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Implication of the SMN complex in the biogenesis and steady state level of the Signal Recognition Particle

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ABSTRACT

Spinal muscular atrophy is a severe motor neuron disease caused by reduced levels of the ubiquitous Survival of MotoNeurons (SMN) protein. SMN is part of a complex that is essential for spliceosomal UsnRNP biogenesis. Signal recognition particle (SRP) is a ribonucleoprotein particle crucial for co-translational targeting of secretory and membrane proteins to the endoplasmic reticulum. SRP biogenesis is a nucleo-cytoplasmic multistep process in which the protein components, except SRP54, assemble with 7S RNA in the nucleolus. Then, SRP54 is incorporated after export of the pre-particle into the cytoplasm. The assembly factors necessary for SRP biogenesis remain to be identified. Here, we show that 7S RNA binds to purified SMN complexes *in vitro* and that SMN complexes associate with SRP in cellular extracts. We identified the RNA determinants required. Moreover, we report a specific reduction of 7S RNA levels in the spinal cord of SMN-deficient mice, and in a *Schizosaccharomyces pombe* strain carrying a temperature-degron allele of SMN. Additionally, microinjected antibodies directed against SMN or Gemin2 interfere with the association of SRP54 with 7S RNA in *Xenopus laevis*

oocytes. Our data show that reduced levels of the SMN protein lead to defect in SRP steady-state level and describe the SMN complex as the first identified cellular factor required for SRP biogenesis.

INTRODUCTION

The SMN protein was discovered because decreased levels of this protein correlate with the phenotypic severity of spinal muscular atrophy (SMA) (1,2), a neuromuscular disease characterized by the degeneration of the lower motor neurons, leading to muscular weakness and atrophy [reviewed in (3)]. The disease is due to recessive mutations or deletions affecting the survival of motor neuron (*SMN1*) gene (4). Two genes, *SMN1* and *SMN2*, code for the SMN protein in humans, the copy number of *SMN2* being a determinant of disease severity (1,5). Indeed, while *SMN1* produces full-length transcripts, *SMN2* mainly produces an alternatively spliced messenger RNA (mRNA) lacking exon 7 (*SMNΔEx7*) (6,7). As the *SMNΔExon7* protein is unstable and rapidly degraded (8,9), *SMN2* cannot fully compensate for the loss of *SMN1* in SMA. The SMN protein is ubiquitously expressed and essential in all eukaryotes that have been tested so far, including *Schizosaccharomyces pombe*. In *Saccharomyces cerevisiae*, an orthologous protein-coding gene has not yet been found. In vertebrates, the SMN protein is predominantly associated with Gemin2–8 and

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Unrip proteins to form a large stable complex called the SMN complex (10–13). SMN and Gemin2 orthologs but no other components of the SMN complex have been found in the fungus *S. pombe* (14–17). The metazoan SMN complex has been proposed to function in universal eukaryotic processes related to RNA metabolism, including transcription, splicing, ribonucleoprotein (RNP) biogenesis and in neuron-specific functions, like neurite and axon outgrowth, growth cone excitability, mRNA transport and the function of the neuromuscular junction [reviewed in (18–21)]. The most well characterized mechanism of action of the SMN complex is in the assembly of the spliceosomal U-rich small nuclear RNP (UsnRNP) (18,19,22–27). Accordingly, SMN deficiency would alter the stoichiometry of snRNAs that might cause widespread and tissue-specific pre-mRNA splicing defects in SMA mice models (28,29), as well as in the *S. pombe* model organism carrying a temperature-degron allele of the SMN protein (30). More recently, the splicing of some, but not all, minor U12-type introns was reported to be inhibited in cells derived from SMA patients, and in mammalian cells and *Drosophila* larvae expressing low levels of SMN, demonstrating a link between SMN deficiency and alterations of splicing events mediated by the minor spliceosome (31,32). However, the identity of the impaired or altered SMN function(s) responsible for SMA is still a matter of debate. This is reinforced by the fact that although the SMN complex has been called the master ribonucleoprotein assembler (33), there is no direct proof of its involvement in assembly mechanisms other than UsnRNPs. For instance, it has been previously suggested that the SMN complex may play a role in box C/D and H/ACA RNP assembly based on its interaction with Fibrillarin, a core component of the C/D box RNPs and with Gar1, a common component of the H/ACA box RNPs (34,35). In addition, a decrease of the levels of U3 small nucleolar RNA (a C/D box snoRNA) was found upon reduction of SMN levels in HeLa cells by RNAi (36). However, whether box C/D and H/ACA RNP assembly depends on the SMN complex, has only been poorly studied. Several data also suggest that the SMN protein alone or the SMN complex may assist the assembly of specific mRNAs into mRNP particles in neurons, as well as their stability, their targeting to the neuronal transport system along neurites and their localized translation in synapses and axonal growth cones (37–40) [reviewed in (20)].

Interestingly, while the signal recognition particle (SRP) is one of the most abundant RNPs in eukaryotic cells, a possible involvement of the SMN complex in its assembly had not been proposed yet. Here, we bring very strong arguments in favor of a role of the SMN complex in its stability and biogenesis. SRP is an ubiquitous RNP that co-translationally delivers most membrane and secretory proteins to the plasma membrane in prokaryotes and to the endoplasmic reticulum in eukaryotes [for reviews, (41–43)]. Mammalian SRP consists of six proteins, SRP9, 14, 19, 54, 68, 72 and a single RNA molecule, i.e. the 7S RNA (Figure 1A). The RNA secondary structure possesses extensive base paired regions, which form a

prominent central helix flanked by a small (or Alu) and a large (or S) domain (Figure 1A) (44). The S-domain, which corresponds to the central region of the RNA, associates with the SRP19 and SRP54 proteins, and a SRP68/72 heterodimer. The Alu-domain comprises both the 3' and 5' terminal RNA regions and binds a SRP9/14 heterodimer. Fungal SRP resembles its mammalian counterpart in that it also consists of six proteins (SRP72p, SRP68p, SRP54p, SRP14p and Sec65p, which are homologous to the mammalian proteins, and Srp21) and a single RNA molecule called *srp7* in *S. pombe* (45–48). Studies in yeasts (49,50), *Xenopus laevis* oocytes (51,52) and mammalian cells (53,54) support a model in which all SRP proteins, except SRP54, are imported to the nucleolus for assembly with 7S RNA. The resulting pre-particle is then exported to the cytoplasm where it incorporates SRP54, leading to the formation of a mature and functional RNP. None of the factors required for the *in vivo* biogenesis of SRP has yet been described.

By screening for possible interactions of *in vitro* transcribed RNAs with purified human SMN complex, we identified the 7S RNA as a possible partner of this complex. We observed that the binding of 7S RNA to purified SMN complexes was strongly competed by U1 and U2 snRNAs. The use of 7S RNA domains taken individually and RNase protection experiments identified the RNA determinants required for interaction with the SMN complex. Moreover, we showed that Gemin5, which is the snRNA binding protein of the SMN complex, interacts *in vitro* with the 7S RNA. These observations motivated us to investigate a possible role of the SMN complex in SRP biogenesis, and accordingly, we detected an association of SRP with the SMN complex in different systems, i.e. *X. laevis* oocytes, HeLa cells and yeast *S. pombe*. Importantly, our analysis of 7S RNA levels by real-time polymerase chain reaction (PCR) in tissues of SMN-deficient mice revealed a significant reduction of 7S RNA in the spinal cord, whereas it remained the same in the brain and the heart. We also observed a decrease in the 7S RNA levels and in the amount of SRP in a *S. pombe* mutant strain carrying a temperature-degron allele of SMN. Altogether, we bring strong data in favor of an *in vivo* association of the SMN complex with SRP and show that the SMN protein may be required for SRP stability. As we show that antibodies directed against either SMN or Gemin2 interfere with the cytoplasmic association of SRP54 with 7S RNA in *X. laevis* oocytes, we propose that the SMN complex functions in SRP biogenesis. The possibility that SRP biogenesis may be altered upon SMN protein deficiency in SMA is discussed.

MATERIALS AND METHODS

DNA constructs and antibodies

Plasmids encoding vertebrate wild-type UsnRNAs, as well as U1 Δ SL1 and U4 Δ Sm, have been described previously (55–57) and so have the construct for production of Flag-tagged Gemin5 (58) and production of GST-SMN (59).

