The Spinal Muscular Atrophy Disease Gene Product, SMN: A Link between snRNP Biogenesis and the Cajal (Coiled) Body

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Abstract. The spliceosomal snRNAs U1, U2, U4, and U5 are synthesized in the nucleus, exported to the cytoplasm to assemble with Sm proteins, and reimported to the nucleus as ribonucleoprotein particles. Recently, two novel proteins involved in biogenesis of small nuclear ribonucleoproteins (snRNPs) were identified, the Spinal muscular atrophy disease gene product (SMN) and its associated protein SIP1. It was previously reported that in HeLa cells, SMN and SIP1 form discrete foci located next to Cajal (coiled) bodies, the so-called "gemini of coiled bodies" or "gems." An intriguing feature of gems is that they do not appear to contain snRNPs. Here we show that gems are present in a variable but small proportion of rapidly proliferating cells in culture. In the vast majority of cultured cells and in all primary neurons analyzed, SMN and SIP1 colocalize precisely with snRNPs in the Cajal body. The presence of SMN and SIP1 in Cajal bodies is confirmed by immunoelectron microscopy and by microinjection of antibodies that interfere with the integrity of the structure. The association of SMN with snRNPs and coilin persists during cell division, but at the end of mitosis there is a lag period between assembly of new Cajal bodies in the nucleus and detection of SMN in these structures, suggesting that SMN is targeted to preformed Cajal bodies. Finally, treatment of cells with leptomycin B (a drug that blocks export of U snRNAs to the cytoplasm and consequently import of new snRNPs into the nucleus) is shown to deplete snRNPs (but not SMN or SIP1) from the Cajal body. This suggests that snRNPs flow through the Cajal body during their biogenesis pathway.

Key words: Cajal (coiled) body • leptomycin B • nucleocytoplasmic transport • SMN protein • spliceosomal small nuclear ribonucleoproteins

SMALL nuclear ribonucleoproteins (snRNPs)[1] are essential mediators of RNA processing events. In particular, the so-called spliceosomal snRNPs play a key role in splicing of pre-mRNA (reviewed by Burge et al., 1999). This class includes the U1, U2, U4, U5, and U6 snRNPs. The five spliceosomal snRNAs are synthesized in the nucleus and, with the single exception of U6 (Vankan et al., 1990), they are rapidly exported to the cytoplasm (Lührmann, 1988; Mattaj, 1988). The U1, U2, U4, and U5 snRNAs contain an evolutionarily conserved structural element composed of a single-stranded, uridylic acid–rich region that is typically flanked by two hairpin loops (Branlant et al., 1982). This motif binds a complex of eight proteins (B, B′, D1, D2, D3, E, F, and G), collectively known as the Sm or core proteins (Lührmann et al., 1990). After assembly of the Sm core domain, the 5′-monomethylated guanosine cap structure of the snRNAs is hypermethylated to yield 2,2,7-trimethylguanosine (m_3G) (Mattaj, 1986). In addition, varying numbers of nucleotides are trimmed from the 3′ end of several of the snRNAs and many internal nucleotides are modified (Reddy and Busch, 1988). Assembly of the Sm core, cap hypermethylation, and 3′ end processing occur in the cytoplasm and are crucial for the subsequent nuclear import of the snRNP particles (Mattaj, 1988; Izaurralde and Mattaj, 1992). The final step in the biogenesis of these particles involves the association of more than 30 snRNP-specific proteins that are found in only one type of snRNP. It remains unclear whether the assembly of these proteins takes place just before or after nuclear import. When and where the internal snRNA modifications are introduced is also uncertain, but a recent study by Yu et al. (1998) indicates that 2′-*O*-meth-

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1. *Abbreviations used in this paper:* m_3G, 2,2,7-trimethylguanosine cap; SIP1, SMN interacting protein 1; SMN, survival motor neurons; snRNP, small nuclear ribonucleoprotein.

ylation and pseudouridylation of U2 snRNA take place in the nucleus and are required for assembly of snRNP-specific proteins.

Transport of snRNP particles from the cytoplasm to the nucleus involves a receptor- and energy-dependent mechanism that is not completely understood. Both the m_3G-cap and a nuclear localization signal located within the Sm core have been implicated, but not all spliceosomal snRNPs have the same m_3G-cap requirements (Fischer et al., 1991; Michaud and Goldfarb, 1992). Differential m_3G-cap requirements are also detected between *Xenopus* oocytes and mammalian somatic cells, presumably due to differences in soluble cytosolic factors (Marshallsay and Lührmann, 1994). Competition experiments indicate that snRNPs are imported by specific receptors not shared by other classes of nuclear proteins (Fischer et al., 1991; Michaud and Goldfarb, 1992; Izaurralde et al., 1997), and the first nuclear import receptor for snRNPs, termed snurportin1, was recently identified (Huber et al., 1998).

