

# Gemin4: A Novel Component of the SMN Complex That Is Found in both Gems and Nucleoli

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**Abstract.** The survival of motor neurons (SMN) protein, the product of the neurodegenerative disease spinal muscular atrophy (SMA) gene, is localized both in the cytoplasm and in discrete nuclear bodies called gems. In both compartments SMN is part of a large complex that contains several proteins including Gemin2 (formerly SIP1) and the DEAD box protein Gemin3. In the cytoplasm, the SMN complex is associated with snRNP Sm core proteins and plays a critical role in spliceosomal snRNP assembly. In the nucleus, SMN is required for pre-mRNA splicing by serving in the regeneration of spliceosomes. These functions are likely impaired in cells of SMA patients because they have reduced levels of functional SMN. Here, we report the identification by nanoelectrospray mass spectrometry of a novel component of the SMN complex that we name Gemin4. Gemin4 is associated in vivo with the

SMN complex through a direct interaction with Gemin3. The tight interaction of Gemin4 with Gemin3 suggests that it could serve as a cofactor of this DEAD box protein. Gemin4 also interacts directly with several of the Sm core proteins. Monoclonal antibodies against Gemin4 efficiently immunoprecipitate the spliceosomal U snRNAs U1 and U5 from *Xenopus* oocytes cytoplasm. Immunolocalization experiments show that Gemin4 is colocalized with SMN in the cytoplasm and in gems. Interestingly, Gemin4 is also detected in the nucleoli, suggesting that the SMN complex may also function in preribosomal RNA processing or ribosome assembly.

**Key words:** gems • nucleoli • SMN • spinal muscular atrophy • snRNP biogenesis

## Introduction

Spinal muscular atrophy (SMA)<sup>1</sup> is a common autosomal recessive disease that is the leading hereditary cause of infant mortality. SMA is characterized by degeneration of motor neurons of the anterior horn of the spinal cord resulting in muscular weakness and atrophy (Pearn, 1980; for review see Melki, 1999). SMA results from deletions or mutations in the survival of motor neurons gene (*SMN*), which is duplicated as an inverted repeat on human chromosome 5 at 5q13 (Brzustowicz et al., 1990; Melki et al., 1990, 1994). The telomeric copy of the

*SMN* gene (*SMN1*) is deleted or mutated in >98% of SMA patients (Lefebvre et al., 1995; for review see Burghes, 1997). The SMN protein is expressed in all tissues of mammalian organisms with particularly high levels expressed in motor neurons (Coover et al., 1997; Lefebvre et al., 1997). In contrast, individuals affected by the most severe form of SMA, Werdnig-Hoffman syndrome or SMA type I, have barely detectable levels of SMN in motor neurons (Coover et al., 1997; Lefebvre et al., 1997).

The SMN protein is part of a multiprotein complex and two other proteins of the complex, Gemin2 (formerly SIP1) and Gemin3 (for component of gems 2 and 3, respectively) thus far have been characterized (Liu et al., 1997; Charroux et al., 1999). SMN, Gemin2, and Gemin3 localize in the cytoplasm and the nucleus of somatic cells. In the nucleus, they are concentrated in bodies called gems, which are similar in size and number to coiled bodies (CBs) and are often associated with them (Liu and

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<sup>1</sup>Abbreviations used in this paper: CB, coiled body; Gemin2, 3, and 4; component of gems number 2, 3, and 4, respectively; ORF, open reading frame; SMA, spinal muscular atrophy; SMN, survival of motor neurons; snRNP, small nuclear ribonucleoprotein (RNP); snoRNP, small nucleolar RNP.

Dreyfuss, 1996; Liu et al., 1997; Charroux et al., 1999). In addition to SMN, Gemin2 and Gemin3, the large cytoplasmic complex of which they are part also contains Sm proteins that are components of spliceosomal small nuclear ribonucleoprotein (snRNPs; Liu et al., 1997; Charroux et al., 1999). SMN interacts directly with the Sm proteins and with Gemin2 and Gemin3 (Liu et al., 1997; Charroux et al., 1999; Pellizzoni et al., 1999). Antibody microinjection experiments in *Xenopus* oocytes revealed that Gemin2 has a critical role in the assembly of snRNPs (Fischer et al., 1997), a process which takes place in the cytoplasm where the Sm proteins combine with snRNAs that were exported from the nucleus (Mattaj and De Robertis, 1985; Mattaj, 1988; Luhrmann et al., 1990). Once properly assembled and modified, the snRNPs recruit the necessary nuclear import receptors and translocate into the nucleus where they function in pre-mRNA splicing (Mattaj, 1986, 1988; Luhrmann et al., 1990; Neuman de Vegvar and Dahlberg, 1990; Zieve and Sauterer, 1990). Transfections of a dominant negative form of SMN (SMN $\Delta$ N27) revealed that SMN also plays a critical role in the cytoplasmic assembly of snRNPs (Pellizzoni et al., 1998). In the nucleus, overexpression of the SMN $\Delta$ N27 protein causes a striking rearrangement of the snRNPs, which accumulate with the mutant SMN $\Delta$ N27 in enlarged gem/coiled body structures (Pellizzoni et al., 1998). SMN has been further shown to be required for pre-mRNA splicing, likely for the regeneration or recycling of snRNPs (Pellizzoni et al., 1998). Of the known components of the SMN complex, the recently described DEAD box protein Gemin3 is the most likely candidate to have the capacity to perform such functions (Charroux et al., 1999). Interestingly, SMN mutants found in SMA patients lack the snRNP regeneration activity likely because of their defective interaction with the Sm proteins as well as with Gemin3 (Pellizzoni et al., 1998, 1999; Charroux et al., 1999).

Here, we report the amino acid sequencing by nano-electrospray mass spectrometry (Wilm et al., 1996) and molecular cloning of a novel component of the SMN complex designated Gemin4 (available from GenBank/EMBL/DBJ under accession number AF173856). Several lines of evidence suggest that Gemin4 participates in the functions of the SMN complex. Together with SMN, Gemin2 and Gemin3, Gemin4 can be isolated in a complex with the spliceosomal snRNP proteins. The presence of Gemin4 in the SMN complex in vivo is a result of its direct interaction with Gemin3 but not with SMN. Gemin4 also interacts directly with several of the spliceosomal snRNP core Sm proteins, including SmB, SmD1-3, and SmE, and is associated with U snRNAs in the cytoplasm of *Xenopus* oocytes. Gemin4 is a novel protein and shows no significant homology to any other protein found in the databases. Its tight association with Gemin3 suggests that it may act as a cofactor of the putative ATPase and/or helicase activity of Gemin3. We have produced mAbs to Gemin4, and show by immunofluorescence microscopy that it colocalizes with SMN in gems. Interestingly, unlike other gems proteins described so far, Gemin4 is also detected in the nucleolus, suggesting that it may have additional functions in ribosome biogenesis.