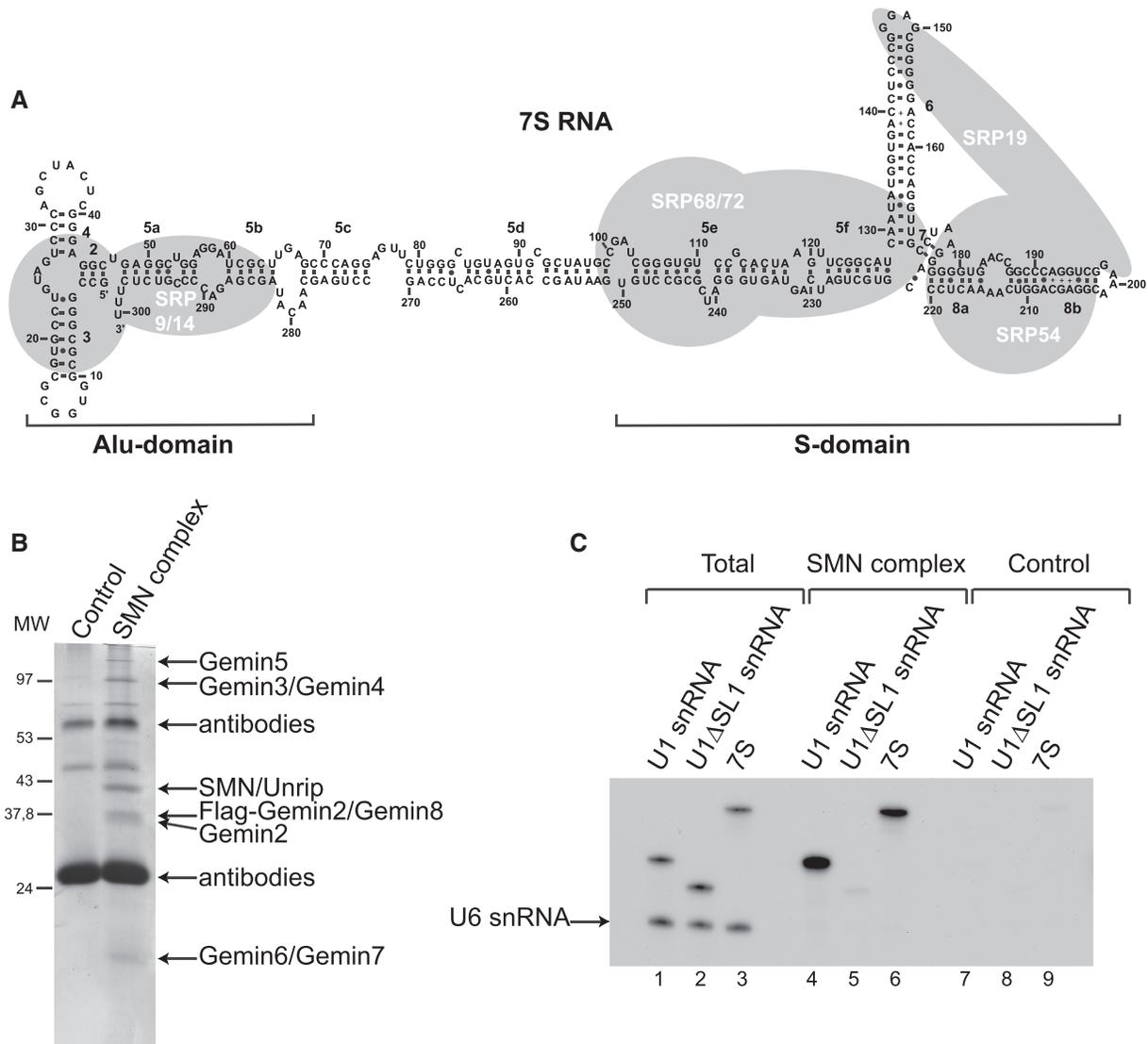


Figure 1. Immunoselected native SMN complexes associate *in vitro* with 7S RNA. (A) Human 7S RNA sequence. The secondary structure was established by previous studies (75,76). Stems are numbered 2 to 8 according to the criteria of previous study (76). (B) Native SMN complexes (SMN complex) were purified by immunoprecipitation with anti-Flag antibodies using total extracts prepared from HeLa TET-off cells that stably expressed Flag-tagged Gemin2. The protein composition of the complexes was analyzed by SDS-PAGE followed by silver staining. The non-specific proteins immunoselected from HeLa TET-off cells that do not express Flag-Gemin2 are shown as a negative control (Control). Proteins were identified according to their molecular weight as referred to molecular weight markers (MW) and to (39) and (68). The identity of the proteins was confirmed by western blotting and mass spectrometry (data not shown). (C) The purified native SMN complex (SMN complex) shown in (B) or the non-specific proteins purified from parental cells (Control) were incubated with *in vitro* transcribed [³²P]UTP-labeled 7S RNA, U1 snRNA and U1ΔSL1 in the constant presence of U6 snRNA as an internal negative control. Subsequently, bound RNAs were isolated after washing and analyzed by electrophoresis on denaturing polyacrylamide gels and autoradiography. Lanes labeled 'Total' represent 10% of input.

For production of GST-tagged Gemin5 in bacteria, a DNA corresponding to the ORF of Gemin5 was generated by PCR amplification and cloned into the pDEST-15 vector with Gateway technology (Invitrogen). As human and *X. laevis* 7S RNA share 87% sequence homology (60), we used human 7S RNA in our experiments. DNA fragment corresponding to the sequence encoding the human 7S RNA was generated by RT-PCR amplification using specific primers and total RNAs from HeLa cells, and cloned downstream of the T7 promoter into the pUC18 vector. The 7S RNA transcript obtained by *in vitro* transcription using this

plasmid contains an additional G at the 5'-end. Construction of deletion mutants of 7S RNA into the pUC18-7S was carried out by PCR amplification using specific primers. The RNA 7S Alu+long stem comprises nucleotides 1–128 and nucleotides 222–300 linked by a GUAA sequence; the RNA 7S Alu+short stem contains nucleotides 1–98 and nucleotides 252–300 linked by an UA sequence; the RNA 7S Alu contains nucleotides 1–65 and nucleotides 282–300 linked by an UA sequence. The S-domain+long stem and the S-domain RNAs comprise nucleotides 48–300 and 101–250, respectively. All the mutated RNAs contain an additional G at the

5'-end. All the constructs were analyzed by automated sequencing.

The entire coding region of fission yeast *smn1+* was amplified by PCR and cloned into the pREP41-NTAP vector (obtained from K. Gould) using standard methods. The plasmid encoding the TAP-SpSMN fusion was transformed into a diploid heterozygous strain carrying a deletion of the *smn1+* gene (BG_1375; Bioneer Corporation) together with plasmid pON177, which allows sporulation. Transformed cells were selected on Edinburgh minimal medium (EMM) plates with appropriate amino acids supplements. After sporulation and dissection, clones bearing a *smn1+* deletion and the pREP41-TAP-SpSMN plasmid were identified by diagnostic PCR and western blot analysis.

The antibodies used in these experiments were as follows: anti-SMN [2B1, (61); 4B3, (62)], anti-Gemin2 (2E17) (23), anti-Gemin3 (12H12) (63), anti-Gemin4 (17D10) (64), anti-Gemin5 (10G11) (58), anti-Gemin6 (20H8) (65), anti-SRP9 (PTG lab), anti-SRP14 (PTG lab and kind gift from Dr Dobberstein), anti-SRP54 (BD transduction laboratories and PTG lab), anti-SRP68 (PTG lab), anti-tubuline (Sigma), anti-Y12 (66), anti-Sox2 (PTG lab), anti-Lin28 (PTG lab) and non-immune (SP2/O) (67).

***In vitro* transcription of RNAs**

In vitro transcription and [³²P]UTP labeling of RNAs were carried out at 37°C for 3 h on 1 µg of linearized plasmid in 15 µl of transcription buffer (TB) (80 mM HEPES-KOH, pH 7.5, 24 mM MgCl₂, 2 mM spermidine, 40 mM DTT) containing 40 U RNasin, 30 U T7 RNA polymerase, 1.3 mM of ATP, CTP, GTP, 66 µM UTP and 10 µCi αUTP [³²P] (GE Healthcare, 3000 Ci/mmol). *In vitro* transcription of unlabeled RNAs was carried out at 37°C for 3 h on 1 µg of linearized plasmid in 30 µl of TB containing 60 U T7 RNA polymerase and 7.5 mM of ATP, CTP, GTP and UTP. RNAs were then purified by electrophoresis on 7 M urea-8% acrylamide gels and precipitated with ethanol.

***In vitro* binding of RNAs to immunoselected SMN complexes**

Native SMN complexes were affinity purified using a HeLa Tet-Off cell line stably expressing Flag-tagged Gemin2 as described previously (39,68). *In vitro* RNA-binding experiments and competition experiments were performed as described earlier (56), except that 20 000 cpm of radioactive RNAs were used instead of 10 000. For competition experiments, purified native SMN complexes were pre-incubated with 250 nM of unlabeled RNAs for 30 min at 4°C. Subsequently, 20 000 cpm of [³²P]UTP-labeled 7S RNA was added to the pre-incubated mixtures and further incubated for 1 h at 4°C. Bound RNAs were isolated and analyzed by electrophoresis on 7 M urea-10% polyacrylamide gels.

RNase protection assay

In vitro transcribed 7S RNA bound or not to purified SMN complexes was incubated in 50 µl of Resuspension

buffer (RSB) 100 buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM MgCl₂) in the presence of 5 µg of tRNA. Digestion was for 6 min at 20°C, in the presence of 2 U of T1 RNase (Ambion), 5 U of T2 RNase (Mobictec) or 0.002 U of V1 RNase (Roche). RNase reactions were stopped by addition of 50 µl of water, 4 µl of EDTA 0.1 M, pH 8 and 20 µg of tRNA, followed by phenol extraction. For production of a ladder, an alkaline hydrolysis of the naked RNA was performed for 3 min at 95°C, using 20 000 cpm of RNA dissolved in 1 µl of 100 mM sodium carbonate, pH 9.2. To identify the nucleotides in the ladder, an RNase T1 digestion was also performed in denaturing conditions. To do so, 20 000 cpm of RNA was incubated with 2 µg of tRNA in Citrate Buffer (20 mM Citrate NaOH, 1 mM EDTA, 7 M urea, 0.05% Xylene cyanol blue, 0.05% bromophenol blue) for 10 min at 65°C; 2 U of RNase T1 was then added and the incubation was followed for 10 min at 65°C.

***In vitro* binding of RNAs to Gemin5**

HeLa cells were transfected with plasmid expressing Flag-tagged Gemin5 using the Calphos Mammalian transfection kit (Clontech) according to the manufacturer's recommendation. Immunoprecipitation of the SMN complex or Flag-Gemin5 alone using anti-Flag antibodies were carried out as described previously (39,68), in the presence or in the absence of 1% Empigen-BB detergent, respectively. Recombinant GST, GST-SMN and GST-Gemin5 proteins were expressed in *Escherichia coli* and purified by affinity chromatography on glutathione-Sepharose beads, as described (39). *In vitro* RNA binding experiments on Flag-Gemin5 or GST-Gemin5 were performed as described previously (56).