In the course of studies on heterogeneous nuclear ribonucleoproteins (hnRNPs), two novel proteins involved in snRNP biogenesis, SMN and SIP1, were discovered (Liu and Dreyfuss, 1996; Liu et al., 1997). The SMN protein is encoded by the Survival Motor Neurons gene, which is deleted or mutated in >98% of patients with Spinal Muscular Atrophy (SMA), and is therefore considered the SMA disease gene (Lefebvre et al., 1998). The SMN gene is duplicated in humans (Lefebvre et al., 1995), but is an essential, single-copy locus in mice (Schrank et al., 1997). SIP1 is a novel protein that forms a stable heteromeric complex with SMN in vivo and in vitro (Liu et al., 1997). In addition to SIP1, SMN binds to RNA (Lorson and Androphy, 1998; Bertrandy et al., 1999) and interacts directly with several snRNP Sm core proteins forming a large SMN-SIP1–snRNP complex (Liu et al., 1997). Importantly, microinjection of anti–SIP1 antibodies in the cytoplasm of *Xenopus* oocytes completely inhibited the Sm core assembly on U1 and U5 snRNAs and, consequently, blocked their nuclear import (Fischer et al., 1997). In addition to playing an essential role in snRNP biogenesis, more recent data suggest that SMN may also be involved in recycling of the nuclear spliceosomal snRNPs after each round of splicing (Pellizzoni et al., 1998).

The SMN-SIP1–snRNP complexes are biochemically detected in both the cytoplasm and the nucleus of somatic cells, and immunolocalization studies revealed SMN and SIP1 proteins concentrated in novel nuclear structures that were called "gemini of coiled bodies" or "gems" (Liu and Dreyfuss, 1996; Liu et al., 1997) because they are frequently located in close proximity to coiled bodies, a subnuclear structure that is highly enriched in snRNPs (for recent reviews see Lamond and Earnshaw, 1998; Matera, 1999).

Coiled bodies were first described as accessory bodies of the nucleolus (Ramón y Cajal, 1903), and later rediscovered by electron microscopists (Hardin et al., 1969; Monneron and Bernhard, 1969; Hervás et al., 1980). The name "coiled body" was introduced because when this subnuclear structure is viewed with the electron microscope it resembles a tangle of coiled threads (Monneron and Bernhard, 1969). At present, coiled bodies have been renamed Cajal bodies (Gall et al., 1999) and are known to contain three major classes of small nuclear ribonucleoproteins. These are the spliceosomal U1, U2, U4, U5, and U6 snRNPs (Carmo-Fonseca et al., 1991, 1992; Huang and Spector, 1992; Matera and Ward, 1993), the U7 snRNA required for 3′ end processing of histone mRNA (Wu and Gall, 1993; Frey and Matera, 1995; Wu et al., 1996), and the U3 and U8 small nucleolar RNAs (snoRNAs) involved in processing of pre–rRNA (Wu et al., 1993; Jiménez-Garcia et al., 1994; Bauer et al., 1994). Although the function of the Cajal body is still unknown, some of these structures are located near the replicative histone gene cluster (Gall et al., 1981; Frey and Matera, 1995), where they may recruit histone processing factors to the sites of histone pre–mRNA transcription (Bellini and Gall, 1998; Abbott et al., 1999). In addition, Cajal bodies associate with the human U3 snoRNA genes (Gao et al., 1997), and with the tandemly repeated human U1 and U2 snRNA genes (Frey and Matera, 1995; Smith et al., 1995). Remarkably, the association with U2 genes requires transcriptionally competent loci, indicating that targeting of the body to this chromosomal site is mediated by nascent snRNAs (Frey et al., 1999).

The discovery that SMN and SIP1, two proteins essential for snRNP biogenesis, are located in close proximity to the Cajal body represented a major breakthrough in unraveling the function of this structure. However, an intriguing question was how SMN plays a direct role in nuclear snRNP metabolism when it is concentrated in gems, which do not contain snRNPs under physiologic conditions (see Dietz, 1998). In the present work, we demonstrate that SMN and SIP1 are normally located in the Cajal body, whereas gems are only detected in a variable but small proportion of rapidly proliferating cells in culture. The Cajal body is shown to represent a unique intranuclear site specifically enriched in snRNPs, SMN, and SIP1, thus suggesting a direct involvement of this structure in the snRNP biogenesis pathway.

