (ProtC-TEV-ProtA)-tagged SSm4 and LSm4, respectively. The PTP tag consists of two protein A domains, a tobacco etch virus (TEV) protease cleavage site, and the protein C (ProtC) epitope (26, 27). The open reading frames of *T.brucei* SSm4 (Tb927.7.6380; positions −123 to 616) and LSm4 (Tb11.01.5535; positions −498 to 379) were PCR amplified from genomic DNA and inserted in frame into vector pC-PTP-NEO immediately upstream of the PTP tag sequence using ApaI and NotI restriction sites. For the transfection of trypanosome cells, 10 μg of each construct was linearized with BmgBI (SSm4) or XbaI (LSm4), respectively, and 1.2×10^8 cells were used for electroporation. Positive transformants were selected by adding 50 μg/ml of G418. The stable expression of the tagged proteins was analyzed by immunoblotting with peroxidase-anti-peroxidase soluble complex antibodies (Sigma).

Tandem affinity purification of PTP-tagged proteins was done as described previously (19, 26, 27), with the following additional modifications. Cells from a 500-ml culture (~1×10^7 cells/ml) were collected and lysed in IPP150 buffer (20 mM Tris-HCl [pH 8.0], 150 mM KCl, 3 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], 0.1% Tween 20, EDTA-free protease inhibitor [Roche]). To affinity purify the U4-specific Sm/LSm proteins solely, the U4/U6 di-snRNP was first split into two halves, using RNase H cleavage of the U4 snRNA by the U4c DNA oligonucleotide, which is complementary to nucleotides 53 to 72 of the trypanosome U4 snRNA. This cleavage reaction separated the U4 snRNA 3′ half (carrying the Sm site and U4-specific Sm/LSm proteins) from the remainder of the di-snRNP. For each IgG purification, 30 μl of IgG Sepharose 6 Fast Flow beads (GE Healthcare) was incubated with cleared lysate for 3 h at 4°C. After washing, bound material was released by cleavage with AcTEV protease (Invitrogen). For anti-ProtC purification through PTP-tagged SSm4, the supernatant was adjusted to 4 mM $CaCl_2$ and incubated with 30 μl of anti-protein C affinity matrix (Roche) for 3 h at 4°C. After washing with PC150 buffer (20 mM Tris-HCl [pH 8.0], 150 mM KCl, 3 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1% Tween 20, EDTA-free protease inhibitor [Roche]), the ProtC-tagged SSm4 was eluted with EDTA/EGTA elution buffer (5 mM Tris-HCl [pH 7.7], 10 mM EGTA, 5 mM EDTA, 1 mM $CaCl_2$, 150 mM KCl, 3 mM $MgCl_2$, 0.01% Tween 20). Affinity-selected proteins were pooled, acetone precipitated, separated on a 15% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue R-250. Protein bands were excised and used for mass spectrometric (MS) analysis.

Mass spectrometric analysis of protein samples. Tandem electrospray ionization (ESI)-MS analysis was performed after the in-gel digestion of proteins and the subsequent extraction of the tryptic peptides with an Orbitrap XL mass spectrometer (Thermo Fisher Scientific) coupled to a nanocapillary liquid chromatography system (Agilent 1100 system) under standard conditions. Fragment spectra were searched against the NCBInr database by using MASCOT as the search engine, as previously described (19).

Steady-state RNA analysis, cesium chloride density gradient centrifugation, and Northern blotting. For the analysis of steady-state snRNA levels, cells were silenced for 1 to 4 days (t_1 to t_4), and total RNA was prepared by using TRIzol reagent (Invitrogen). As a control, RNA from uninduced cells (t_0) was prepared. Five micrograms of total RNA from each time point was treated with 5 units of RQ1 DNase (Promega) for 30 min at 37°C, phenolized, and ethanol precipitated. Equal amounts of RNA were separated by 10% denaturing polyacrylamide gel electrophoresis and analyzed in parallel by Northern blotting with a mixture of snRNA-specific, digoxigenin (DIG)-labeled probes (2) and by silver staining.

For snRNP analysis, cleared *T. brucei* cell lysates after 3 days of silencing (t_3) and from uninduced cells (t_0) were prepared in buffer D (20 mM HEPES-KOH [pH 8.0], 100 mM KCl, 15 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride [PMSF], EDTA-free protease inhibitor [Roche]) by disrupting the cells with a PT 3100 cell homogenizer (Kinematica) and subsequent sonication. A CsCl stock solution (1.55 g/ml), supplemented with 0.5 mM DTT and 0.5 mM PMSF, was mixed 4:1 with the cell lysate. Centrifugation was done at 90,000 rpm for 20 h at 4°C with a TLX tabletop ultracentrifuge (Beckman) by using a precooled TLA 120.2 rotor. After centrifugation, the gradient was fractionated from top to bottom (10 100-μl fractions). Each fraction was adjusted to 1% SDS, and RNA was prepared by phenolization and ethanol precipitation, followed by 10% denaturing polyacrylamide gel electrophoresis and Northern blotting with a mixture of snRNA-specific, DIG-labeled probes.