***Xenopus laevis* oocyte microinjections**

Pieces of *X. laevis* ovary were dissected, treated with 5000 U collagenase and individual stage VI oocytes were prepared for microinjection as described (69). Injections and culture of oocytes were carried out in OR2 medium (5 mM HEPES-KOH, pH 7.8, 82.5 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 1 mM Na₂HPO₄) containing 1 mM CaCl₂ as described previously (70). For nuclear injection, oocytes were previously centrifuged for 15 min at 1000g to allow the visualization of the germinal vesicle (71). In a typical injection experiment, 18.4 nl of [³²P] UTP-labeled RNA (1 × 10⁶ cpm/µl for each RNA) were injected into the nucleus of the oocytes. After a 6-h incubation, oocytes were manually dissected to prepare nuclear and cytoplasmic fractions in RSB 150 G buffer [10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM MgCl₂, glycerol 10% (v/v)] for further analysis. For the antibody inhibition experiments, oocytes were first injected with 18.4 nl of antibody (1 µg/µl) into the cytoplasm 1 h before they received a second injection of 18.4 nl of [³²P] UTP-labeled RNA into the nucleus.

Immunoprecipitation experiments and analysis

For a typical immunoprecipitation experiment using *X. laevis* extracts, nuclear, cytoplasmic or total fractions from 20 oocytes were homogenized in 300 µl of RSB 150

buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2.5 mM MgCl₂) and the insoluble material was pelleted by centrifugation. The supernatant was incubated with antibodies bound to protein A or G-Sepharose (Amersham). Immunoprecipitations and analysis of bound RNAs were performed as previously described (55). For immunoprecipitation using HeLa cells, cytoplasmic extracts were prepared as previously described (72) and incubated with specific antibodies bound to protein A or G-Sepharose (Amersham) for 2 h at 4°C in RSB 100 buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2.5 mM MgCl₂) containing 0.01% NP-40. The beads were washed with the same buffer and the immunoprecipitated proteins were analyzed by SDS-PAGE and western blotting as previously described (23). The immunoprecipitated RNAs were analyzed by RT-PCR using the oligonucleotides described in (73) for analysis of the mRNA coding GAPDH, and the following oligonucleotides for the analysis of 7S RNA: forward 5'-GGAGTTCTGGGCTGTAGTGC-3', reverse 5'-GATCAGCACGGGAGTTTT-3'.

Fission yeast *in vivo* cross-linking, extract preparation and immunoprecipitation

Fission yeast cells bearing a *smn1+* deletion and the pEP41-TAP-SpSMN plasmid were grown in EMM2-Leu medium and cross-linked by addition of formaldehyde (1% final concentration) for 30 min at 28°C with continuous shaking. Adding glycine to 125 mM final concentration terminated the reaction. After incubation for 5 min, cells were washed twice with PBS and frozen in liquid nitrogen. Cells were resuspended in RNA IP Buffer (50 mM Tris-HCl, pH 8, 1 mM EDTA, 1% NP40, 0.5% Na-deoxycholate, 150 mM NaCl) and broken using a FreezerMill SPEX6770 grinder. After thawing on ice and homogenization with a tight dounce, the extract was centrifuged at 15 000g for 20 min. TAP-tag purification was performed as described previously (74) with an overnight Tobbaco Etch Virus (TEV) protéase (Promega) incubation at 4°C. After digestion with RQ1 RNase free DNase (Promega) according to manufacturer's procedure and incubation with proteinase K for 30 min at 37°C, the reverse cross-linking reaction was carried out at 70°C for 45 min. The samples were phenol-chloroform extracted and the RNA ethanol-precipitated. Yeast extract preparation and native gel electrophoresis, as well as RT-PCR analysis were performed as previously described [(30) and (31), respectively]. Primer sequences and PCR regimes are available upon request.

RNA isolation from mice and real-time RT-PCR

Ten-day-old SMN Δ 7 mice ($n = 5$) and age-matched wild-type controls ($n = 5$) were anesthetized (10 mg/kg xylazine, 100 mg/kg ketamine) and perfused intracardially with 0.1 mol/l PBS. The brain, the liver and the spinal cord tissues were removed and total RNA was extracted using the Nucleospin RNA II kit (Macherey Nagel). Total RNA was treated for DNA contamination by DNase RQ1 (Promega) (1 U/ μ g of RNA) for 30 min at 37°C. RNAs were then phenol/chloroform extracted and ethanol precipitated. First strand cDNA synthesis was carried

out using 1 μ g of DNA-free RNA. This was added to 0.5 μ g random hexamer in nuclease-free water. This mixture was heated at 65°C for 10 min and then cooled on ice for 10 min to allow primers to anneal. A master mix was added that contained 0.4 mM dNTPs, 40 U RNAsin (Fermentas), 1x M-MLV reaction buffer and 200 U of M-MLV (Promega), in a total reaction of 25 μ l. The samples were then incubated at 37°C for 1 h. About 1 μ l of diluted cDNA (2- to 100-fold dilution) was used for each real-time PCR reaction. The reactions were carried out on a LightCycler (Roche) using SYBR Green I Master mix (Roche). Specific primers were used to generate cDNA: 7S forward 5'-GGAGTTCTGGGCTGTAGTGC-3', 7S reverse 5'-GATCAGCACGGGAGTTTT-3', RP29 forward 5'-GCCTATGTCCTTCGCGTACT-3' and RP29 reverse 5'-CTGAAGGCAAGATGGGTCAC-3'. The efficacy of each primer pairs was determined and was comprised between 1.8 and 2. As a control of the amplification specificity, melting curve analysis was performed for each PCR experiments. Each run included negative controls. Quantification was performed using LightCycler software (Roche). Data were calculated according to the $\Delta\Delta$ CT method. Results were expressed as mean \pm standard error of the mean of three measurements. Differences between $2^{\Delta\Delta$ CT values for the RNA target versus $2^{\Delta\Delta$ CT values for the RNA reference were analyzed using unpaired Student's *t*-test; $P < 0.05$ was considered significant.

RESULTS

Association of *in vitro* produced 7S RNA with immunoselected SMN complexes

First we tested whether the 7S RNA component of human SRP may interact with the SMN complex. To this end, we carried out *in vitro* binding assays using various [³²P]UTP-labeled RNAs and immunoselected SMN complexes, in conditions that were previously established to study the SMN complex–UsnRNA interactions (55). To isolate SMN complexes, we used a cell line that stably expressed Flag-tagged Gemin2. A total cell extract was incubated with anti-Flag antibodies linked to agarose beads as previously described (39,68). We verified that the material retained on the beads contained all the known components of the SMN complex, namely Gemin2–8 and unrip, and that no Sm proteins and other proteins known to interact with the SMN complex were present in detectable amounts (39). The purified SMN complexes bound to agarose beads (SMN complex) and, as a negative control, agarose beads incubated with a cellular extract prepared from the parental HeLa Tet-Off cells (Control) (Figure 1B) were incubated with *in vitro* transcribed RNAs. In addition to the tested [³²P]UTP-labeled 7S RNA, U1 snRNA and U1 Δ SL1, which does not contain the stem-loop 1 necessary for specific interaction of U1 snRNA with the SMN complex (55), were used as positive and negative controls, respectively. *In vitro* transcribed U6 snRNA was added in each of the assays as an internal negative control (Figure 1C). As expected, the immunoselected

SMN complexes associated with U1 snRNA (Figure 1C, lane 4), but not with U1 Δ SL1 (Figure 1C, lane 5). Interestingly, 7S RNA was retained on the SMN complexes (Figure 1C, lane 6). This interaction was likely to be specific, because our negative control U6 snRNA was not immunoselected in any of the assays (Figure 1C, lanes 4–6), and also because 7S RNA did not bind to the beads pre-incubated with extracts from HeLa Tet-Off cells (Figure 1C, lane 9). Therefore, these first results indicated that 7S RNA may interact with the SMN complex.

U1 and U2 snRNAs compete with 7S RNA for binding to immunoselected SMN complexes

Although all UsnRNAs bind to the SMN complex in the course of UsnRNP assembly, competition experiments have shown that all of them do not bind to the same site on this complex (56). Therefore, having demonstrated that 7S RNA can interact *in vitro* with the SMN complex, it was important to test whether one or several UsnRNAs can compete with 7S RNA for binding to the SMN complex. Competition binding experiments were performed in conditions previously established for UsnRNAs (56). Purified SMN complexes bound to agarose beads (SMN complex) were incubated with trace amounts of [³²P]UTP-labeled 7S RNA and an excess of non-radioactive U1, U1 Δ SL1, U2, U4, U5 or 7S RNAs. After incubation, bound RNAs were analyzed by denaturing polyacrylamide gel electrophoresis. As shown in Figure 2, non-radioactive 7S RNA competed with itself and non-radioactive U1 and U2 snRNAs significantly competed with labeled 7S RNA. This competition was specific because U1 Δ SL1 was unable to fully compete with 7S RNA. The binding level of labeled 7S RNA in the presence of non-radioactive U4 or U5 snRNA was almost comparable with the one in the presence of non-radioactive U1 Δ SL1, indicating that U4 and U5 snRNAs did not significantly compete with labeled 7S RNA. These data suggested that U1, U2 and 7S RNAs share the same binding site or have partially overlapping binding sites on

the SMN complex, whereas U4 and U5 snRNAs have different binding sites.

The overall 7S RNA is important for efficient interaction with the SMN complex

In order to delineate the region(s) of 7S RNA necessary for its interaction with the SMN complex, we individually produced 7S RNA domains (Figure 3A) and tested their ability to interact with purified SMN complex. As shown in Figure 3B, the RNAs corresponding to the Alu domain alone or the Alu domain extended with helix 5 did not bind to purified SMN complex. RNAs corresponding to the S-domain and the S-domain extended with helix 5 had the capability to interact albeit at very low level compared with wild-type RNA. Therefore, the entire 7S RNA molecule seems to be required for efficient interaction. To go into more details, we analyzed the regions of 7S RNA that are protected against RNase activities when it is bound to the SMN complex (Figure 4). *In vitro* transcribed 7S RNA bound to the purified SMN complex or free 7S RNA were digested by T1, T2 or V1 RNases. RNase protections were observed in three regions of the RNA: in the upper loop of the Alu domain, in the helix 5d region (nucleotides 86–103) and in one strand of helix 8 (nucleotides 174–198). It is interesting to note that when the 7S RNA was bound to the SMN complex, the sensitivity of residues G₇₆, G₂₀₉ and G₂₁₀ to T1 RNase digestion was increased. These data suggest that upon binding to the SMN complex, the 7S RNA undergoes a conformational change leading to destabilization of the two base pairs G₂₀₉-C₁₉₀ and G₂₁₀-C₁₈₉.