Materials and Methods

Cell Culture

HeLa cells (human epitheloid carcinoma, cervix), Huh7 cells (human hepatoma), and WI-38 cells (human female lung diploid fibroblasts) were grown as monolayers in minimum essential medium supplemented with 2 mM L-glutamine, 50 IU/ml penicillin, 50 mg/ml streptomycin, and 10% fetal calf serum (GIBCO BRL). Primary cultures of Schwann cells were prepared as described by Brockes et al. (1979). In brief, sciatic nerves were dissected from 3-d-old Sprague-Dawley rats, and mixed cultures of Schwann cells and endoneurial fibroblasts were maintained in minimum essential medium supplemented with 10% fetal bovine serum for 1 d. To reduce fibroblast contamination, the culture was then incubated for 2 d in the presence of 10^{-5} M cytosine arabinose. On the fourth day, the cells were transferred to polylysine-coated coverslips and maintained for further 3 d in the presence of 10% fetal bovine serum, 2 μM forskolin, and 20 μg/ml bovine pituitary extract.

Immunofluorescence and Immunoelectron Microscopy of Cultured Cells

For indirect immunofluorescence cells were grown on 10 × 10-mm glass coverslips. The cells were washed twice in PBS, fixed with 3.7% formaldehyde (freshly prepared from paraformaldehyde) in PBS for 10 min at room temperature, and subsequently permeabilized with 0.5% Triton X-100 in PBS for 20 min at room temperature. Alternatively, cells were first permeabilized with 0.5% Triton X-100 in CSK buffer (100 mM NaCl,

300 mM sucrose, 10 mM PIPES, 3 mM $MgCl_2$, 1 mM EGTA, pH 6.8; Fey et al., 1986) containing 0.1 mM PMSF for 1 min on ice, and subsequently fixed with 3.7% formaldehyde in CSK buffer, for 10 min at room temperature. After fixation and permeabilization, the cells were rinsed in PBS containing 0.05% Tween-20 (PBS-Tw), incubated for 30 min with primary antibodies diluted in PBS, washed in PBS-Tw, and incubated for 30 min with the appropriate secondary antibodies conjugated to fluorescein (FITC), Texas red, or indodicarbocyanine (Cy5) (Jackson ImmunoResearch Laboratories, Inc.). Finally, the coverslips were mounted in VectaShield (Vector Laboratories) and sealed with nail polish.

For immunoelectron microscopy, cells were fixed with 3.7% formaldehyde in PBS, ~20 min. First, the medium was washed twice with PBS and the fixative was added to the culture dishes for 10 min; then, the cells were scraped using a rubber policeman and centrifuged at 7,000 *g* for 10 min. The cell pellets were sequentially dehydrated with continuous low-speed stirring, in 30% methanol at 4°C for 5 min, 50% methanol at 4°C for 5 min, 75% methanol at −20°C for 5 min, and 90% methanol at −20°C for 30 min. This was followed by mixtures of 90% methanol/Lowicryl K4M (1:1 and 1:2 vol/vol, for 1 h each at −20°C). Finally, the cell pellets were embedded in Lowicryl K4M for 24 h at −20°C and transferred to gelatine capsules. Polymerization was induced by UV irradiation for 5 d at −20°C, followed by one more day at room temperature. Ultrathin sections on gold grids were sequentially incubated with 0.1 M glycine in PBS (15 min), 5% BSA in PBS (15 min), and finally the primary antibody was diluted in 1% BSA, 0.1 M glycine in PBS (1 h at room temperature). After washing, the sections were incubated with appropriate secondary antibody conjugated to either 5 or 10 nm gold particles diluted 1:25 in 0.1% BSA in PBS (45 min at room temperature). Finally, the sections were washed and stained with 10% aqueous uranyl acetate. As controls, sections were treated as described, but omitting the primary antibody.