## Materials and Methods

### Production of Proteins In Vitro

Proteins were labeled with [<sup>35</sup>S]methionine by an in vitro coupled transcription-translation reaction (Promega Biotech). His-tagged Gemin4 (amino acids 611–1,058) and His-tagged SmB fusion proteins were expressed from pET28a in *Escherichia coli* strain BL21(DE3) and purified on nickel columns according to the manufacturer's recommendations. GST, GST-Gemin3, and GST-Gemin4 fusion proteins were expressed from pGEX-5X-3 (Pharmacia) in *E. coli* strain BL21 and purified using glutathione-Sepharose (Pharmacia) according to the manufacturer's protocol.

### Production of mAbs to Gemin4

Anti-Gemin4 antibody 22C10 was prepared by immunizing Balb/C mice with a His-tagged COOH-terminal fragment of Gemin4. Hybridoma production, screening, and ascites fluid production were performed as previously described (Choi and Dreyfuss, 1984).

### Immunoprecipitation and Immunoblotting

Immunoprecipitations of in vitro translated proteins were carried out in the presence of 1% Empigen BB buffer as previously described (Choi and Dreyfuss, 1984). Coimmunoprecipitations were carried out using total HeLa lysate in the presence of 0.5% Triton X-100 as previously described (Pinol-Roma et al., 1988). For immunoblotting, proteins were resolved on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Schleider and Schuell, Inc.) using a BioTrans Transblot apparatus (model B; Gelman Science) according to the manufacturer's instructions. The membranes were incubated in blocking solution (PBS 5% nonfat milk) for at least 1 h at room temperature, rinsed with cold PBS, and incubated in blocking solution with primary antibody for at least 1 h at room temperature. Membranes were washed three times in PBS containing 0.05% NP-40, followed by incubation with peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, Inc.), which were visualized by an ECL Western blotting kit (Amersham) after three additional washes with PBS containing 0.05% NP-40.

### Cell Culture

HeLa cells were cultured in DME supplemented with 10% FBS (both from GIBCO BRL).

### Immunofluorescence Microscopy

Immunofluorescence staining was carried out essentially as previously described (Choi and Dreyfuss, 1984). Double-labeled immunofluorescence experiments were performed by separate, sequential incubations of each primary antibody diluted in PBS containing 3% BSA, followed by the specific secondary antibody coupled to either FITC or Texas red. All incubations were carried out at room temperature for 1 h. Laser confocal fluorescence microscopy was performed with a Leica TCS 4D confocal microscope. Images from each channel were recorded separately and, where indicated, the files were merged. Antibodies used in these experiments were as follows: mouse IgG1 monoclonal anti-Gemin4 (22C10; this work); mouse IgG1 monoclonal anti-SMN (2B1; Liu and Dreyfuss, 1996); rabbit polyserum anti-p80 coilin (R288; Andrade et al., 1991); mouse IgG3 monoclonal anti-Sm (Y12; Lerner et al., 1981); human autoimmune antibody against fibrillarin 1881 (Reimer et al., 1987); and rabbit affinity-purified anti-SMN exon 7 epitope antibody (Liu et al., 1997).

### In Vitro Protein-binding Assay

Purified GST or GST fusion proteins (2  $\mu$ g) bound to 25  $\mu$ l of glutathione-Sepharose beads were incubated with 10<sup>6</sup> cpm of in vitro translated protein in 1 ml of binding buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 2 mM EDTA, 0.1% NP-40, 2  $\mu$ g/ml leupeptin and pepstatin A, and 0.5% aprotinin). After incubation for 1 h at 4°C, the resin was washed five times with 1 ml of binding buffer. The bound fraction was eluted by boiling in SDS-PAGE sample buffer and run on SDS-PAGE. The gels were fixed for 30 min and the signal was enhanced by treatment with Amplify solution (Amersham). For direct in vitro binding, we used 5  $\mu$ g of purified His-tagged SmB and a binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM EDTA, 0.05% NP-40, 2  $\mu$ g/ml leupeptin and pepstatin A,

and 0.5% aprotinin). The binding experiment was performed as described above, except that bound His-tagged SmB proteins were immunodetected by Western blot using a rabbit polyclonal anti-His tag antibody (Santa Cruz Biotechnology).

### Gel Filtration

Total HeLa cell extract prepared in RSB-100 was loaded on a Superose 6 HR 10/30 column (Pharmacia). The column was washed with RSB-100 at a flow rate 0.5 ml/min. Fractions (1 min) were collected, and 1:20 of each fraction was resolved on SDS-PAGE followed by Western blotting.

### Oocyte Injections and Immunoprecipitation of RNA-Protein Complexes

Injections were carried out as previously described (Fischer et al., 1993). In brief, oocytes were incubated for 3 h in modified Barth's solution containing 0.2% collagenase type II (Sigma Chemical Co.). Defolliculated stage V and VI oocytes were collected and usually used the day after for micro-injection. In a typical injection experiment, 30 nl of  $^{32}\text{P}$ -labeled RNA ( $10^6$  cpm/ $\mu\text{l}$ ; total concentration of 0.7  $\mu\text{M}$ ) was injected into the cytoplasm. For immunoprecipitation of RNA-protein complexes (Fisher et al., 1993), the injected oocytes were homogenized in 300  $\mu\text{l}$  of ice-cold PBS, pH 7.4. The insoluble fraction was pelleted by centrifugation, and the clear supernatant was transferred into a new 1.5-ml Eppendorf tube containing antibodies bound to protein G-Sepharose (Pharmacia). This mixture was incubated with constant shaking for 1 h at 4°C and subsequently washed five times with 1 ml of ice-cold PBS. Bound RNAs were isolated by phenol extraction for 1 h, precipitated with ethanol, and analyzed by denaturing gel electrophoresis.

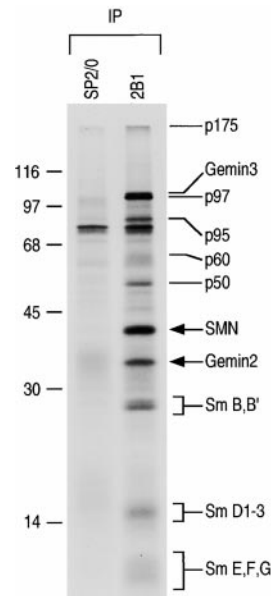
### Plasmid DNA and In Vitro Transcription

In vitro transcription of  $^{32}\text{P}$ -labeled RNAs was carried out as described in Fisher et al. (1993) from plasmids encoding for U1, U2, U4, and U5 snRNAs. The plasmid encoding the chicken  $\delta$ -crystallin pre-mRNA was previously described (Pellizzoni et al., 1998). The plasmid encoding the chicken  $\delta$ -crystallin mRNA was constructed by elution of the chicken  $\delta$ -crystallin mRNA from polyacrylamide gel followed by reverse transcriptase-PCR and subcloning into pSP65 (pSP1415m; Promega Corp). The plasmids used for in vitro transcription and translation of SMN, Gemin2, Gemin3, and the Sm proteins have been previously described (Charroux et al., 1999; Pellizzoni et al., 1999).