Gemin5 directly binds 7S RNA *in vitro*

Even though multiple binding sites for UsnRNAs seem to exist within the SMN complex (56), Gemin5 had been shown to be the protein of the SMN complex that directly interacts with all the UsnRNAs (57). Therefore, we tested whether Gemin5 was also able to interact with 7S RNA. To this end, we prepared total extracts from 293T cells that were transiently expressing Flag-tag Gemin5 (Flag-Gemin5). These extracts were incubated with anti-FLAG antibodies bound to agarose beads in the presence of Empigen (Figure 5A). SMN complexes are disrupted under these conditions, Gemin5 being therefore efficiently dissociated from them (57) (Figure 5A). As previously described, Empigen-treated Flag-Gemin5 was able to bind to U1 and U4 snRNAs, but not to U4 Δ Sm and U6 snRNA (57), and the data revealed the binding of 7S RNA to Flag-Gemin5 (Figure 5B). To exclude the possibility that some minor components in cell extracts that had been co-immunoselected with Flag-Gemin5 conferred this binding specificity to 7S RNA, GST-tagged Gemin5 was expressed and purified from *E. coli* by immobilization on Glutathione sepharose beads. In contrast to a GST-SMN fusion protein and GST alone, the bound GST-tagged Gemin5 was able to interact with 7S RNA (Figure 5C), indicating that Gemin5 binds directly to 7S RNA without requiring any other components of the SMN complex or other cellular factors.

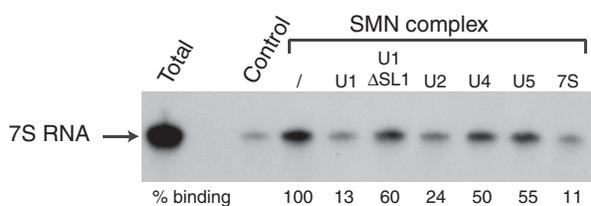


Figure 2. U1 and U2 snRNAs compete with 7S RNA for binding to the SMN complex. Purified native SMN complexes (SMN complex) were pre-incubated with an excess of unlabeled U1, U1 Δ SL1, U2, U4, U5 and 7S. A pre-incubation was also done without the presence of any RNA (/) as a control. Non-specific proteins purified from parental cells (Control) were also pre-incubated. Then, *in vitro* transcribed [³²P]UTP-labeled 7S RNA was added to the pre-incubated mixtures and further incubated. Bound RNAs were isolated after washing and analyzed by electrophoresis on denaturing polyacrylamide gels and autoradiography. Lanes labeled 'Total' represent 10% of input. The intensity of signal in each condition was quantified using the Typhoon 9410 (Amersham).

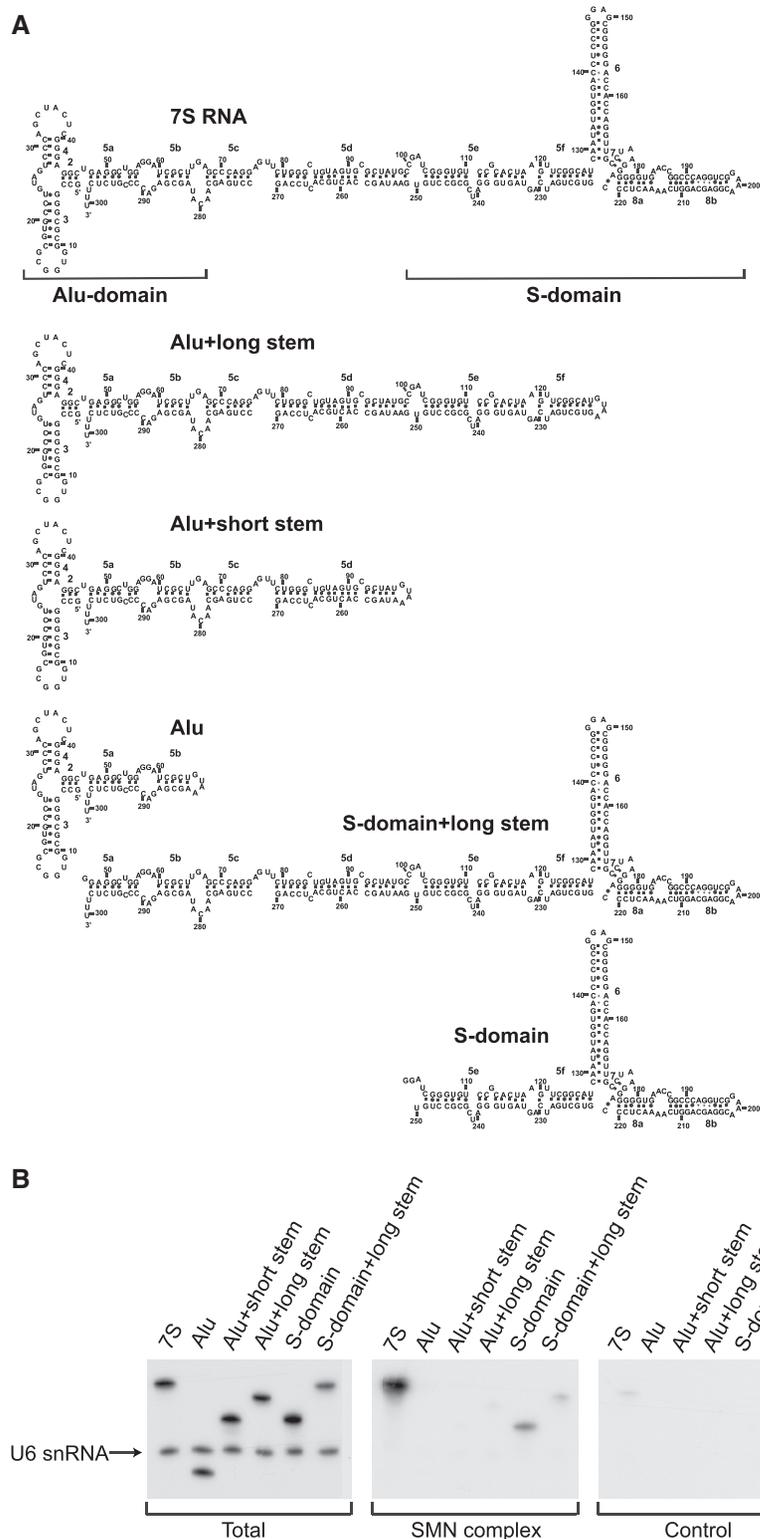


Figure 3. The overall 7S RNA molecule is required for efficient interaction with the purified SMN complex *in vitro*. **(A)** Schematic representation of the 7S RNA domains used in the study. **(B)** The purified native SMN complex (SMN complex) or the non-specific proteins purified from parental cells (Control) were incubated with *in vitro* transcribed [³²P]UTP-labeled 7S RNA WT or the individual 7S RNA domains, in the constant presence of U6 snRNA as an internal negative control. Subsequently, bound RNAs were isolated after washing and analyzed by electrophoresis on denaturing polyacrylamide gels and autoradiography. Lanes labeled 'Total' represent 10% of input.