Reconstitution of recombinant Sm cores and His tag pulldown assay. His-tagged *T. brucei* Sm subcomplexes (SmD1/SmD2, SmD3/SmB, and SmE/SmF/SmG) were purified as described previously (33). In each case, the first cistron bears an N-terminal His_6 tag. For the bicistronic expression of the His-tagged SSm4/SmB subcomplex, the SSm4 open reading frame was PCR amplified from *T. brucei* genomic DNA and used to replace the SmD3 open reading frame in the SmD3/SmB pQE30 expression construct. His-tagged SSm4/SmB was expressed in M15[pREP4] cells (Qiagen), and recombinant proteins were purified by Ni-nitrilotriacetic acid (NTA) affinity chromatography with an ÄKTApurifier high-pressure liquid chromatography system (GE Healthcare).

For reconstitution, 100 pmol purified His-tagged Sm subcomplexes (SmD3/SmB, SmD1/SmD2, and SmE/SmF/SmG for the canonical Sm core and SSm4/SmB, SmD1/SmD2, and SmE/SmF/SmG for the U4 Sm core) were mixed in equimolar amounts in 10 μl of 5× high-salt reconstitution buffer (100 mM Tris-HCl [pH 7.5], 2 M NaCl, 25 mM $MgCl_2$, 5 mM DTT). Ten micrograms of *T. brucei* total RNA (prepared with TRIzol reagent; Invitrogen) was then added to give a total volume of 50 μl. The reconstitution reaction mixtures were incubated at 30°C for 30 min and then at 37°C for 15 more minutes. Reconstituted Sm cores were recovered by His tag pulldown: 25 μl of packed Ni-NTA agarose beads (Qiagen) was incubated for 3 h at 4°C in 0.5 ml of 1× high-salt reconstitution buffer. After washing with the same buffer, the beads were resuspended in 1× proteinase K buffer (100 mM Tris-HCl [pH 7.5], 150 mM NaCl, 12.5 mM EDTA, 1% SDS), and the RNA was recovered by phenolization and ethanol precipitation. Purified RNA was analyzed by 10% denaturing polyacrylamide gel electrophoresis, followed by Northern blotting, using a mixture of DIG-labeled snRNA probes.

Immunofluorescence. Approximately 2×10^7 to 4×10^7 *T. brucei* cells expressing PTP-tagged SSm4 were harvested, washed, and resuspended in 1 ml of phosphate-buffered saline (PBS). Twenty-five microliters was fixed onto coverslips with 4% formaldehyde in PBS for 30 min at 4°C. After blocking with 1%