Immunocytochemistry of Rat Neurons

Male rats of the Sprague-Dawley strain were kept on a 12-h day/night lighting regime with lights out at 8:00. All animals were killed by overdose of pentobarbital. For immunofluorescence, the rats were perfused through the ascending aorta with 3.7% formaldehyde (freshly prepared from paraformaldehyde) in PBS, pH 7.4, for 15 min at room temperature. Supraoptic nuclei were dissected out of 500-μm-thick hypothalamic slices and trigeminal ganglia were removed and cut into small fragments. These tissue fragments were washed in PBS for 1 h and individually transferred to a drop of PBS on a siliconized slide. Then, a coverslip was applied on top of the slide and the tissue was squashed by percussion with a histologic needle. The preparation was then frozen in dry ice and the coverslip was removed using a razor blade. The slides with adhered neurons were sequentially dehydrated in 96% and 70% ethanol at 4°C for 10 min and rinsed in PBS. Before immunostaining, the samples were sequentially incubated with 0.5% Triton X-100 in PBS for 10 min, 0.1 M glycine in PBS for 30 min, and 0.01% Tween 20 in PBS for 5 min.

For immunoelectron microscopy, animals were perfused with 4% formaldehyde (freshly prepared from paraformaldehyde) in 0.12 M phosphate buffer, pH 7.4. The hypothalamic supraoptic nucleus was dissected out from brain slices and left in the same fixative for 3 h at 4°C, dehydrated in increasing concentrations of methanol at −20°C, and embedded in Lowicryl K4M at −20°C. Ultrathin sections were immunostained as described above.

Microscopy and Image Processing

Confocal microscopy was performed with either a Zeiss laser scanning microscope LSM 410 or a BioRad MRC-1024, using excitation wavelengths of 488 nm (for FITC), 543 nm (for Texas red), and 633 nm (for Cy5). Each channel was recorded independently and pseudocolor images were generated and superimposed. Ultrathin sections were examined with a JEOL CXII electron microscope operated at 80 Kv.

For quantitative analysis, images were obtained from untreated and leptomycin B–treated cells, and double labeled with probes for U2 snRNA (green staining) and coilin (red staining). Each image was decomposed in RGB (y_R = red, y_G = green) and segmented according to Fwu and Djuriç (1996) into four classes: C_0 = extracellular background, C_1 = intracellular background (i.e., cytoplasm and nucleoli), C_2 = nucleoplasm, C_3 = Cajal bodies. The average intensities for $y_G \in C_3$ (m_2) and $y_G \in C_2$ (m_1) were estimated. Finally, the ratio m_2/m_1 was determined.

Results

SMN Is Localized in the Cajal Body

Immunofluorescence microscopy using anti–SMN antibodies reveals bright intranuclear foci and additional diffuse cytoplasmic labeling (Fig. 1 A), as previously reported by Liu and Dreyfuss (1996). Labeling of the cytoplasm is more evident when cells are first fixed with formaldehyde, and then permeabilized with Triton X-100, whereas the intranuclear foci are equally stained in cells treated with detergent either before or after fixation.

Double-labeling experiments using anti–SMN and anticoilin antibodies show that in some HeLa cells the SMN protein is present in dot-like structures closely related but distinct from Cajal (coiled) bodies (Fig. 1, B and C, arrow), the so-called gemini of coiled bodies or gems (Liu and Dreyfuss, 1996). Gems are also often detected independent from Cajal bodies (Fig. 1 C, arrowhead). Most important, gems are only visible in a small proportion of HeLa cells (1–10%). In the vast majority of cells analyzed, SMN colocalizes precisely with coilin (Fig. 1, D and E). Similar results were observed in both HeLa and Huh7 cells (human cervical carcinoma and hepatoma derived epithelial cell lines, respectively) using either anti–SMN monoclonal antibody 2B1 (Liu and Dreyfuss, 1996), or serum 95020 (Lefebvre et al., 1997) (Fig. 1, D–G). Triple-labeling experiments using anti–SMN, anti–coilin, and anti–Sm antibodies (Fig. 1, H–K) demonstrate that snRNPs colocalize with SMN in Cajal bodies (arrows), but not in gems.

Since colocalization studies based on computer-superimposed images of fluorochrome-coupled antibody labeling are constrained by the resolution limits of light microscopy, immunoelectron microscopy was also performed. Cells were fixed in formaldehyde, embedded in Lowicryl K4M at −20°C, and ultrathin sections were incubated with anti–SMN and anti–coilin antibodies. Fig. 2 depicts thin sections through the nucleus of Huh7 cells. In Fig. 2 A, the section was incubated with anticoilin antibody and appropriate secondary antibody conjugated to 10-nm gold particles. The gold particles decorate the characteristic entangled threads of a Cajal body. A similar labeling pattern (i.e., concentration of gold particles over a Cajal body) is observed in B, which depicts a thin section incubated with anti–SMN antibody. Fig. 2, C and D, shows Cajal bodies simultaneously labeled by anticoilin and anti–SMN antibodies (detected with 5- and 10-nm gold particles, respectively). This demonstrates that SMN is present in the Cajal body of cultured cell lines.