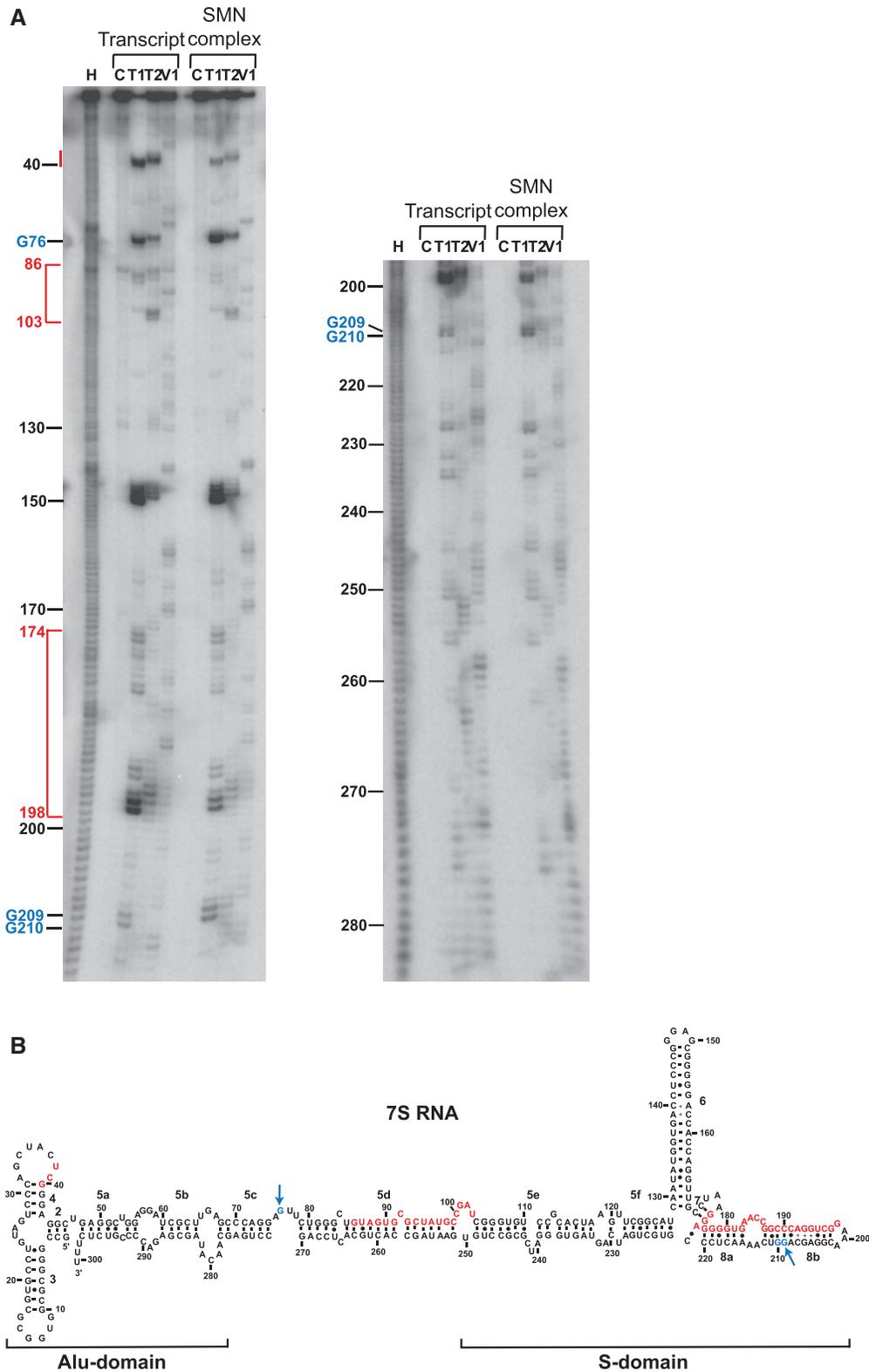


Figure 4. The SMN complex protects three regions of the 7S RNA. (A) *In vitro* transcribed 7S RNA was incubated in the absence (Transcript) or in the presence (SMN complex) of the purified SMN complex. The RNA was then digested by RNases T1, T2 and V1 as described in the ‘Materials and Methods’ section. The cleavage products were analyzed by electrophoresis on denaturing polyacrylamide gels. An alkaline hydrolysis is shown (H). Nucleotides are numbered on the left and were designed by the use of RNase T1 in denaturing conditions and by a longer fractionation on polyacrylamide gel to identify residues in the 5'-end (data not shown). (B) The protected regions within the 7S RNA are indicated in red, and the G residues that were more sensitive to T1 RNase cleavages are indicated by blue arrows.

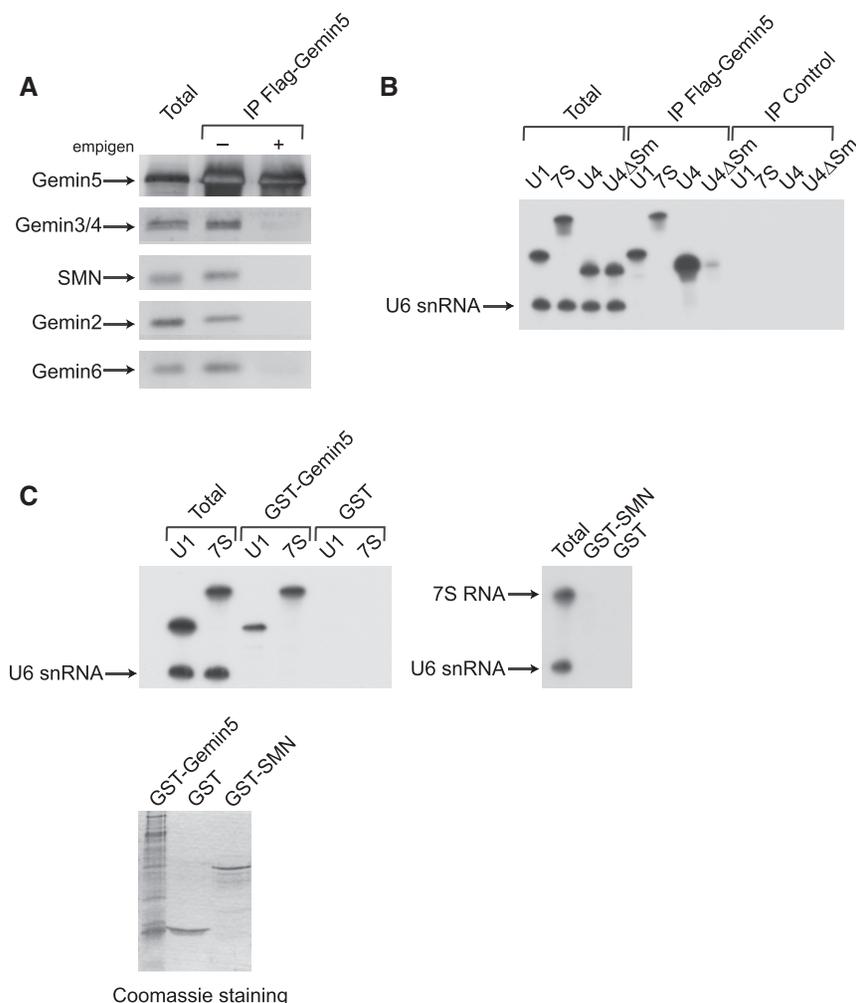


Figure 5. Gemin5 binds to 7S RNA. (A) Immunoprecipitation experiments using anti-Flag antibody were carried out on total extracts prepared from HeLa cells transiently expressing Flag-tagged Gemin5 in the absence (–) or in the presence (+) of 1% Empigen-BB detergent. The immunoprecipitated proteins were analyzed by SDS–PAGE and western blotting using antibodies directed against the indicated proteins. In total, 5% of the extracts used in the experiments is shown. (B) After immunoprecipitations as in (A) in the presence of 1% Empigen-BB detergent, purified Flag-Gemin5 was incubated with [³²P]UTP-labeled 7S RNA, U4 snRNA or U1 snRNA, in the constant presence of U6 snRNA used as an internal negative control. (C) Recombinant GST-Gemin5, GST-SMN or GST were expressed in *E. coli* and bound to glutathione-Sepharose beads. The proteins were incubated with [³²P]UTP-labeled 7S RNA or U1 snRNA, in the constant presence of U6 snRNA, used as an internal negative control. The recombinant proteins used in the study have been analyzed by SDS–PAGE and coomassie staining. In panels B and C, bound RNAs were isolated after washing and analyzed by electrophoresis on denaturing polyacrylamide gels. Lanes labeled ‘Total’ represent 10% of the input.

The SMN complex associates with SRP in HeLa cell extracts

To determine whether the 7S interaction with the SMN complex has a biological significance, we next tested whether the SMN complex was able to associate with SRP *in cellulo*. Our strategy was to immunoprecipitate HeLa cell extracts with anti-SRP9 (Figure 6A) or anti-SRP54 (Figure 6B) antibodies and to test for the presence of proteins of the SMN complex in the immunoprecipitated material. We also used an anti-SMN antibody 4B3 and tested whether SRP co-immunoprecipitated with SMN (Figure 6C). The immunoprecipitated proteins (IP) and proteins from an aliquot of the respective extracts (Total) were fractionated by SDS–PAGE and analyzed by western blotting. The anti-SRP9 and anti-SRP54 antibodies efficiently immunoprecipitated

endogenous SRP9 and SRP54 proteins, respectively, as well as other SRP proteins (SRP68 and SRP14), indicating that the entire SRP particle was immunoprecipitated. Importantly, consistent with an association of SRP with the SMN complex *in vivo*, all the components of the SMN complex that were tested (SMN and Gemin2, 3, 4 and 5) were found to co-immunoprecipitate with SRP9 (Figure 6A) and SRP54 (Figure 6B). In the reverse experiment, the anti-SMN antibody immunoprecipitated the SRP proteins that were tested, i.e. SRP9, SRP19 and SRP68. The 7S RNA was also associated with SMN, whereas the mRNA coding for Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) did not and was used as a negative control (Figure 6C). Our results support the conclusion that the SMN complex associates with the SRP in HeLa cells.