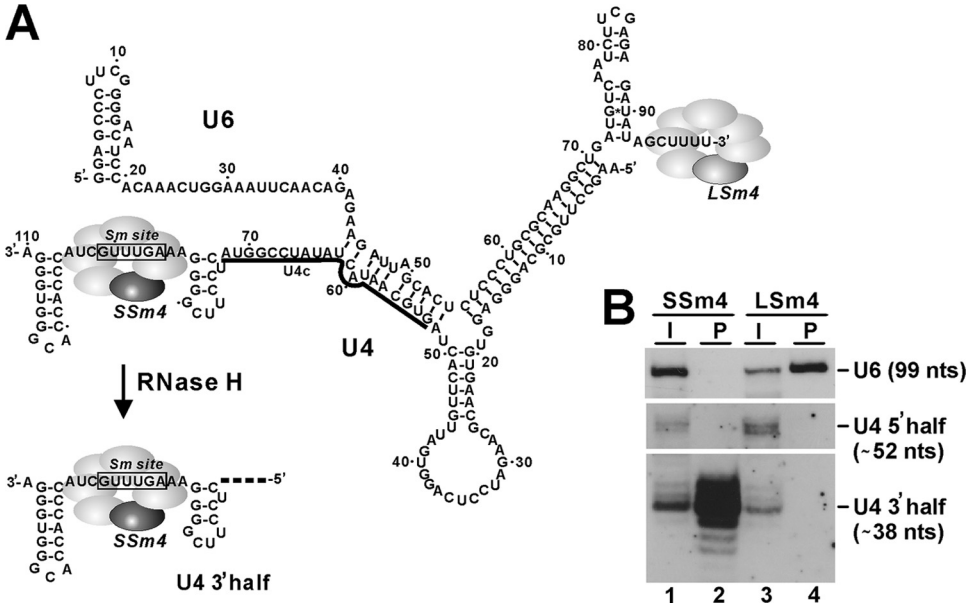

FIG. 1. The *T. brucei* SSm4 protein is associated with the 3′-terminal region of the U4 snRNA. (A) Schematic model of the RNase H-mediated cleavage of the U4 snRNA. Shown are the sequence and secondary structure of the trypanosome U4/U6 snRNA duplex with the U4-specific Sm site (boxed) and the target sequence of the antisense DNA oligonucleotide U4c (nucleotides [nts] 53 to 72 of the U4 snRNA) (underlined). The binding of the oligonucleotide followed by RNase H cleavage dissociates the U4/U6 duplex into two parts: the U4 snRNA 3′ half (at least nucleotides 73 to 110), carrying the U4 Sm site, and the U4 snRNA 5′ half (at least nucleotides 1 to 52) (not shown). The dashed line represents the potential RNase H cleavage region in the U4 snRNA. (B) Coprecipitation of the 3′ half of the U4 snRNA with PTP-tagged SSm4. Lysates from *T. brucei* cells stably expressing PTP-tagged SSm4 or PTP-tagged LSm4 were subjected to oligonucleotide U4c-mediated RNase H cleavage. Following affinity purification through IgG Sepharose beads, the copurified RNAs (P) (lanes 2 and 4) as well as 2.5% of the inputs (I) (lanes 1 and 3) were analyzed by Northern blotting using probes against U6 snRNA (top) or U4 snRNA (middle and bottom). Because different exposures had to be used, only the sections of the Northern blots that contain full-length U6 snRNA and the 5′ and 3′ halves of the U4 snRNA are shown (sizes are in nucleotides).

cold-water fish gelatin (Sigma-Aldrich), cells were incubated with rabbit anti-protein A primary antibody (1:40,000; Sigma-Aldrich). Following washing with PBS containing 0.05% Tween 20, 4′,6-diamidino-2-phenylindole (DAPI; 1 mg/ml) and goat anti-rabbit Alexa Fluor 594 secondary antibody (1:400; Invitrogen) were added. After washing, the coverslips were mounted in Immu-Mount (Thermo Scientific). A Zeiss Axioskop 20 microscope and Axio Vision software were used for imaging.

RESULTS AND DISCUSSION

***T. brucei* SSm4 is a specific component of the trypanosome U4 Sm core.** SSm4 (Tb927.7.6380) from *T. brucei* had initially been identified as LSm2 through a bioinformatic screen of the *T. brucei* genome for the complete set of Sm/LSm proteins (13). Although considered a component of the U6-specific LSm2-8 core in that initial study, Tkacz et al. (30) presented evidence for this protein being part of the U4 snRNP and renamed it SSm4 based on the specific degradation of U4 snRNA after RNAi knockdown.

To clarify whether SSm4 is part of the U4-specific Sm core exclusively and does not belong to the LSm core of U6 or to other spliceosomal snRNPs, we applied to following strategy. The 3′ half of the U4 snRNA with the Sm site was separated in the U4/U6 di-snRNP from the rest of the RNP by using RNase H cleavage mediated by DNA oligonucleotide U4c, which is directed against the stem I region of the U4 snRNA (Fig. 1A). In our earlier work on trypanosome snRNPs, we demonstrated that after U4c-mediated RNase H cleavage, the major stable protein binding domain resides in the 3′-terminal fragment of the U4 snRNA (6). This procedure should allow us to clearly distinguish the U4 Sm domain, located in the 3′-terminal region of the U4 snRNA, and the remainder of the RNP, i.e., the 5′ fragment of U4 snRNA and the U6 snRNP with its LSm domain, from each other. The RNase H cleavage reaction was carried out with extract from a *T. brucei* cell line, which stably expresses PTP-tagged SSm4 (see Materials and Methods). After pulldown through the PTP tag of SSm4 by IgG Sepharose, coprecipitated RNA was prepared and analyzed by Northern blotting using U4- and U6-specific probes (Fig. 1B, lane 2). The total input fraction was analyzed in parallel (lane 1). As an additional specificity control, the same procedure was done with lysate from a cell line stably expressing PTP-tagged LSm4 (lanes 3 and 4), which is a bona fide U6-specific LSm core component (13; N. Jaé and A. Bindereif, unpublished data). Clearly, only SSm4 but not LSm4 precipitated a small fragment of ~38 nucleotides, which represents the 3′ fragment of the U4 snRNA with the Sm site (Fig. 1B, compare lanes 1 and 2 with lanes 3 and 4). Significantly, no detectable U6 snRNA or the U4 5′-terminal fragment copurified in the PTP-SSm4 pulldown, as shown by U4- and U6-specific probes (Fig. 1B, lanes 1 and 2). In contrast, pulldown through PTP-tagged LSm4 efficiently recovered the U6 snRNA from total RNA but recovered no detectable U4 snRNA fragments (Fig. 1B, lanes 3 and 4), demonstrating the U6 snRNA specificity of LSm4.