## Results

### Gemin4, a Novel Component of the SMN Complex

Immunoprecipitations from [ $^{35}\text{S}$ ]methionine-labeled HeLa cell lysates with anti-SMN mAbs revealed the presence of several protein components in the SMN complex (Liu et al., 1997). Proteins that coimmunopurify with anti-SMN antibodies (Fig. 1) include Sm proteins, Gemin2 (Liu et al., 1997), and the recently described DEAD box protein Gemin3 (Charroux et al., 1999). In addition to these proteins, there are bands of 175, 97, 95, 60, and 50 kD that coimmunopurified with SMN (Fig. 1). In this paper, we describe the molecular cloning and characterization of the 97-kD polypeptide. The p97 band was digested in gel by trypsin, and the resulting peptides were sequenced by nanoelectrospray mass spectrometry as described previously (Shevchenko et al., 1996; Wilm et al., 1996). Searching the databases with several peptides from this band (using the peptide sequence tag algorithm) we identified a human expressed sequence tag sequence (clone no. R55454) (Shevchenko et al., 1996). Several additional cDNA clones were obtained by hybridization screening of a human leukemia 5'-STRETCH PLUS cDNA library using the expressed sequence tag clone as a probe. We iso-



**Figure 1.** Immunoprecipitation of the SMN complex with mAb against SMN. Immunoprecipitations using anti-SMN mAb 2B1 and [ $^{35}\text{S}$ ]methionine-labeled HeLa cell cytoplasmic extract. The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. Antibody 2B1 (lane 2B1) immunoprecipitates SMN, Gemin2, Gemin3, Sm proteins B, B', D1-3, E, F, and G, and a group of proteins indicated as p175, p97, p95, p60, and p50. The SP2/0 lane shows the background of immunoprecipitation (lane SP2/0). The position of the molecular mass markers is indicated on the left in kilodaltons.

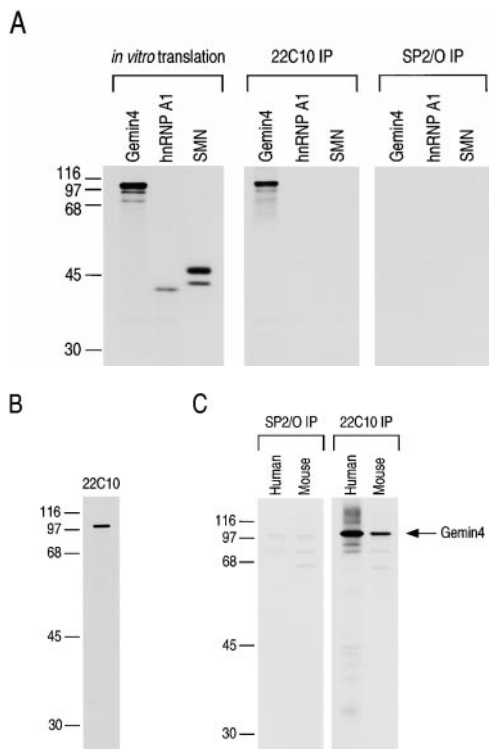
lated 20 independent partial cDNA clones with insert sizes ranging from 0.8 to 3.1 kb, all of which contained overlapping regions of the same open reading frame (ORF). A cDNA clone containing the longest ORF was constructed, and conceptual translation of its nucleotide sequence revealed a potential initiator methionine preceded by an in-frame stop codon. This cDNA encodes a putative protein of 1,058 amino acids with a calculated molecular mass of 119.9 kD and an estimated pI of 5.68. Database searches with the full-length clone did not reveal significant homology to any other protein or any recognizable motifs. Thus, this is a full-length cDNA clone that encodes a novel component of the SMN complex (see below), which we termed Gemin4 for component of gems number 4.

### Production of Monoclonal Antibodies to Gemin4

To investigate the interaction of Gemin4 with SMN and to characterize Gemin4 further, we generated mAbs by immunizing mice with a bacterially produced, purified recombinant His-tagged Gemin4 fragment (from amino acids 611 to 1,058). One hybridoma, 22C10, was selected for additional studies. Several lines of evidence demonstrate that this hybridoma indeed produced mAb that recognizes Gemin4 specifically. First, 22C10 immunoprecipitated Gemin4 produced by in vitro transcription and translation from the Gemin4 cDNA but not similarly produced hnRNP A1 or SMN proteins (Fig. 2 A). Second, on an immunoblot of total HeLa lysate, 22C10 recognized a single protein of ~97 kD (Fig. 2 B). Finally, 22C10 specifically immunoprecipitated a single protein of ~97 kD from [ $^{35}\text{S}$ ]methionine-labeled HeLa and mouse 3T3 cell lysates (Fig. 2 C), suggesting that Gemin4, like SMN, is conserved in vertebrates.

### Gemin4 Is Localized both in Gems and in Nucleoli

Indirect immunofluorescence laser confocal microscopy using mAb 22C10 was performed on HeLa cells to determine the subcellular localization of Gemin4. Fig. 3, A and



**Figure 2.** mAb 22C10 is specific for Gemin4. (A) Myc-tagged Gemin4, hnRNP A1, and SMN proteins were produced in rabbit reticulocyte lysate in the presence of [ $^{35}$ S]methionine. The labeled proteins were immunoprecipitated using mAb 22C10, and the immunoprecipitated material was analyzed by SDS-PAGE and autoradiography. The left panel shows 10% of the in vitro translated proteins. (B) Immunoblotting using mAb 22C10 on total HeLa extract. (C) Immunoprecipitations using anti-Gemin4 mAb 22C10 and [ $^{35}$ S]methionine-labeled HeLa or mouse 3T3 cell extracts. The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. The position of the molecular mass markers is indicated on the left in kilodaltons.

C, shows that anti-Gemin4 antibodies display intense staining of prominent discrete bodies in the nucleus, and these are also readily discernible by differential interference contrast (Fig. 3 D). In addition, the 22C10 antibody stained nucleoli (see below). To determine if the discrete nuclear structures stained by 22C10 are gems or CBs, we performed double label immunofluorescence experiments using antibodies against Gemin4 and either p80-coilin, as a marker of CBs (Andrade et al., 1991), or SMN, as a marker of gems (Liu and Dreyfuss, 1996). In many cell lines, the antibody staining of gems and CBs entirely overlap, however, in the HeLa PV strain used here these two bodies are frequently found separate from each other (Liu and Dreyfuss, 1996; Matera and Frey, 1998). In a recent report, Carvalho et al. (1999) have also observed that gems are often detected separately from CBs. The nuclear structures that contain Gemin4 are clearly distinct from CBs but are completely colocalized with SMN in gems (Fig. 3, B, C, and E). The colocalization of Gemin4 with SMN strongly supports the conclusion that these two proteins exist as a complex in the cell. Thus, Gemin4 is the fourth constituent of gems described so far. Like staining