We next compared the localization of SMN and coilin in primary tissue cells (Fig. 3). The study was focused on neurons from either the hypothalamic supraoptic nucleus or trigeminal ganglia, because neuronal cells contain prominent Cajal bodies (Lafarga et al., 1983). A characteristic feature of neuronal cells is that, in addition to large Cajal bodies (Fig. 3, B and E, arrows), they contain coilin in numerous small cap-like structures that form a ring around the nucleolus (Fig. 3, B and E, arrowheads; Samtama et al., 1996; Lafarga et al., 1998). Analysis of the subnuclear distribution of SMN and coilin in >1,000 neurons revealed that SMN localizes precisely in the Cajal body (Fig. 3, A–C, ar-

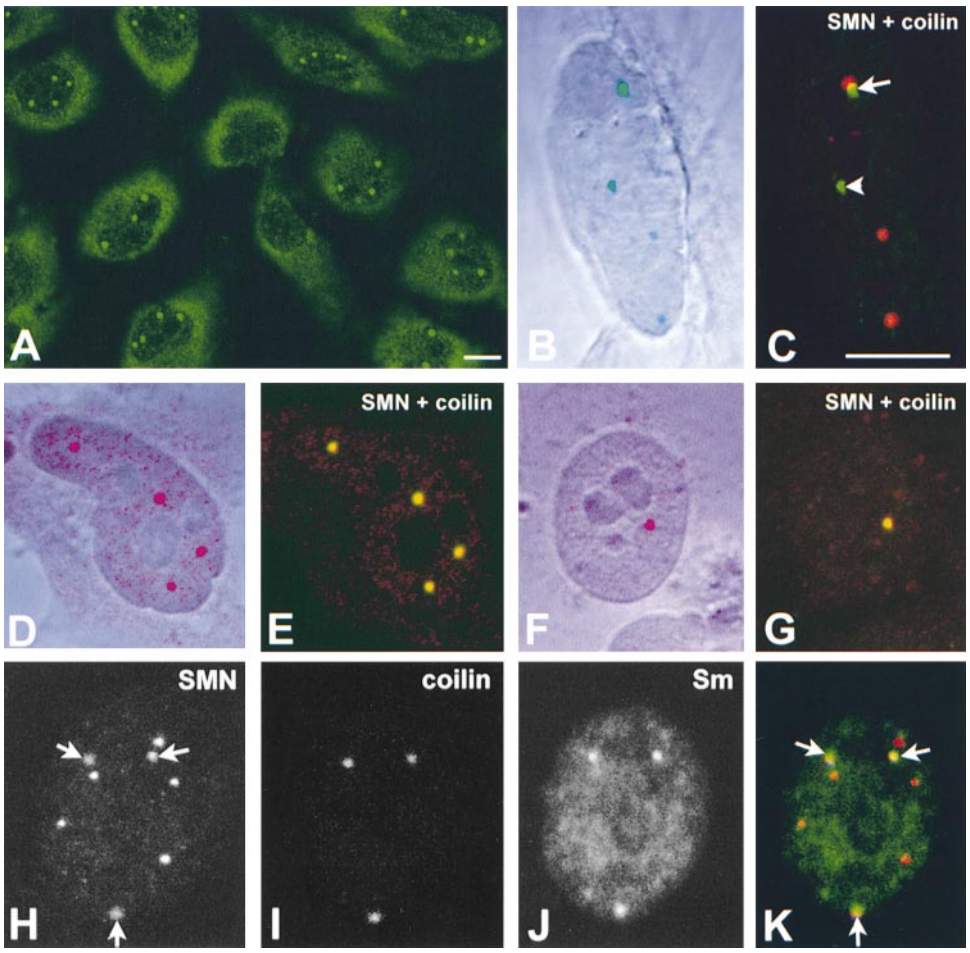


Figure 1. Localization of SMN in human cell lines. (A) HeLa cells were fixed in formaldehyde, permeabilized with Triton X-100, and immunostained with anti–SMN monoclonal antibody 2B1. Note the cytoplasmic labeling with additional nuclear foci. Staining of the cytoplasm is more difficult to detect when cells are extracted with detergent before fixation (B–G). (B and D) Phase contrast images of HeLa cells with superimposed SMN labeling produced by either monoclonal antibody 2B1 (B, green foci) or serum 95020 (D, red foci). (C and E) The corresponding cells double labeled with anticoilin antibodies. The arrow and the arrowhead in C indicate gems; one is adjacent to a Cajal body (arrow), whereas the other is independent (arrowhead). The yellow color observed in E demonstrates a precise