Our results indicate that SSm4 is a specific component of the

TABLE 1. SSm4-associated proteins identified by mass spectrometry

Sm/LSm protein	Molecular mass (kDa)[a]	Open reading frame[a]	Reference(s)
SmB	12.3	Tb927.2.4540	18
SmD1	11.7	Tb927.7.3120	18
SmD2	12.5	Tb927.2.5850	18
SmE	9.6	Tb927.6.2700	18
SmF	8.3	Tb09.211.1695	18
SmG	8.9	Tb11.01.5915	18
SSm4[b]	23.2	Tb927.7.6380	13 (as LSm2), 30 (as SSm4), this study (SSm4)

[a] See the GeneDB website (http://www.genedb.org/genedb/tryp/).
[b] Initially identified as U6-specific "LSm2" (13), later renamed "SSm4" by Tkacz et al. (30), and independently established in this study as an Sm protein specific to the U4 Sm core.

U4 Sm core and is not present in the U6 snRNP portion of the U4/U6 di-snRNP. Nevertheless, it was not clear at this point whether only one single canonical Sm protein or an entire Sm subcomplex was replaced in the trypanosome U4 snRNP. To clarify this question, the experiment described above was repeated as a two-step affinity purification based on stably expressed, PTP-tagged SSm4, followed by mass spectrometric analyses of copurified proteins. Proteins copurifying with SSm4 are listed in Table 1; except for SmD3, all canonical Sm proteins (SmB, SmD1, SmD2, SmE, SmF, and SmG) copurified with SSm4. Taken together, these findings established biochemically that SSm4 replaces SmD3 in the heptameric Sm core structure of the U4 snRNP and is not part of the LSm ring of the U6 snRNP.

Nuclear-localized SSm4 is essential for cell growth and splicing. The expression of SSm4 was silenced in *T. brucei* by a doxycycline-inducible RNAi system in which double-stranded RNA (dsRNA) is produced in the form of a stem-loop construct (32). Stably transfected cells showed normal growth when RNAi was not induced (Fig. 2A). Upon the knockdown of SSm4, cell growth was rapidly affected (Fig. 2A): 2 to 3 days after RNAi induction, the growth inhibition was already apparent, indicating that SSm4 is essential for cellular functions