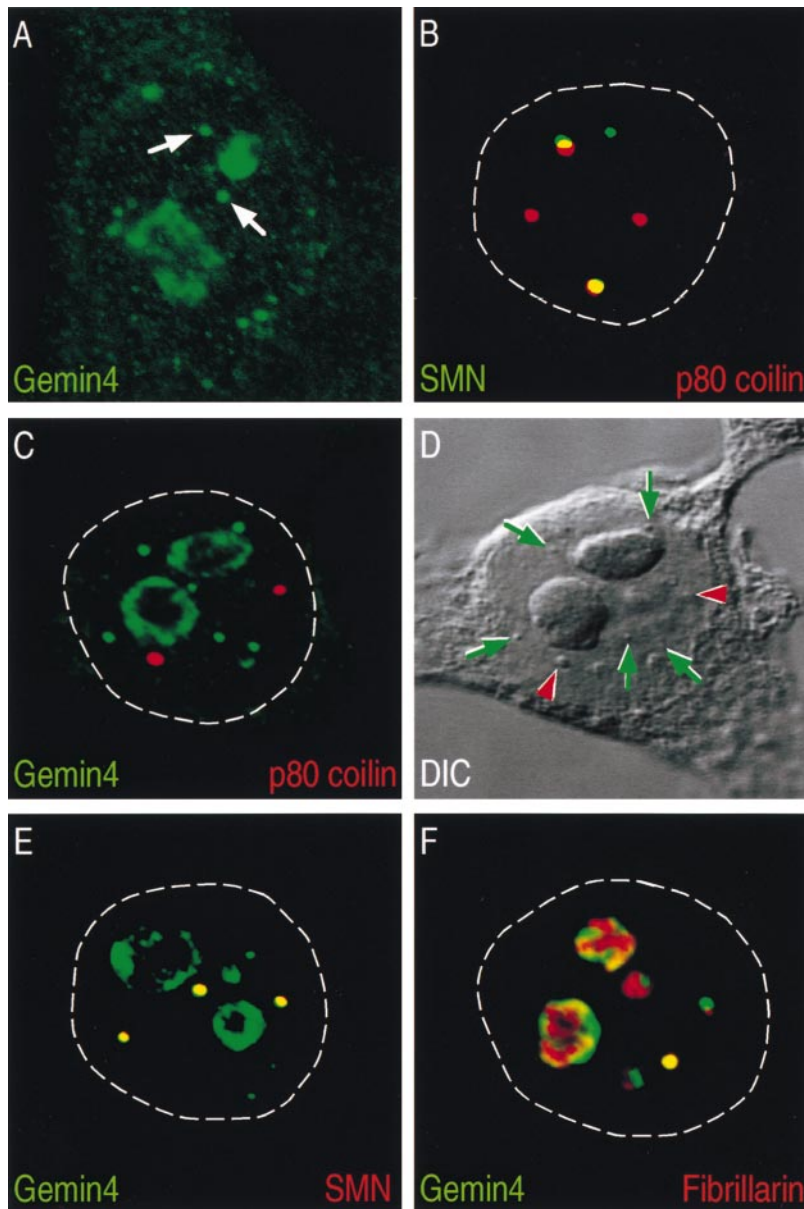
with antibodies to SMN, Gemin2 and Gemin3, 22C10 shows a diffuse cytoplasmic staining for Gemin4 (Fig. 3 A).

To further study the nucleolar staining of Gemin4, we performed double label immunofluorescence experiments using the Gemin4 antibody 22C10 and the fibrillarin antibody 1881 as a marker of the nucleolus (Raska et al., 1990). The staining, shown in Fig. 3 F, indicates that Gemin4 and fibrillarin do not colocalize, but both show distinct nucleolar patterns. This demonstrates that Gemin4 is present in the nucleolus but excluded from the dense fibrillar compartment of the nucleolus (Ochs and Smetana, 1991). Thus, Gemin4 represents the first component of the SMN complex that localizes both in gems and nucleoli (see Discussion).

### *Gemin4 Is in a Complex with SMN, Gemin2 and Gemin3*

To characterize the Gemin4-containing complex, we tested for its presence in the SMN complex in vivo by coimmunoprecipitation and Western blotting experiments. The anti-Gemin4 mAb 22C10 was used for immunoprecipitation from HeLa cell total extracts, and these were resolved by SDS-PAGE, immunoblotted, and probed with the anti-SMN antibody 2B1 (Liu and Dreyfuss, 1996). As shown in Fig. 4 A (lane 22C10 IP), 2B1 readily detects SMN in the 22C10 immunoprecipitates, indicating that SMN is coimmunoprecipitated with Gemin4. Because SMN forms a stable complex with Gemin2 in vivo and in vitro (Liu et al., 1997), we also investigated whether Gemin2 could be coimmunoprecipitated with Gemin4. As shown in Fig. 4 A, the anti-Gemin2 mAb 2S7 clearly detects Gemin2 in the anti-Gemin4 immunoprecipitates (lane 22C10 IP). We also examined whether Gemin4 can be coimmunoprecipitated with Gemin3, which is a recently identified novel component of the SMN complex (Charroux et al., 1999). Fig. 4 A shows that, like SMN and Gemin2, Gemin4 is present in the anti-Gemin3 (lane 11G9 IP) immunoprecipitate (Charroux et al., 1999). In a reciprocal experiment, the Gemin4 protein could also be coimmunoprecipitated by the anti-SMN, the anti-Gemin2, and the anti-Gemin3 mAbs (Fig. 4 B). No SMN, Gemin2, Gemin3, or Gemin4 proteins were detected in the control nonimmune (SP2/O) immunoprecipitate (data not shown and lane SP2/O, Fig. 4 B). These results suggest that SMN, Gemin2, Gemin3, and Gemin4 are associated in vivo in one or more complexes that can be immunoprecipitated by either anti-SMN, anti-Gemin2, anti-Gemin3, or anti-Gemin4 antibodies.

Further support for the existence in vivo of a single complex that contains SMN, Gemin2, Gemin3, and Gemin4 was obtained from gel filtration experiments. Total HeLa extract was fractionated on a Superose 6 HR 10/30 high performance gel filtration column; fractions were resolved by SDS-PAGE, blotted, and probed with anti-SMN, anti-Gemin2, anti-Gemin3, and anti-Gemin4 antibodies. SMN, Gemin2, Gemin3, and Gemin4 comigrated and showed a peak at  $\sim 800$  kD, indicating that they are components of a large macromolecular complex (Fig. 4 B). Gemin4 is also detected in a second complex of  $\sim 550$  kD that lacks SMN and Gemin2 but does contain a faster migrating form of Gemin3. Thus, there appear to be at least two different Gemin4-containing complexes.



**Figure 3.** The Gemin4 protein colocalizes with SMN in gems and is detected in nucleoli. (A) Laser confocal image of indirect immunofluorescence on HeLa cells using mAb 22C10 against the Gemin4 protein, arrows in A indicate gems. (B and C) Superimposed laser confocal images of double label immunofluorescence microscopy experiments using antibodies against p80 coilin (B and C, red), SMN (B, green), and Gemin4 (C, green). (D) Differential interference contrast image of the same cell shown in C, arrows indicate gems and arrowheads indicate CBs. (E) Superimposed laser confocal images of double label immunofluorescence microscopy experiments using mAb against Gemin4 (green) and a rabbit affinity-purified antibody against exon 7 epitope of the human SMN protein (red). (F) Superimposed laser confocal images of double label immunofluorescence microscopy experiments using mAbs against Gemin4 (green) and against fibrillarin (red). Dashed lines demarcate the nucleus. A is a z-series composite stack of confocal sections that shows the cytoplasmic staining of Gemin4. B, C, E, and F, are confocal sections through the nucleus that underestimate the cytoplasmic staining of Gemin4 and/or SMN.