colocalization of SMN (red staining) and coilin (green staining). (F) Phase contrast image of an Huh7 cell with superimposed SMN staining (red focus); (G) the same cell double labeled for SMN (red staining) and coilin (green staining); the yellow color indicates colocalization of both proteins in the same focus. (H–K) HeLa cell triple labeled for SMN (H), coilin (I), and Sm (J) proteins. This experiment was performed using mouse anti–SMN monoclonal antibody 2B1 (detected with Texas red), rabbit anticoilin serum 204.3 (detected with Cy5), and human anti–Sm autoimmune serum C45 (detected with FITC). Superimposition of the three images (K) reveals that SMN colocalizes with Sm in Cajal bodies (arrows), but not in gems (red foci). Bars, 10 μm.

row), but it is not detected in the perinucleolar structures (Fig. 3, A–C, arrowheads). Double-labeling experiments using anticoilin antibodies and monoclonal antibody 4G3 directed against the U2 snRNP protein B″ (Habets et al., 1989) confirmed that snRNPs are highly enriched in the Cajal bodies (Fig. 3, D–F, arrow), but not in the perinucleolar rings (Fig. 3, D–F, arrowheads). Thus, SMN and snRNPs colocalize specifically in the Cajal body.

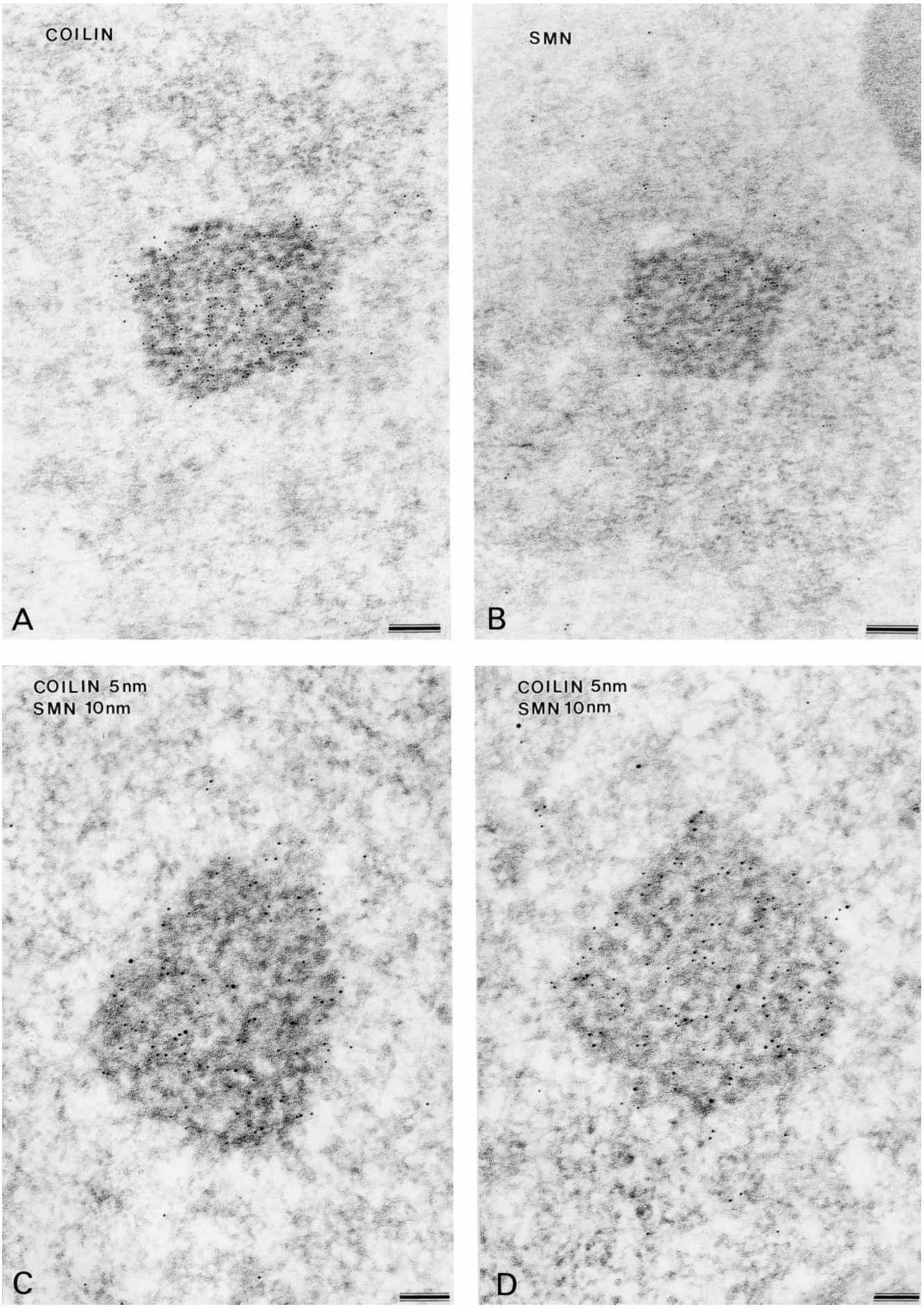
Staining of primary Schwann cells kept in culture further revealed localization of SMN in the Cajal body (Fig. 3 G). However, when these cells were stimulated to proliferate by addition of forskolin and pituitary factors (Brockes et al., 1979), ~10–15% of the nuclei contained SMN in gems (Fig. 3, H and I).