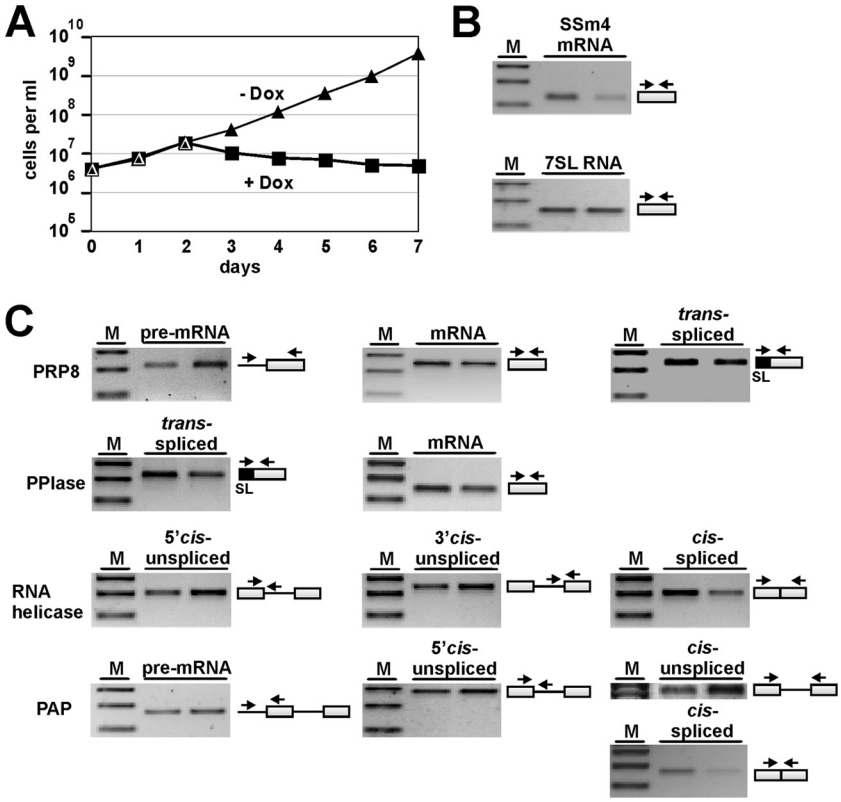

FIG. 2. SSm4 is essential for cell viability and splicing. (A) Growth curve of a representative procyclic *T. brucei* cell line in which RNAi of SSm4 expression was induced by the addition of doxycycline. For 7 days, cells were grown in the absence of doxycycline (−Dox; line with triangles) or in the presence of doxycycline (+Dox; line with squares). Every day, cell numbers were determined, and cultures were diluted back to 2×10^6 cells/ml. (B) RNAi-mediated knockdown of SSm4 mRNA expression. SSm4 mRNA levels were measured by semiquantitative RT-PCR of uninduced cells (middle lane) and of cells after 3 days of silencing (right lane). As a control, 7SL RNA expression was assayed for the same RNA samples. M, markers (100, 200, and 300 bp). (C) The following *T. brucei* genes (GeneDB open reading frames in parentheses) were analyzed for splicing defects by RT-PCR, comparing RNA from uninduced cells (middle lanes) and cells after 3 days of SSm4 silencing (right lanes): *PRP8* (Tb09.211.2420), *PPIase* (peptidyl-prolyl *cis-trans* isomerase; Tb927.8.690), a putative RNA helicase (Tb927.8.1510), and *PAP* [poly(A) polymerase; Tb927.3.3160]. RNA samples correspond to those described above for B. For the detection of mRNAs and pre-mRNAs, combinations of SL-, intergenic region-, exon-, and intron-specific primers were used. Primer positions and orientations are schematically represented on the side. *cis*-Spliced introns were assayed for RNA helicase and *PAP* genes; both spliced and unspliced introns could be detected from the same RT-PCR only for the *PAP* gene (two sections are shown with different exposures to visualize both products). For the RNA helicase, the unspliced intron was too long to be detected by the two exon primers. M, markers (100, 200, and 300 bp; for *cis*-unspliced *PAP*, 800 and 900 bp).

and growth in *T. brucei.* As shown by semiquantitative RT-PCR, the level of SSm4 mRNA was strongly reduced after 3 days of induction; as a control, 7SL RNA levels analyzed with the same RNA samples did not change (Fig. 2B).

Next, we assayed for splicing defects after RNAi knockdown of SSm4 (Fig. 2C), which may correlate with the growth inhibition phenotype observed. Total RNA prepared from uninduced cells or after 3 days of induction was analyzed by semiquantitative RT-PCR for *trans*- and *cis*-splicing defects by using combinations of SL-, intergenic region-, exon-, and intron-specific primers to detect unspliced pre-mRNA or SL-*trans*-spliced, *cis*-spliced, or total mRNA (schematically illustrated in Fig. 2C). The following genes were analyzed: *PRP8* and *PPIase* for *trans* splicing, a putative RNA helicase gene, and the poly(A) polymerase (*PAP*) for both *trans* and *cis* splicing. Clearly, after SSm4 silencing, signals for pre-mRNAs (*PRP8* and *PAP*) as well as for *cis*-unspliced pre-mRNAs (RNA helicase and *PAP*) increased, reflecting pre-mRNA accumulation. Conversely, signals for total mRNAs (*PRP8* and *PPIase*), SL-*trans*-spliced mRNAs (*PRP8* and *PPIase*), and *cis*-spliced mRNAs (RNA helicase and *PAP*) decreased. These results were confirmed by real-time RT-PCR assays (see Fig. S1 in the supplemental material). Taken together, these splicing assays indicate that SSm4, a specific component of the U4 snRNP, is essential for *in vivo trans* and *cis* splicing in trypanosomes. Differences in the severities of the splicing defects are most likely due to different RNA stabilities and expression levels.