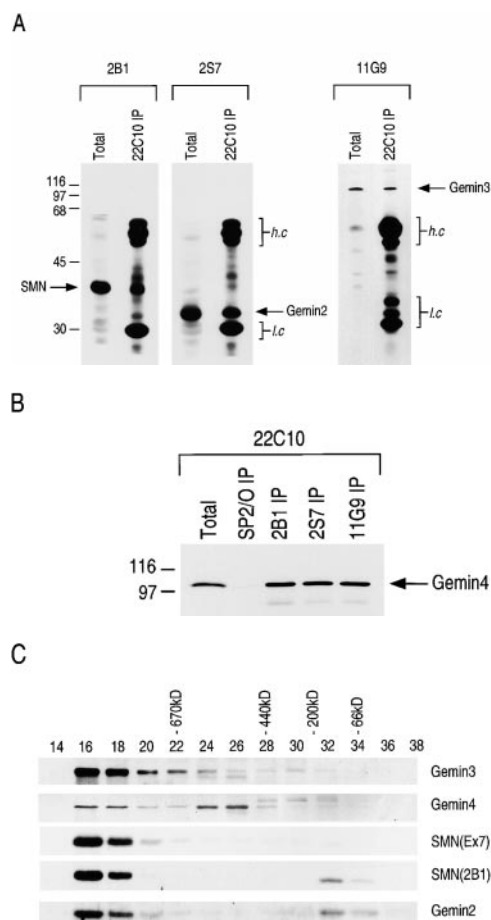
A second peak containing SMN and Gemin2, but lacking Gemin3 and Gemin4, is observed at lower molecular mass fractions that correspond to ~100 kD. The SMN protein present in these fractions was not detectable with a rabbit polyclonal antibody specific to the peptide sequence encoded by exon 7 of SMN (Liu et al., 1997; Fig. 4 B). Thus, this smaller SMN–Gemin2 complex contains the oligomerization-deficient form of SMN lacking amino acids encoded by exon 7, which is most likely produced by the SMN2 gene (see Discussion; Gennarelli et al., 1995; Lefebvre et al., 1995; Pellizzoni et al., 1999).

#### *Gemin4 Interacts Tightly with the DEAD Box Protein Gemin3*

To investigate the interactions of Gemin4, we performed *in vitro* protein binding assays using Gemin4 and several constituents of the SMN complex. For this assay, purified

GST or GST-Gemin4 fusion immobilized on glutathione-Sepharose were incubated with [<sup>35</sup>S]methionine-labeled SMN, Gemin2, Gemin3, and Gemin4 produced by *in vitro* transcription and translation in rabbit reticulocyte lysate. As shown in Fig. 5 A, full-length Gemin3 is the only protein of the SMN complex that binds specifically to immobilized GST-Gemin4 (Fig. 5 A). No binding to GST alone was observed (data not shown).

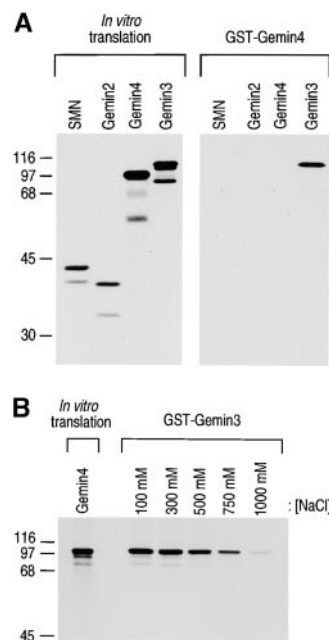
To further investigate this interaction, Gemin3 was expressed as a fusion protein with GST, and Gemin4 was produced and labeled with [<sup>35</sup>S]methionine in rabbit reticulocyte lysate. GST-Gemin3 fusion was incubated with labeled Gemin4 in the presence of increasing salt concentrations. As shown in Fig. 5 B, full-length Gemin4 bound to GST-Gemin3, and this binding appears to be avid because it is not disrupted at 750 mM NaCl. No binding of Gemin4 to GST alone was detected (data not shown).



**Figure 4.** Gemin4 is in a complex with SMN, Gemin2, and Gemin3. (A and B) SMN, Gemin2, Gemin3, and Gemin4 can be coimmunoprecipitated *in vivo*. (A) mAbs against Gemin4 coimmunoprecipitate SMN, Gemin2, and Gemin3. Immunoprecipitation from total the HeLa extract was done with mAb anti-Gemin4 (22C10) and the immunoprecipitated proteins were analyzed by Western blot using 2B1 (anti-SMN), 2S7 (anti-Gemin2), or 11G9 (anti-Gemin3) antibodies. (B) mAbs against SMN, Gemin2, and Gemin3 coimmunoprecipitate Gemin4. Immunoprecipitation from total HeLa extract was done with mAb anti-SMN (lane 2B1 IP), anti-Gemin2 (lane 2S7 IP), anti-Gemin3 (lane 11G9 IP), or SP2/O (lane SP2/O IP) as a negative control. The immunoprecipitated proteins were analyzed by Western blot using the anti-Gemin4 mAb 22C10. The position of the molecular mass markers is indicated on the left (in kD) and the positions of the light chain (l.c) and heavy chain (h.c) of the antibodies used for immunoprecipitation are indicated. (C) Gel filtration analysis of SMN complexes. Total HeLa extract was fractionated on a Superose 6 HR 10/30 column. The fractions were analyzed by SDS-PAGE, and SMN, Gemin2, Gemin3, and Gemin4 proteins were detected by Western blotting. The fraction number and the molecular mass standards are indicated.

### Gemin4 Is Associated with Spliceosomal snRNPs in the Cytoplasm

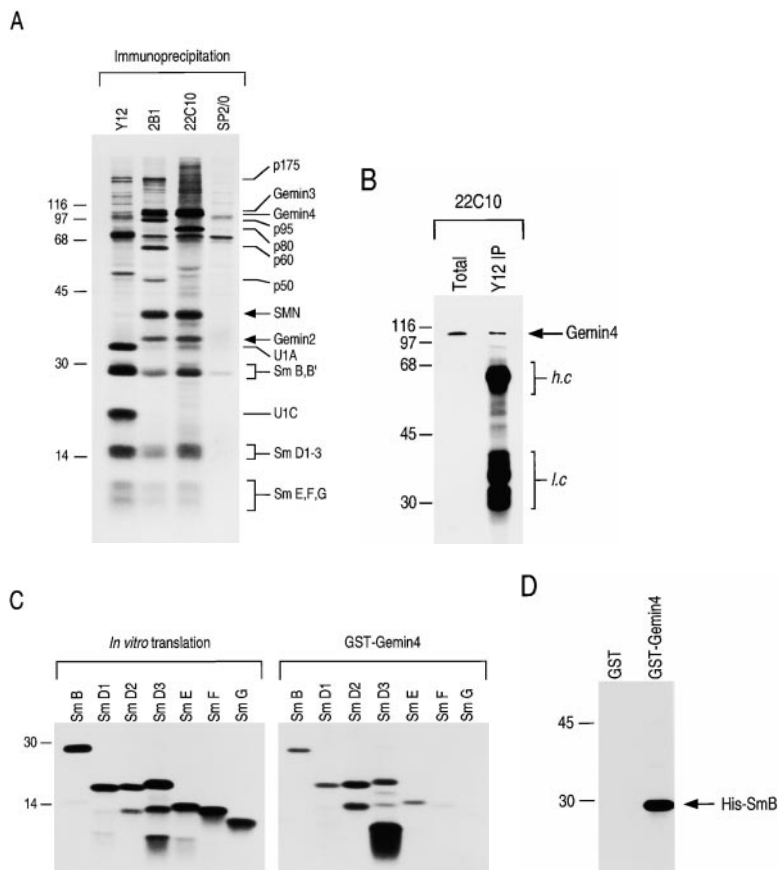
To further characterize the Gemin4 complex, immunoprecipitations using anti-Gemin4 mAbs from [<sup>35</sup>S]methionine-labeled HeLa cells were carried out, and the immunoprecipitated proteins were analyzed by SDS-PAGE. As references for these immunoprecipitations, we also per-