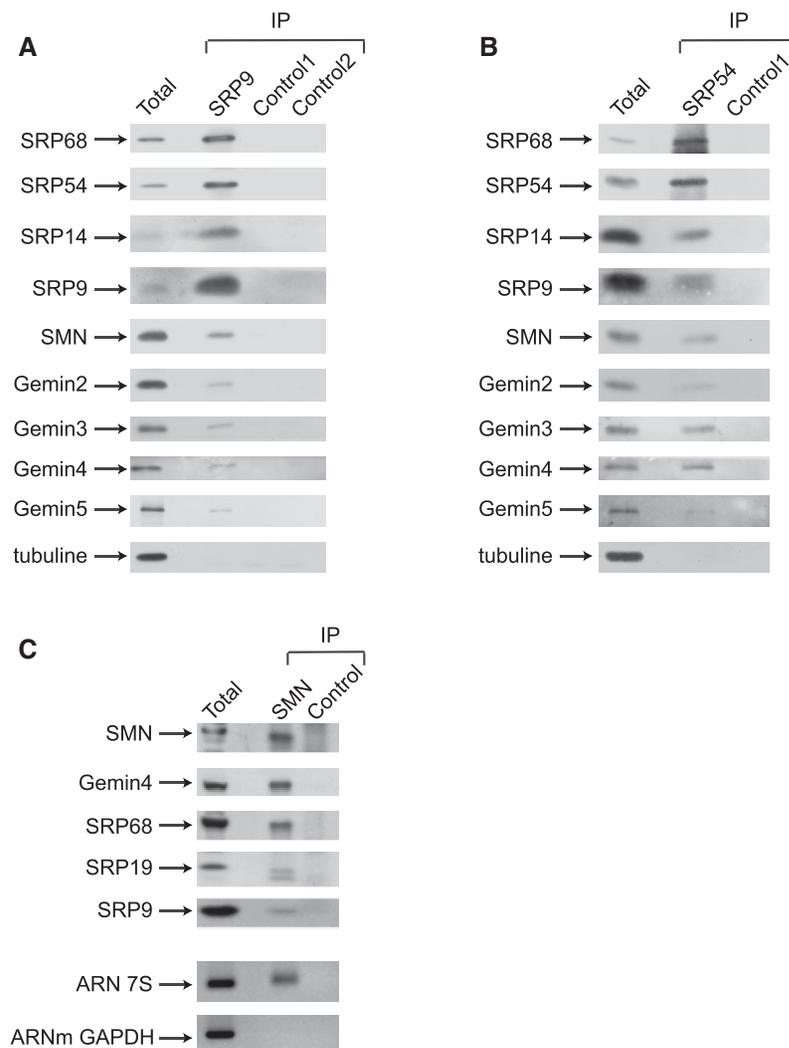


Figure 6. The SMN complex associates with SRP in HeLa cell extracts. IP experiments were carried out on HeLa cytoplasmic extracts using anti-SRP9 (A), anti-SRP54 (B), or anti-SMN 4B3 (C) antibodies bound to A-sepharose [in (A) and (B)] or G-dynabeads [in (C)]. The unrelated anti-Sox2 and anti-Lin28 antibodies were used as negative controls in (A) (Controls 1 and 2, respectively). A-sepharose alone was used as control in (B) and negative control mouse IgG (DAKO) in (C). The immunoprecipitated proteins were analyzed by SDS-PAGE and western-blotting with antibodies to the indicated proteins; 3% of the inputs are shown (Total). The immunoprecipitated RNAs were analyzed by RT-PCR in (C).

SMN and Gemin2 associate with the 7S RNA in the cytoplasm of *X. laevis* oocytes

Our observations that 7S RNA can interact with purified SMN complexes and that SRP associates with the SMN complex in human cells suggested a possible involvement of the SMN complex in SRP biogenesis. As microinjection of vertebrate UsnRNAs in *X. laevis* oocytes had been one key approach to demonstrate the role of the SMN complex in UsnRNP assembly (22,55), we decided to use this approach to test our hypothesis. This was as much justified as human and *X. laevis* 7S RNA microinjections in *X. laevis* oocytes had also been previously used for *in cellulo* study of SRP assembly (51,52). Previous microinjection of 7S RNA in the nucleus of *X. laevis* oocytes, showed that it is first localized in the nucleolus before being exported to and stably accumulated in the

cytoplasm (51,52). Importantly, in *X. laevis* oocytes, the SMN and Gemin2 proteins are detected in the cytoplasm but not in nuclei (22). As a first step in the demonstration of our hypothesis, we had to test whether the *X. laevis* SMN complex can associate with 7S RNA in oocytes. To this end, we produced an *in vitro* transcribed [³²P]UTP-labeled human 7S RNA that we injected into the nucleus of oocytes and after 6 h of incubation, the oocytes were manually dissected. More than 50% of the injected 7S RNA was found to be transported to the cytoplasm (C), whereas the remaining part was still in the nucleus (N) (Figure 7A, lanes 1 and 2). We performed IPs on both the nuclear and cytoplasmic fractions, using antibodies that were able to recognize specifically the endogenous SMN, Gemin2 and SRP54 proteins and non-immune antibodies (IP control). We used nuclear

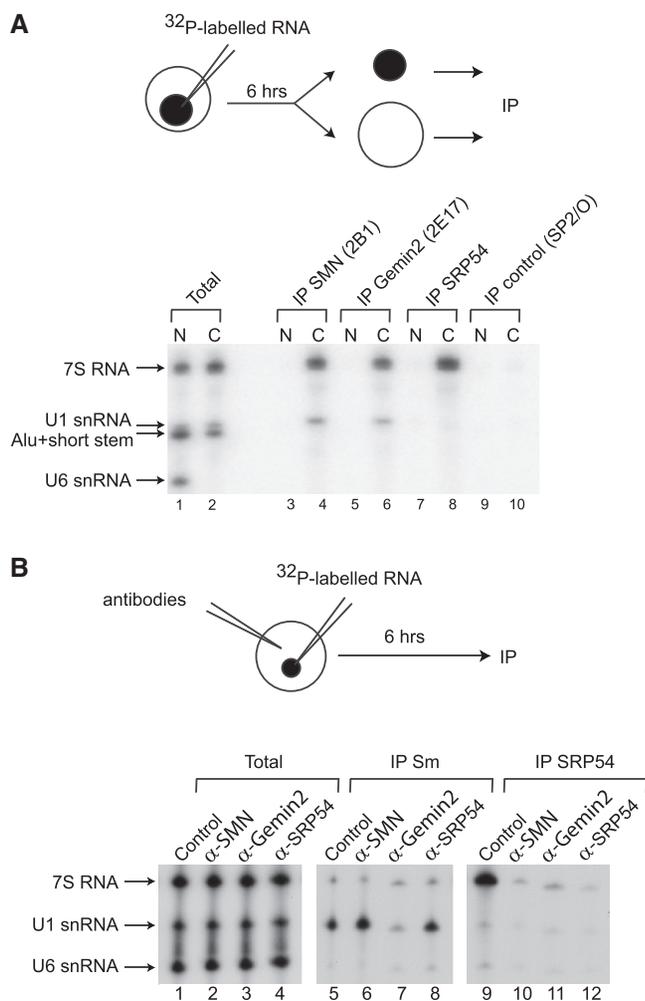


Figure 7. Anti-SMN and anti-Gemin2 antibodies interfere with the binding of SRP54 on 7S RNA in the cytoplasm of *X. laevis* oocytes. (A) A mixture of [³²P]UTP-labeled U1, U6, 7S and 7S 5'-3'-regions RNAs was injected into the nucleus of *X. laevis* oocytes. After incubation for 6 h, the oocytes were manually dissected into nuclear (N) and cytoplasmic (C) fractions. IPs were then carried out from both fractions with the anti-SMN (2B1), anti-Gemin2 (2E17), anti-SRP54 or control non-immune (SP2/O) antibodies. Bound RNAs were analyzed by electrophoresis on a denaturing polyacrylamide gel and autoradiography. About 10% of each fraction was loaded on the gel (Total). (B) Either non-immune (SP2/O), anti-SRP54, anti-Gemin2 (2E17) or anti-SMN (2B1) antibodies were injected into the cytoplasm of *X. laevis* oocytes. The same oocytes were nuclear injected 1 h later with a mixture of [³²P]UTP-labeled U6 snRNA and 7S RNA. After 6 h, total cellular extracts were prepared from these oocytes. Immunoprecipitations were then carried out with the anti-Sm core (Y12) (IP Y12) or anti-SRP54 (IP SRP54) antibodies. RNAs were analyzed by electrophoresis on a denaturing polyacrylamide gel and autoradiography. About 10% of each injected RNA was loaded on the gel (Total).

fractions as negative controls in these assays. In addition to 7S RNA, we microinjected two other radiolabeled RNAs: (i) *X. laevis* U1 snRNA was used as a positive control because it has been shown to associate with the SMN complex in the cytoplasm of *X. laevis* oocytes (22) and (ii) a truncated version of human 7S RNA (Alu+short stem) was used as a negative control, because this RNA failed to bind efficiently to the purified SMN complexes in

our *in vitro* experiments (Figure 3). In agreement with previous data (51), a portion of the injected 7S Alu+short stem was transported to the cytoplasm (Figure 7A, lanes 1 and 2). Microinjected human U6 snRNA, which is known to be retained in the nucleus (77), was used to control the quality of the dissections. As expected, (i) anti-SMN and anti-Gemin2 antibodies immunoprecipitated U1 snRNA from the cytoplasmic fraction (Figure 7A, lanes 4 and 6, respectively), but not the nuclear fraction (Figure 7A, lanes 3 and 5, respectively) and (ii) anti-SRP54 and non-immune antibodies did not immunoprecipitate U1 snRNA (Figure 7A, lanes 7–10). In contrast, we found that the radiolabeled 7S RNA, which had been transported to the cytoplasm, was immunoprecipitated by anti-SRP54 antibodies (Figure 7A, lane 8), indicating the interaction of SRP54 with the injected RNA. Radiolabeled 7S RNA was also efficiently immunoprecipitated from the cytoplasmic fraction when using anti-SMN and anti-Gemin2 antibodies, whereas the 7S Alu+short stem was not (Figure 7A, lanes 4 and 6). These results demonstrated a cytoplasmic association of 7S RNA with SMN and Gemin2 proteins after its injection in the nuclei of *X. laevis* oocytes, which suggested a possible role of the SMN complex in the cytoplasmic step of SRP assembly.