To determine the cellular localization of SSm4, cells expressing PTP-tagged SSm4 were analyzed by indirect immunofluorescence using the protein A epitope of the PTP tag (Fig. 3). As a result, we detected SSm4 predominantly in the nucleoplasm, concentrated in a few areas within the nucleoplasm and excluding the nucleolus (compare Fig. 3B [DAPI staining], C [SSm4 immunofluorescence], and D [merged image]). This finding is consistent with the nuclear localization of several other protein splicing factors in *T. brucei* (for example, see references 12, 14, 16, 22, and 30).

SSm4 is essential for U4 snRNA and U4/U6 di-snRNP integrity. To study the biochemical basis of the general splicing defect upon SSm4 knockdown in more detail, we first analyzed changes in the steady-state levels for all spliceosomal snRNAs during the silencing of SSm4 over a time course of 4 days (Fig. 4A). Total RNA was prepared after silencing for 1, 2, 3, and 4 days or from uninduced cells as a control. The steady-state levels of U2, SL, U4, U6, U5, and U1 snRNAs were determined by Northern blot hybridization. Strikingly, after the first and second days of SSm4 silencing, the U4 snRNA-specific signal had already strongly decreased, and after 3 days, no U4 snRNA could be detected anymore. Furthermore, a significant accumulation of SL RNA could be detected over the time course of 4 days, correlating with the reduction of *trans*-splicing activity observed after knockdown. In contrast, the U2, U6, U1, and U5 snRNAs did not change significantly, consistent with the findings described previously by Tkacz et al. (30). As an additional loading and specificity control, the levels of rRNAs and tRNAs were analyzed for the same samples by silver staining (Fig. 4B). No significant changes were observed for these major RNA species. In sum, our data demonstrate that SSm4 is specifically required for the integrity of the U4 snRNA.

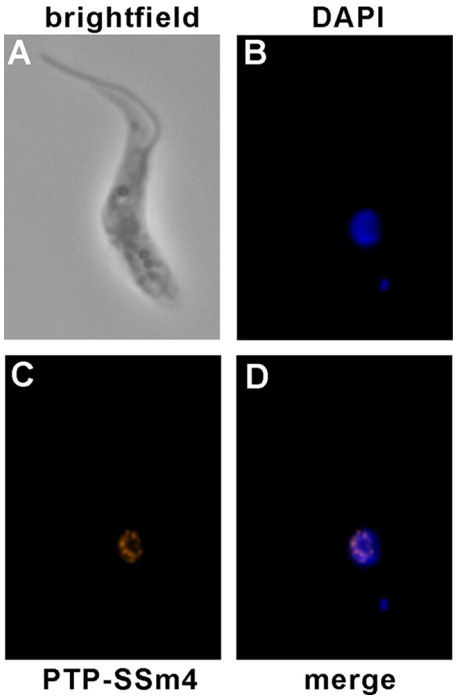

FIG. 3. SSm4 is predominantly nuclear. Shown is the localization of PTP-SSm4 in *T. brucei*. (A and B) Cells stably expressing PTP-tagged SSm4 were fixed (A), and nuclear as well as kinetoplast DNAs were stained with DAPI (B). (C) PTP-tagged SSm4 was detected by anti-protein A primary and Alexa 594 secondary antibodies. (D) In addition, a superimposition of both fluorescence signals is shown.

Next, we assessed the integrity of the spliceosomal snRNPs by CsCl density gradient fractionation. Total cell extract was prepared from uninduced *T. brucei* cells or cells after 3 days of RNAi induction, and the distribution of snRNAs across the CsCl gradient was analyzed by Northern blotting (Fig. 4C). Characteristically, most snRNP core particles are stable under these conditions and distribute within gradient fractions 1 to 8, depending on their RNA-protein ratios; in contrast, free RNA sediments in bottom fractions 9 and 10 (5, 6, 17). Without RNAi induction (t_0; Fig. 4C, left), the U4/U6 core snRNP was concentrated in fractions 3 and 4. In contrast, after 3 days of knockdown (t_3; Fig. 4C, right), the U4/U6 core snRNP was almost undetectable, and no significant amounts of U4 snRNA could be found throughout the gradient, consistent with the steady-state snRNA analysis (Fig. 4A). On the other hand, the U6 snRNA in uninduced cells distributed between the U4/U6 (fractions 3 and 4; Fig. 4C, left) and free U6 snRNPs (fractions 9 and 10), the latter of which are unstable under these conditions (5). After the knockdown of the U4-specific SSm4, the U6 snRNA completely shifts to the bottom fractions (fractions 9 and 10) (Fig. 4C, right), reflecting free U6 snRNA and/or free U6 snRNPs.