**Figure 5.** Gemin4 interacts directly with the DEAD box protein Gemin3 *in vitro*. (A) Gemin4 interacts with Gemin3 *in vitro*. *In vitro* translated [<sup>35</sup>S]methionine-labeled myc-tagged SMN, Gemin2, Gemin3, and Gemin4 proteins were incubated with purified GST-Gemin4 as described in Materials and Methods. Bound proteins were analyzed by SDS-PAGE and fluorography. The *in vitro* translation panel shows 5% of the input. The position of the molecular mass markers is indicated on the left in kilodaltons. (B) Gemin4 interacts tightly with Gemin3. *In vitro* translated [<sup>35</sup>S]methionine-labeled myc-Gemin4 was incubated with purified GST-Gemin3 in increasing salt concentrations as indicated (see Materials and Methods). Bound proteins were analyzed by SDS-PAGE and fluorography. The *in vitro* translation panel shows 10% of the input.

formed immunoprecipitation with the anti-Sm mAb Y12 (Lerner and Steitz, 1979; Lerner et al., 1981) and with the anti-SMN mAb 2B1. As shown in Fig. 6 A, several proteins are coimmunoprecipitated with anti-Gemin4 antibodies, and the pattern is very similar to the one obtained with anti-SMN antibodies. Besides SMN, Gemin2, Gemin3, and Gemin4, the Sm core proteins B/B', D1-3, E, F, and G proteins were also coimmunoprecipitated with the 22C10 anti-Gemin4 antibody. Additional proteins coimmunoprecipitated specifically with anti-SMN (at 175, 95, 60, and 50 kD) or anti-Gemin4 (at 80 kD) mAbs. Note that the U1-specific A protein, but not the U1-specific C protein, coimmunoprecipitates with SMN and Gemin4. This indicates that the SMN complexes are not associated with mature snRNP particles.

The *in vivo* association of Gemin4 with the Sm proteins was confirmed by a coimmunoprecipitation and Western blot experiment. The anti-Sm mAb Y12 was used for immunoprecipitation from HeLa cell total extracts, and the immunoprecipitate was analyzed by SDS-PAGE and an immunoblot with the anti-Gemin4 antibody 22C10. As shown in Fig. 6 B, 22C10 readily detects Gemin4 in the Y12 immunoprecipitate, demonstrating that Gemin4 is associated with the Sm proteins *in vivo*. To investigate whether Gemin4 interacts with Sm proteins directly, purified recombinant GST-Gemin4 protein was used for binding assays with *in vitro* produced [<sup>35</sup>S]methionine-labeled Sm proteins. Fig. 6 C shows that the Sm proteins B, D1, D2, D3, and E bind to GST-Gemin4, whereas there is no detectable binding to GST alone (data not shown). To determine if Gemin4 is able to interact directly with the Sm proteins and not via other components of the SMN complex that may be present in the rabbit reticulocyte lysate, recombinant His-tagged SmB protein was produced and



**Figure 6.** Gemin4 is associated with snRNPs in vivo. (A) Immunoprecipitations of [ $^{35}$ S]methionine-labeled total HeLa cell extract using mAbs specific to SMN (2B1), Gemin4 (22C10), and the snRNP Sm core proteins (Y12). The immunoprecipitated proteins were analyzed by SDS-PAGE and autoradiography. The several proteins immunoprecipitated are indicated on the right. (B) Gemin4 is coimmunoprecipitated with Sm proteins. Immunoprecipitation from the total HeLa extract was performed with mAb anti-Sm (Y12) and the immunoprecipitated proteins were analyzed by Western blotting using 22C10 (anti-Gemin4). The position of the molecular mass markers is indicated on the left (in kD). The positions of the light chain (l.c) and heavy chain (h.c) of the antibody used for immunoprecipitation are indicated. The total lane shows 2% of the input. (C) Gemin4 interacts with several Sm proteins in vitro. In vitro translated [ $^{35}$ S]methionine-labeled myc-tagged Sm proteins B, D1, D2, D3, E, F, and G were incubated with purified GST-Gemin4 as described in Materials and Methods. Bound proteins were analyzed by SDS-PAGE and fluorography. The in vitro translation panel shows 5% of the input. The position of the molecular mass markers is indicated on the left in kilodaltons. (D) Gemin4 interacts directly with SmB in vitro. Recombinant His-tagged SmB protein was incubated with purified GST or GST-Gemin4 proteins as described in Materials and Methods. Bound proteins were analyzed by SDS-PAGE and Western blotting with an anti-His tag antibodies.

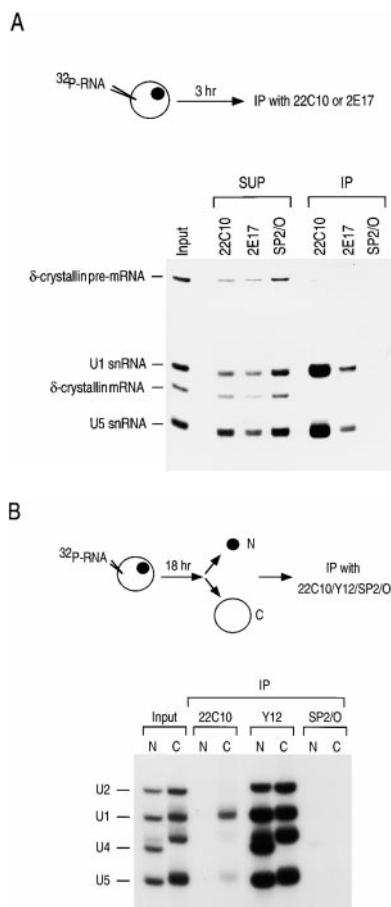
incubated with GST or GST-Gemin4. Bound proteins were resolved by SDS-PAGE, and analyzed by Western blotting with a rabbit polyclonal antibody specific to the 6xHis-tag. As shown in Fig. 6 D, SmB binds specifically to Gemin4 but not to GST. We conclude that Gemin4 interacts directly with SmB.