Anti-SMN and anti-Gemin2 antibodies strongly interfere with SRP54 association to 7S RNA in *X. laevis* oocytes

The cytoplasmic step of SRP assembly consists in SRP54 association to 7S RNA. To test whether the SMN complex might be involved in this step in the cytoplasm of *X. laevis* oocytes, we microinjected anti-SMN (2B1), anti-Gemin2 (2E17) or anti-SRP54 antibodies in the cytoplasm of oocytes and tested their effects on the association of SRP54 with 7S RNA which was microinjected 1 h later in the nucleus. Microinjection of non-immune antibodies (SP2/O) was used as a negative control, and U6 snRNA and U1 snRNA were co-injected with 7S RNA. After 6 h of incubation, total extracts were prepared and immunoprecipitations were carried out using either the anti-Sm core protein antibody (Y12) (66) to monitor the assembly of Sm proteins on U1 snRNA, or anti-SRP54 antibodies to test for SRP54 binding to the 7S RNA. As expected, Y12 antibodies did not immunoprecipitate U6 snRNA and 7S RNA, which both do not associate with Sm proteins (Figure 7B, lanes 5–8) and anti-SRP54 antibodies did not immunoprecipitate U6 and U1 snRNAs that do not interact with SRP54 (Figure 7B, lanes 9–12). Pre-injection of oocytes with non-immune (SP2/O, Control) or anti-SRP54 antibodies, did not alter Sm protein association with nuclear-injected U1 snRNA as evidenced by its efficient immunoprecipitation with Y12 antibodies (Figure 7B, lanes 5 and 8). As expected (22), the anti-Gemin2 (2E17) antibody inhibited the Sm core assembly on U1 snRNA (Figure 7B, lane 7), while as previously described (22), the anti-SMN antibody (2B1) unexpectedly slightly stimulated the assembly (Figure 7B, lane 6). When the oocytes were pre-injected with non-immune antibody (Control) (Figure 7B, lane 9), the injected 7S RNA was efficiently immunoprecipitated

with anti-SRP54 antibody. In contrast, after pre-injection of anti-SRP54 antibody in the cytoplasm of oocytes (Figure 7B, lane 12), the injected 7S RNA was not immunoprecipitated with anti-SRP54 antibody. Thus, upon binding to its SRP54 epitope, the anti-SRP54 antibody abolished the association of SRP54 with 7S RNA. We observed that the association of 7S RNA with SRP54 was inhibited in oocytes that were pre-injected with anti-SMN (2B1) or anti-Gemin2 (2E17) antibodies (Figure 7B, lanes 10 and 11). Thus, we could conclude that occlusion of SMN or Gemin2 epitopes by antibodies interfered with the association of SRP54 to microinjected 7S RNA. Taken together, these data were strong evidence in favor of a role of the SMN complex in the last step of SRP biogenesis, i.e. association of SRP54 to 7S RNA in the cytoplasm.

7S RNA associates with SMN in *S. pombe* and its level is decreased in a mutant carrying a temperature-degron allele of SMN

Another approach to test for a possible role of the SMN complex in SRP assembly was to use yeast genetic methods. As mentioned in the 'Introduction' section, although the SMN complex is not present in *S. cerevisiae*, an SMN protein is present in *S. pombe* and a tdSMN strain carrying a thermosensitive-degron SMN allele had been produced (30). On the other hand, SRP is highly conserved in evolution and the sequence of the *S. pombe* 7S RNA gene (*srp7*) had been reported (45,46). The *S. pombe* 7S RNA is 254-nt long and shows ~50% of sequence identity to its human counterpart, its secondary structure being strikingly similar to the proposed structure of human 7S RNA (46). Therefore, *S. pombe* turned to be an excellent model to investigate the possible involvement of the SMN complex in SRP biogenesis. First, we determined whether SMN also associates with 7S RNA in *S. pombe*. To this end, after *in vivo* RNA-protein cross-linking with formaldehyde of a *S. pombe* strain expressing a TAP-tagged version of the fission yeast SMN protein, we performed immunoprecipitation experiments under stringent conditions using IgG Sepharose beads (78). RT-PCR analysis of RNAs covalently cross-linked to and co-purified with TAP-SMN revealed that 7S RNA and, as expected, U2 snRNA were present in the pellet (Figure 8A), whereas other RNAs, such as U3 snoRNA and tRNA^{Ser} showed no significant co-purification. These observations revealed an association of the SMN protein with the RNA component of SRP in *S. pombe* cells.

Next, as SRP assembly was expected to be required for 7S RNA stability, we tested whether SMN was required for the accumulation of the *S. pombe* 7S RNA. To do so, northern blot analyses were performed on RNA extracted from a *S. pombe* wild-type strain and the tdSMN strain carrying a temperature-degron allele of SMN (30). After a temperature shift from 25°C to 37°C for 8 h, the 7S RNA level decreased by 40% in the mutant strain, whereas no temperature-related change was found in the wild-type strain (Figure 8B). As expected, a decrease of the spliceosomal U2 snRNA was also observed upon

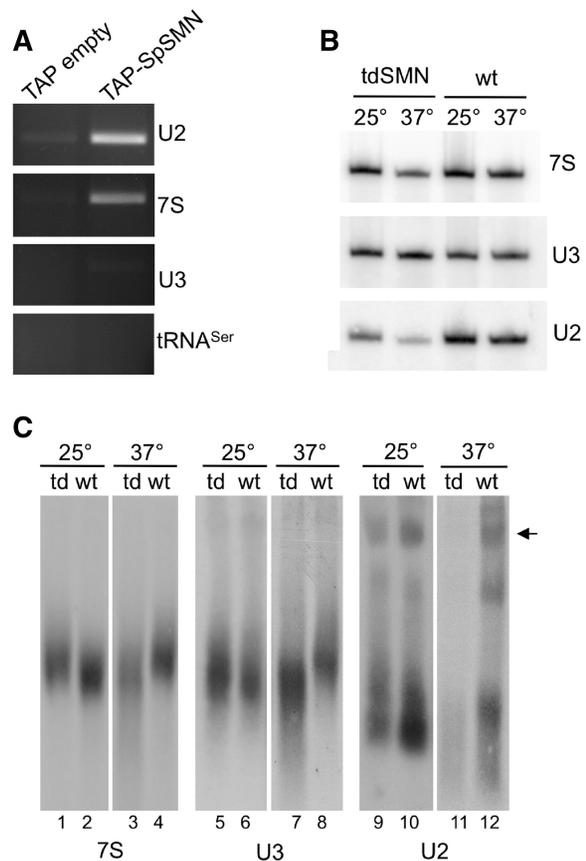


Figure 8. The SMN protein associates with the 7S RNA *in vivo* in fission yeast cells and is required for the stability of the SRP complex. (A) Quantitative RT-PCR experiments were performed for the indicated RNA species using RNA affinity-purified from extracts prepared from fission yeast cells carrying a TAP-SMN construct or an empty TAP vector as control. Experiments were done in duplicate. (B) Northern blot analysis of RNA isolated from wild-type and temperature-sensitive tdSMN cells before and after shift for 8 h at 37°C. The RNA was separated on 6% denaturing polyacrylamide gels, subjected to northern blot and hybridized with oligonucleotide probes for the indicated RNA. (C) Analysis of RNP complexes in wild-type and temperature-sensitive tdSMN cells by native gel electrophoresis. Extracts were prepared from cells grown before and after a 8-h shift at 37°C and 20 µg of extract were separated on 4% native gels. The RNA was subjected to northern blot analysis and hybridized with probes for the indicated RNAs. The arrow points to the position of the U2/U5/U6 tri-snRNP.

temperature shift, whereas the level of U3 snoRNA appeared unchanged (Figure 8B) (30). RNP levels were also analyzed using native gel electrophoresis that has previously been used to study RNP complexes in fission yeast (30,79). As shown in Figure 8C (panels at right), the U2 snRNP, as well as the U2/U5/U6 tri-snRNP (indicated by an arrow) can be observed in extracts prepared from wild-type and tdSMN cells at 25°C, but were strongly destabilized after a 8-h shift to 37°C in the tdSMN strain (lane 11), as expected from the requirement of SMN for spliceosomal snRNPs assembly (30). A decrease in the level of the SRP particle was also observed in the tdSMN cells upon shift to 37°C (Figure 8C, compare lanes 1 and 3), whereas no change in the amount of the 7S RNP occurs in wild-type cells (compare lanes 2 and 4). Finally, similar ratios of U3

snoRNP were found in extracts prepared from the tdSMN and wild-type cells before and after shift to the non-permissive temperature (Figure 8C, middle panels), suggesting that the assembly of this snoRNP does not depend on the SMN complex in *S. pombe*. It should be noted that the level of the U3 particle appeared to increase slightly after shift to 37°C in the tdSMN background (Figure 8C, lane 7). Given that splicing is strongly inhibited at 37°C in the tdSMN cells (30), this might represent a compensatory response to defective splicing of pre-mRNAs coding for U3-associated proteins. Altogether, our data demonstrated that the SMN protein associates with 7S RNA and has an essential role in the steady-state level of SRP in *S. pombe* cells, which reinforces the idea of its possible implication in SRP biogenesis.

The steady-state level of 7S RNA is significantly decreased in spinal cord of SMA mice

Based on the data we obtained in both *X. laevis* oocytes and in *S. pombe*, we hypothesized that reduced SMN levels in mammalian cells might also affect the accumulation of 7S RNA. Therefore, we tested whether 7S RNA level is affected in an animal model of SMA. To this end, we analyzed total RNA from three different tissues of 10-day-old SMN-deficient mice (SMN2^{+/+}, SMNdelta7^{+/+}, Snn^{-/-}). This severe SMA mouse model (corresponding to a SMA type II phenotype, Jackson's laboratory, #5025) has a mean life expectancy of 13 days (80). Study on the spinal cord was particularly of high interest because: (i) it contains the motor neurons, which are the cells mainly affected in SMA, and (ii) due to a specific regulation of exon 7 inclusion in the SMN2 mRNA of motor neurons, expression of the full-length functional SMN protein is strongly reduced in SMA motor neurons as compared with other cells (81). Therefore, we compared the levels of 7S RNA in total RNA from spinal cord, brain and heart of five model mice and five controls corresponding to transgenic mice (SMN2^{+/+}, SMNdelta7^{+/+}, Snn^{+/+}), by using reverse transcription with specific oligonucleotides followed by real-time PCR. Interestingly, the 7S level was markedly decreased in spinal cord (~60%), but not in brain and heart (Figure 9). Our results support the conclusion that a marked decrease of the 7S RNA levels is linked to SMN deficiency in spinal cord of SMA mouse model.