Regarding the other snRNAs/snRNPs, the U2 snRNP is characteristically unstable under the stringent conditions of CsCl gradients and cannot be analyzed (5). The increase in SL RNA steady-state levels (Fig. 4A) corresponded to a higher peak in the SL RNP (fractions 2 and 3; Fig. 4C). Finally, after knockdown, the U1 snRNP peak is slightly shifted (from fraction 1 to fractions 1 to 3), probably reflecting a destabilization

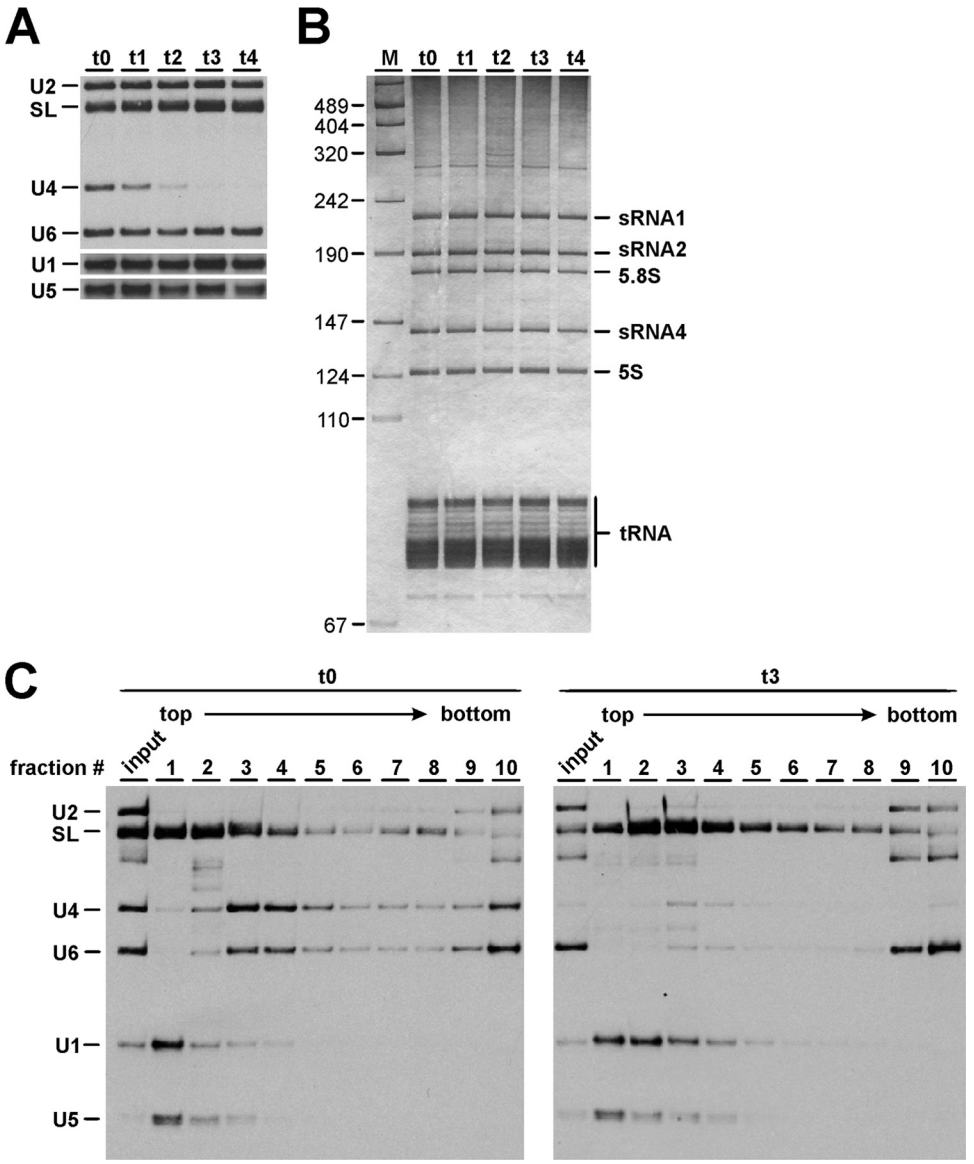

FIG. 4. SSm4 is specifically required for U4 snRNA integrity and is essential for U4/U6 di-snRNP stability. (A and B) Effects of SSm4 silencing on snRNA steady-state levels. (A) Equal amounts of *T. brucei* total RNA were analyzed by denaturing polyacrylamide gel electrophoresis and Northern blotting after 1, 2, 3, and 4 days of RNAi silencing (t_1 to t_4, as indicated). In addition, total RNA from uninduced cells (t_0) was used. RNA positions for U2, SL, U4, U6, U1, and U5 snRNAs are marked on the left. (B) As an additional control, equal amounts of total RNA were analyzed for each time point (as indicated) by silver staining. Positions of major RNAs (rRNAs and tRNAs) are marked on the right. M, markers in nucleotides. (C) CsCl density gradient fractionation of *T. brucei* extract. Total cell extract derived from uninduced cells and from cells after 3 days of SSm4 silencing was subjected to isopycnic CsCl gradient centrifugation, and gradient fractions 1 to 10 (from top to bottom) were assayed by Northern blotting, detecting U2, SL, U4, U6, U1, and U5 snRNAs (positions marked on the left). For comparison, 10% of the inputs are shown.