To determine whether Gemin4 was associated with snRNAs in vivo, we used the *Xenopus* oocyte that provides a particularly advantageous system in which to study spliceosomal snRNP biogenesis by use of microinjection (Mattaj and De Robertis, 1985; Mattaj, 1986). We first wished to determine whether Gemin4 is present in *Xenopus* cells and whether it can be recognized by 22C10 antibody. The 22C10 mAb showed both gems and nucleolar staining on *Xenopus* XL177 somatic cells, strongly suggesting that Gemin4 is conserved in *Xenopus* (data not shown). However, immunoblotting with the anti-human Gemin4 mAb 22C10 on *Xenopus* tissue culture cells or on *Xenopus* oocyte lysates did not detect any protein (data not shown). To determine if Gemin4 is associated with U snRNAs in vivo, various  $^{32}$ P-labeled RNAs including chicken  $\delta$ -crystallin mRNA, chicken  $\delta$ -crystallin pre-mRNA, and the spliceosomal snRNAs U1 and U5 were produced by in vitro transcription and a mixture of these RNAs was microinjected into the cytoplasm of oocytes. After 3 h, immunoprecipitations were carried out with anti-Gemin4 (22C10) and, as a positive control, anti-Gemin2 (2E17) antibodies (Fischer et al., 1997). Fig. 7 A shows that only U1 and U5 snRNAs are efficiently and specifically precipitated, indicating that they associate with

Gemin4. A similar, but less efficient, immunoprecipitation of U1 and U5 snRNAs was observed with the anti-Gemin2 antibody. We further asked whether the other spliceosomal U snRNAs, U2 and U4, were associated with Gemin4 as well, and whether the association of Gemin4 with mature U snRNPs was also observed in the nucleus. To do so, a mixture of  $^{32}$ P-labeled U snRNAs were injected into the cytoplasm of oocytes followed by an 18-h incubation (Fig. 7 B). After this incubation period, ~50% of the injected snRNA was imported into the nucleus while the rest remained in the cytoplasm. Immunoprecipitations from the nuclear and cytoplasmic fractions were carried out with either anti-Gemin4 antibody, anti-Sm antibody as a positive control, or SP2/O as a negative control. The coimmunoprecipitated RNAs were analyzed by gel electrophoresis. As previously reported, U1, U2, U4, and U5 were efficiently immunoprecipitated by Y12 in approximately equal amounts from the nucleus and the cytoplasm (Fig. 7 B; Mattaj, 1986; Fisher and Luhrmann, 1990). In contrast, Gemin4 associated more efficiently with U1 and U5 than U2 and U4, and this association was only observed in the cytoplasm (Fig. 7 B).

We conclude that the Gemin4, like SMN and Gemin2, is stably associated with U1 and U5 snRNAs in the cytoplasm of *Xenopus* oocytes but not after these snRNAs have been assembled into snRNPs and imported into the nucleus. Thus, like SMN and Gemin2 (Fisher et al., 1997), Gemin4 likely dissociates from the spliceosomal snRNPs either immediately before nuclear entry or shortly thereafter.





**Figure 7.** Gemin4 is associated with spliceosomal U snRNAs in the cytoplasm of *Xenopus* oocytes. (A) Immunoprecipitation of spliceosomal U snRNAs with anti-Gemin2 and anti-Gemin4 antibodies. A mixture of different in vitro synthesized  $^{32}\text{P}$ -labeled RNAs were injected into the cytoplasm of oocytes. 3 h later, immunoprecipitations were carried out with either anti-Gemin4 (22C10) or anti-Gemin2 (2E17) or control antibodies (SP2/O). Immunoprecipitated RNA (IP) was analyzed by gel electrophoresis. In the supernatant (SUP) panel 2.5% of each immunoprecipitation is shown. (B) Anti-Gemin4 antibodies can immunoprecipitate U1 and U5 snRNAs only from the cytoplasm. The indicated  $^{32}\text{P}$ -labeled RNAs were injected as a mixture into the cytoplasm of oocytes. After incubation for 18 h, the oocytes were dissected into nuclear (N) and cytoplasmic (C) fractions, and RNAs from both fractions were immunoprecipitated (IP) with either anti-Gemin4 (22C10), anti-Sm (Y12), or control antibodies (SP2/O). 2.5% of the input is shown.

## Discussion

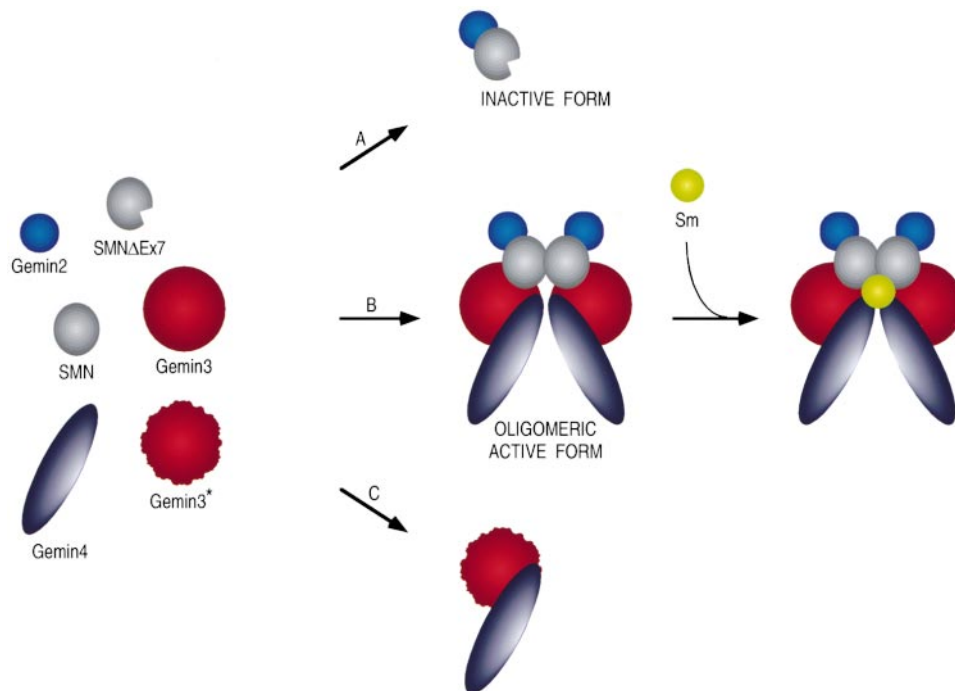
Using a biochemical approach to characterize additional components of the SMN complex, we have identified a novel protein termed Gemin4. Gemin4 does not contain any known or recognizable protein motifs. Several lines of evidence suggest that Gemin4, together with SMN, Gemin2, and Gemin3, function as a complex in vivo. SMN, Gemin2, Gemin3, and Gemin4 can be coimmunoprecipitated, and are present in a large complex that also contains the spliceosomal snRNP core Sm proteins. Like SMN and Gemin3, Gemin4 interacts directly with several snRNP Sm core proteins, including B/B', D1-D3, and E and is associ-

ated with U1 and U5 snRNAs in the cytoplasm of *Xenopus* oocytes, where snRNP assembly takes place (Liu et al., 1997; Fischer et al., 1997; Charroux et al., 1999; Pellizzoni et al., 1999). We have previously shown that the SMN complex plays a critical role in spliceosomal snRNP assembly in the cytoplasm, and is required for pre-mRNA splicing in the nucleus (Fischer et al., 1997; Pellizzoni et al., 1998). Thus, it is likely that Gemin4 also plays an important role in these processes. Note, that so far, we have not been able to detect any effect of anti-Gemin4 antibody 22C10 on snRNP assembly and/or snRNP import into the nucleus upon microinjection into *Xenopus* oocytes (data not shown).