DISCUSSION

7S RNA and SRP associate specifically with the SMN complex

In this study, we found that the 7S RNA can bind to purified SMN complexes. The specificity of this 7S interaction is strongly supported by our finding of an association of 7S RNA with the SMN protein in both *S. pombe* and human cells and the specific association of micro-injected 7S RNA with the SMN and Gemin2 proteins in *X. laevis* oocytes. As we also detected an association of SRP proteins with the SMN complex in HeLa cells, not

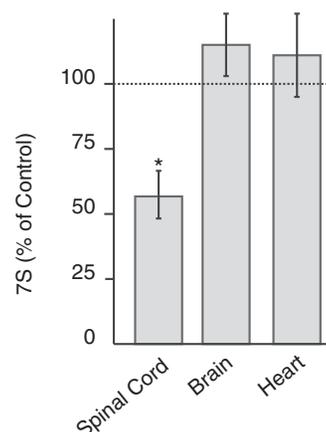


Figure 9. Analysis of endogenous 7S RNA level in tissues of SMA mice. Total RNA from the spinal cord, brain and heart of post-natal Day 10 control and SMA mice were analyzed by real-time RT-PCR. The level of 7S RNA was monitored by five independent biological replicates ($n = 5$). The relative amount of 7S RNA in SMA mice tissues is plotted as percent of the controls. RPS29 mRNA was used to normalize the RNA input. The level of 7S RNA in spinal cord was significantly reduced in SMA mice, whereas its levels in brain and heart remain the same ($P < 0.05$).

only 7S but the entire SRP particle is likely able to interact with the SMN complex.

Studies performed on UsnRNAs have revealed a great complexity of the SMN complex RNA recognition mechanisms. Indeed, in spite of the presence of two highly conserved motifs in all UsnRNAs, which bind the Sm proteins (82), namely the Sm site (AUUUUUG) and the 3' terminal stem-loop structure, the SMN complex carries distinct determinants for the selective recognition of the various UsnRNAs (55,56,83,84). More precisely, the two common UsnRNA motifs are required for recognition of U4 and U5 snRNAs. The 3' terminal stem-loop structure but not the Sm site is required for binding of U2 snRNA, and the high-affinity binding site for the SMN complex on U1 snRNA lies outside of the two conserved motifs, i.e. in stem-loop 1. A 3' stem-loop structure is also crucial for minor UsnRNA binding (84). Competition experiments using the entire SMN complex revealed at least two UsnRNA binding sites on the SMN complex, one for U4 snRNA and one for U1 snRNA. In addition, both U1 and U4 snRNAs can fully compete with U2 and U5 bindings, revealing an even higher complexity of the organization of the UsnRNA binding sites within the SMN complex (56). Our study extends this level of complexity, because we showed that the SMN complex can specifically recognize 7S RNA, which contains none of the UsnRNA canonical motifs. Moreover, the binding site for 7S RNA at least partially overlaps the U1 and U2 binding sites, as these two RNAs, but not U4 and U5, specifically compete with 7S binding on the SMN complex. RNAs corresponding to the Alu domain alone or the Alu domain extended with helix 5 did not bind to purified SMN complex, whereas RNAs corresponding to the S-domain and the S-domain extended with helix 5 had the capability to interact albeit at very low level compared with wild-type RNA. These results indicate that the recognition

determinants depend on either the entire 7S RNA structure or on multiple domains spread over the RNA molecule. This is confirmed by the fact that three regions spanning the overall 7S RNA (one in the Alu domain, a second in helix 5 and a third in helix 8) were buried within the SMN complex when the RNA was bound.

Gemin5 interacts with all the RNA targets of the SMN complex containing the Sm or Lsm motif (57,84,85). The binding of 7S RNA to the SMN complex is probably also mediated by Gemin5, because we showed the specific interaction of Gemin5 with 7S RNA. Nevertheless, we cannot exclude the possibility that other proteins of the SMN complex contribute to 7S recognition.

Decreased SMN amounts can generate a reduction of the 7S RNA levels

Our observation of an association of both the 7S RNA and the SRP with the SMN complex could reflect an implication of the SMN complex in either SRP biogenesis or activity. It is now clearly proven that the SMN complex plays an essential role for assembly of Sm- and Lsm-containing RNPs, i.e. minor and major UsnRNPs, U7 snRNP and *Herpesvirus saimiri* HSURs (18,19,22–27,86–88). As a result, SMN deficiency was found to alter the repertoire of UsnRNAs in cells and tissues derived from SMA mice, in HeLa cells depleted of SMN by RNAi, as well as in the *S. pombe* mutant strain carrying a temperature-degron allele of SMN (28–30,32,89,90). Similarly, an implication of the SMN complex in SRP biogenesis was expected to result in SRP assembly defects in SMN-deficient cells. Furthermore, due to the degradation of misassembled 7S RNA molecule, a reduced level of 7S was expected in SMN-deficient organisms. Accordingly, we found that the expression of the degron-SMN allele in *S. pombe* decreased the 7S RNA cellular level. Using a mouse model for severe SMA, we also observed a marked decrease of 7S RNA in the spinal cord, a mainly affected tissue in SMA, whereas its level was unchanged in the brain and heart. It should be pointed out that in mice, a low level of SMN does not also cause a uniform reduction of UsnRNAs. Indeed, the reduction is rather cell and/or snRNA-specific (28,29,32,89). Thus, our studies report for the first time a defect in the level of another type of RNA than UsnRNAs in SMN-deficient model organisms. This observation favors the hypothesis of a role of the SMN complex in SRP assembly versus the hypothesis for a role of the SMN complex in SRP activity. Consistent with this hypothesis, we found that microinjection of anti-SMN (2B1) or anti-Gemin2 (2E17) antibodies in the cytoplasm of *X. laevis* oocytes strongly interfere with the cytoplasmic association of 7S RNA with SRP54. Therefore, we propose that the SMN complex plays a yet-to-be determined role in the cytoplasmic incorporation of SRP54 in SRP. Interestingly, our results suggest that upon 7S RNA binding to the SMN complex, helix 8 is in interaction with the complex and may undergo a conformational change. Indeed, G₂₀₉ and G₂₁₀ that form two side-by-side base pairs in helix 8 were

more sensitive to cleavages by T1 RNase (Figure 4). This might indicate that the two base pairs are slightly destabilized, or become more accessible in the RNA tertiary structure, when the RNA is bound to the SMN complex. Interestingly, helix 8 is important for individual binding of SRP19 and SRP54 to 7S RNA *in vitro* (91–94). Moreover, these two base pairs are crucial for the SRP19-dependent binding of SRP54 (95). Therefore, the recognition of helix 8 by the SMN complex and its subsequent conformational change may be important for the SMN complex-mediated SRP assembly.

A defect in SRP biogenesis may contribute to SMA

Although reduced levels of SMN cause SMA, it is still unclear why a defect in a ubiquitously expressed protein causes a selective death of the alpha-motor neurons. A defect in the assembly of UsnRNPs is proposed to cause disease onset leading to abnormal splicing of specific mRNAs and disease progression (28–32, 87,96,97). Due to the reduction in the levels of minor UsnRNAs observed in several tissues of SMA mice and to the defect in the assembly of the minor tri-snRNP observed in SMA-derived lymphocytes, one can reasonably think that minor splicing pathway could be specifically affected in SMA, and this may concern gene(s) of high importance for motor neurons activity (28,29,31,89). However, as microarray studies revealed splicing changes in various tissues from SMA mice, it is still not clear whether these alterations have a direct pathogenic role in SMA, or are indirect or secondary changes due to disease progression (29,98). Recently, it has been shown that SMN deficiency perturbs splicing of specific U12 intron-containing genes in mammalian cells and in *Drosophila* larvae (32).

Western blot analysis indicated that the amounts of SMN in different tissues of the SMN Δ ex7 mouse model used in this study are very low and barely detectable (29,80,99). Therefore, this low level makes difficult a precise comparison of SMN quantities between tissues. However, a recent study (81) proposed that one reason for the preferential vulnerability of motor neurons to SMA could be a lower expression of full-length SMN from the *SMN2* gene in motor neurons compared with other cell types that would be due to a less efficient retention of exon7 in these cells. Thus, a lower level of SMN protein in this tissue might explain the reduction of 7S RNA levels in the spinal cord that we detected in the SMA mice. The SMN levels, albeit very low, might be sufficient to maintain normal 7S RNA levels in other tissues tested.

Our observations open the possibility that reduced SRP levels may contribute to SMA onset and/or severity. The effect of reduced levels of SRP in mammalian tissues, including the spinal cord, has not been studied yet in animal models. However, a crucial importance of SRP has been shown for several microorganisms. SRP is essential for growth in bacteria and in *S. pombe* (100,101). It is not essential in *S. cerevisiae*, but its absence considerably slows down cell growth, represses protein synthesis and induces a heat shock response (47,102,103). Tests on the

effects of SRP deficiency were performed in mammalian cells using the possibility to reduce SRP protein expression. Reduced SRP14 expression in cultured mammalian cells has been shown to limit protein secretion and cell growth, but was not lethal (104). Furthermore, no growth defect was observed after silencing of SRP54 and SRP72 expression (105,106). However, due to the high stability and high level of expression of SRP, the level of SRP remaining in the cell in such RNA interference experiments and the existence of SRP-independent pathways (107–109) might have been sufficient to ensure protein secretion. Although synthesis, secretion and trafficking of neurotransmitters, neurotrophic factors, ligands and membrane receptors in neurons are crucial mechanisms for neuronal morphogenesis, synapse formation and for the regulation of the synaptic strength, little is currently known on the abundance and the functional importance of SRP in neurons. Most membrane and secreted proteins are synthesized in the cell body of neurons via protein translocation into the endoplasmic reticulum (ER) and are subsequently transported into the dendrites and axons. Neuronal proteins that are synthesized locally in dendrites and axons likely make use of the ER- and Golgi-like structures that have been detected within neurites, so that their synthesis likely also depends upon SRP [for recent reviews, (110,111)]. Hence, the reduced level of SRP that we detected in the spinal cord of the SMA mice model is expected to have a strong negative effect on motoneuronal development and function and consequently on the SMA pathology.

In conclusion, the human SMA disease forms a continuum of severity that may be explained by the reduced levels of several macromolecular machineries having diverse functions, including UsnRNP and SRP. Our data open a new area of research in the perspective of understanding the causes of the SMA pathology, as well as a new conceptual base in the view to decipher how the essential SRP is assembled *in vivo*.

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