of protein components, and the U5 snRNA showed no apparent changes in its distribution. Taken together, these results demonstrate that SSm4 is essential for U4/U6 di-snRNP stability.

SSm4 is sufficient for specifying U4 Sm core assembly *in vitro*. To address the question of whether the U4-specific Sm core is responsible for the U4 snRNA binding specificity, we used an *in vitro* reconstitution approach (see Fig. 5 for a schematic outline). His-tagged Sm subcomplexes of the trypanosome Sm cores were expressed in *Escherichia coli* cells and purified: SmD1/SmD2, SmD3/SmB, and SmE/SmF/SmG for the canonical Sm core and SmD1/SmD2, SSm4/SmB, and SmE/SmF/SmG for the U4-specific Sm core. Equimolar amounts of the three respective subcomplexes were incubated together with total RNA from *T. brucei* under high-salt conditions (400 mM NaCl), and reconstituted Sm cores were recovered by His tag pulldown. Coprecipitated RNAs were analyzed by denaturing polyacrylamide gel electrophoresis and Northern blotting, using a mixed probe that detects U2, SL, U4, U6, U1, and U5 snRNAs (Fig. 5). Under these stringent conditions, only the U5 snRNA but none of the other snRNAs efficiently reconstituted with the canonical Sm core (Fig. 5,

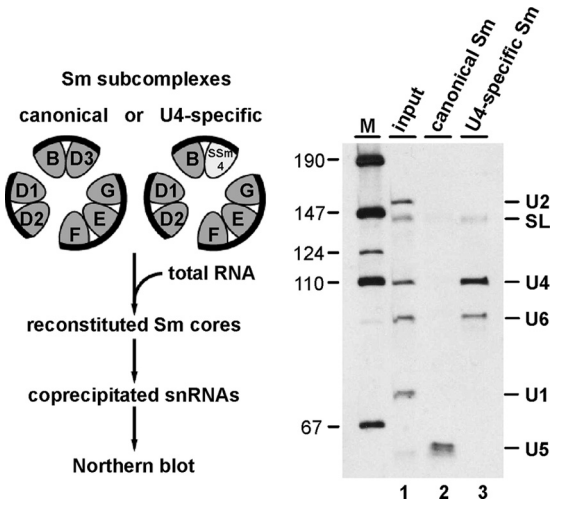

FIG. 5. SSm4 is sufficient for specifying U4 Sm core assembly *in vitro*. Shown is canonical and U4 snRNP-specific Sm core assembly with *T. brucei* total RNA (the *in vitro* reconstitution procedure is schematically outlined on the left). His-tagged Sm subcomplexes of the canonical Sm core (SmD1/SmD2, SmD3/SmB, and SmE/SmF/SmG; lane 2) and the U4-specific Sm core (SmD1/SmD2, SSm4/SmB, and SmE/SmF/SmG; lane 3) were incubated with *T. brucei* total RNA in reconstitution buffer containing 400 mM NaCl. In addition, 5% of RNA input is shown (lane 1). RNA assembled *in vitro* into Sm cores was recovered by His tag pulldown under stringent conditions and analyzed by Northern blotting, detecting U2, SL, U4, U6, U1, and U5 snRNAs (positions are marked on the right). M, markers in nucleotides.

compare lanes 1 and 2). In contrast, if instead of the canonical Sm core, the U4-specific Sm core was used, in which only a single subunit, SmD3, is replaced by SSm4, U4 snRNA was most efficiently reconstituted; the minor amount of U6 snRNA that coprecipitated most likely reflects U4-U6 snRNA base pairing (Fig. 5, compare lanes 1 and 3). Except for very minor amounts of SL RNA, none of the other snRNAs was detectable. Under low-stringency conditions, the other snRNAs reacted with both canonical and U4-specific Sm cores (data not shown). In sum, we conclude that the exchange of a single Sm polypeptide, SmD3 with SSm4, is sufficient to switch the RNA binding specificity between canonical and U4-specific Sm sites.

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