Unlike Gemin2 and Gemin3, Gemin4 does not interact with SMN directly and its presence in the SMN complex is probably the result of its direct and stable interaction with Gemin3. The observation that Gemin4 and the DEAD box protein Gemin3 interact with each other directly and avidly suggests that they function together. Previous studies have shown that the RNA helicase activity of the translation initiation factor eIF4A, also a DEAD box protein, is dependent on the presence of a second initiation factor, eIF4B. Interestingly, in a series of preliminary experiments we have so far not been able to detect RNA helicase or RNA-dependent ATPase activity for recombinant Gemin3 (Charroux et al., 1999). It is possible that such activity will only manifest itself when Gemin3 is associated with other proteins such as Gemin4.

Gel filtration experiments revealed the presence of two SMN complexes in HeLa cells. The high molecular mass complex is the most abundant and contains all the components of the SMN complex thus far identified (SMN, Gemin2, Gemin3, and Gemin4) and likely represents an active form of the complex (see below). The large size of this complex is likely the result of the capacity of SMN to form large oligomers (Pellizzoni et al., 1999). The second complex probably represents a monomeric, COOH-terminal-truncated form of SMN (SMN $\Delta$ Ex7) associated with Gemin2 (Pellizzoni et al., 1999). While SMN1 produces only full-length mRNA, SMN2 mainly produces an alternatively spliced form of SMN mRNA lacking exon 7 (Gennarelli et al., 1995; Lefebvre et al., 1995). Exon 7 skipping is due to the presence of a single nucleotide change in the SMN2 gene compared with SMN1 (Lorson et al., 1999), and the ratio of alternatively spliced versus full-length SMN2 mRNA correlates with the severity of SMA (Gavrilov et al., 1998). Nevertheless, no evidence for the presence of SMN protein lacking exon 7-encoded amino acids in vivo has been reported. The absence of the amino acid sequence encoded by exon7 is thought to generate a nonfunctional SMN protein that lacks the capacity to oligomerize and, thus, cannot interact with Sm proteins (Burghes, 1997; Lorson et al., 1998; Pellizzoni et al., 1999). The absence of Gemin3 and Gemin4 from the SMN $\Delta$ Ex7-Gemin2 complex probably results from the defective interaction of SMN $\Delta$ Ex7 with Gemin3 (Charroux et al., 1999). We have shown that SMN $\Delta$ Ex7 is a nonfunctional protein that is incapable, unlike wild-type SMN, of regenerating splicing extracts in vitro (Pellizzoni et al., 1998). Thus, the loss of function of SMN $\Delta$ Ex7 is likely due to its defective interaction with the Sm proteins as well as with the Gemin3-Gemin4 complex (Charroux et al., 1999;





**Figure 8.** A schematic representation of the SMN complexes. (A) The monomeric SMNΔEx7, associates with Gemin2 to form a dimeric complex. SMNΔEx7 lacks the ability to self-associate as well as to interact with Gemin3 and the Sm proteins (Lorson et al., 1998; Charroux et al., 1999; Pellizzoni et al., 1999). This monomeric SMNΔEx7–Gemin2 complex is very likely an inactive complex (see text for more details). (B) Monomeric SMN, associated with Gemin2 which binds to SMN but not itself (Liu et al., 1997; data not shown), contains a low affinity binding site for the Sm proteins (Pellizzoni et al., 1999). Gemin3 associates with Gemin4 (this work). SMN self-associates, forming at least a SMN–Gemin2 dimeric complex (Pellizzoni et al., 1999). Gemin3–Gemin4 complex associates with SMN–Gemin2 dimeric complex via an

SMN–Gemin3 interaction (Charroux et al., 1999). In this oligomeric conformation, a binding site with a much higher affinity for the Sm proteins is formed. The oligomeric form likely represents the active form of the SMN complex (see text for more details). (C) Gemin4 interacts with Gemin3\* to form a dimeric complex that lacks SMN and Gemin2 (see text for details). Gemin3\* represents the faster migrating form of Gemin3 observed in Fig. 4 B. Only one Sm snRNP protein is included in the drawing to simplify the model.

Pellizzoni et al., 1999). Therefore, we suggest that the SMNΔEx7–Gemin2 complex represents an inactive form of the SMN complex, whereas the high molecular mass complex containing SMN, Gemin2, Gemin3, and Gemin4 represents the active complex that can bind substrates such as the Sm proteins and carry out the functions of the complex (Fig. 8; Fischer et al., 1997; Pellizzoni et al., 1998, 1999).

Higher eukaryotic nuclei contain numerous morphologically distinct substructures or nuclear bodies (for reviews see Singer and Green, 1997; Lamond and Earnshaw, 1998; Matera, 1999). SMN, Gemin2, Gemin3, and Gemin4, in addition to their general localization in the cytoplasm, are found in the nucleus, where they are concentrated in gems (Liu and Dreyfuss, 1996; Liu et al., 1997). Gems are similar in size and number to CBs, and these two bodies are often found either entirely merged or in close proximity (Liu and Dreyfuss, 1996; Liu et al., 1997; Matera and Frey, 1998). CBs are highly enriched in snRNPs and snoRNPs and, together with gems, appear to be involved in snRNP biogenesis and metabolism (Gall et al., 1995; Lamond and Earnshaw, 1998; Pellizzoni et al., 1998; Matera, 1999; Sleeman and Lamond, 1999). In addition to its localization in gems, Gemin4 is in the nucleoli and, thus, represents the first component of the SMN complex present in this nuclear compartment. Using a different polyclonal anti-SMN antibody from 2B1, others have observed a strong nucleolar immunolocalization of SMN in mouse and human CNS tissues (Francis et al., 1998). However, SMN has not been detected in nucleoli of HeLa cells. Interestingly, SMN interacts with fibrillarin (Liu and Dreyfuss, 1996), a common component of small nucleolar RNPs (snoRNPs).

Fibrillarin is found in the nucleoli and in CBs and may be the snoRNP's functional equivalent of the core Sm proteins of spliceosomal snRNPs (Tyc and Steitz, 1989; Maxwell and Fournier, 1995; Smith and Steitz, 1997). Because of their close association with nucleoli, it has been suggested that CBs participate in snoRNP biogenesis and/or metabolism (Raska et al., 1990). Given the close association between CBs and gems and the interaction between SMN and fibrillarin, it is possible that the SMN complex also plays a role in snoRNP assembly and ribosomal RNA metabolism. Therefore, Gemin4 may function to connect SMN to both UsnRNA biogenesis and rRNA biogenesis.

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