In conclusion, these results show that SMN may either be present in discrete focal structures that are not enriched in snRNPs (i.e., gems) or colocalize with snRNPs in the Cajal body. The association of SMN with Cajal bodies is observed in both cultured cell lines and primary neurons, whereas gems are only detected in a small proportion (<15%) of rapidly proliferating cells in culture. Although gems tend to be more frequently detected during the G1 stage of the cell cycle (T. Carvalho, unpublished data), the overall proportion of nuclei containing gems is variable depending on cell type and passage number.

SMN Is Targeted to Preformed Cajal Bodies

In agreement with data from earlier studies (Ferreira et al., 1994), remnants of Cajal bodies are visible in the cytoplasm of mitotic HeLa cells (Fig. 4). We have previously reported that these mitotic Cajal bodies are labeled with

Figure 2 (continues on facing page). Localization of SMN by immunoelectron microscopy. Huh7 cells were embedded in Lowicryl, sectioned, and immunogold labeled with antibodies directed against coilin (A, rabbit serum 204.3) or SMN (B, mouse monoclonal antibody 2B1). (C and D) 0.1 μm. Sections double labeled using anticoilin (5-nm gold particles) and anti–SMN (10-nm gold particles) antibodies. Bars: (A and B) 0.2 μm; (C and D) 0.1 μm.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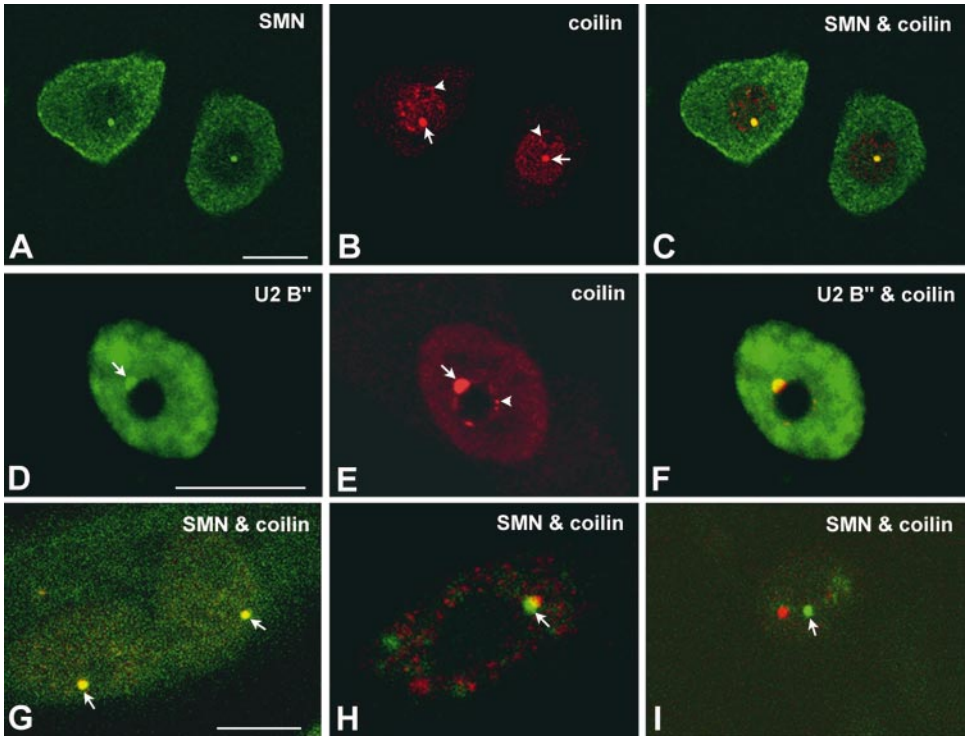


Figure 3. Localization of SMN in primary neurons. (A–F) Squash preparations from rat trigeminal ganglia were double labeled with antibodies directed against either SMN (A, green staining) and coilin (B, red staining) or U2snRNP B″ (D, green staining) and coilin (E, red staining). (C and F) Superimpositions of red and green images. The yellow color indicates that SMN and B″ proteins are localized in Cajal bodies (arrows), but not in the perinucleolar rings (arrowheads). (G–I) Primary Schwann cells from rat sciatic nerve were kept in culture and stimulated to proliferate by addition of forskolin and pituitary extract. The cells were double labeled with antibodies directed against SMN (green staining) and coilin (red staining). Although most cells reveal colocalization of the two proteins (G), some (~15%) contain gems that are located either independent of or in close proximity to a Cajal body (H and I, arrows). Bars, 10 μm.

antibodies directed against both snRNP proteins and the m3G cap, and by snRNA-specific antisense probes, indicating that they contain assembled snRNP particles (Ferreira et al., 1994). Here we show that these structures are additionally labeled by anti–SMN antibodies (Fig. 4, A–C), indicating that the association of SMN and snRNPs with Cajal bodies persists during mitosis. As cells progress from telophase to G1, Cajal bodies reappear in the nucleus (Fig. 4 D, arrows), but SMN remains exclusively in the cytoplasm (Fig. 4 D, arrowheads). The presence of SMN in Cajal bodies is detected later in G1, indicating that there is a lag period between assembly of the Cajal body and localization of SMN therein. This suggests that SMN is targeted to preformed Cajal bodies.

To further investigate whether the Cajal body plays a role in the intranuclear localization of SMN, microinjection experiments were performed with antibodies that promote a specific disappearance of the Cajal body (Almeida et al., 1998). HeLa cells were microinjected in the nucleus with purified anticoilin monoclonal antibodies and allowed to incubate for 24 h. The cells were then briefly permeabilized with Triton X-100, fixed with formaldehyde, and sequentially incubated with fluorescein-conjugated anti–mouse IgG (to detect the injected antibody), rabbit polyclonal anti–SMN antibody 95020, and Texas red–conjugated anti–rabbit IgG. As depicted in Fig. 5 A, cells injected with the anticoilin monoclonal antibody 1D4-delta are devoid of Cajal bodies. Staining of these

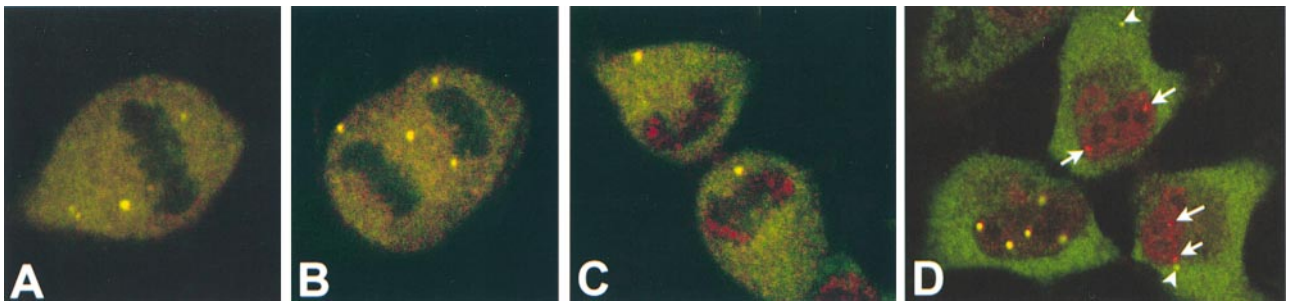


Figure 4. Localization of SMN during mitosis. HeLa cells were fixed in formaldehyde, permeabilized with Triton X-100, and double labeled with anti–SMN monoclonal antibody 2B1 (green staining) and anticoilin antibody (red staining). Metaphase (A), anaphase (B), and telophase (C) cells contain foci in the cytoplasm that appear yellow, demonstrating perfect colocalization of SMN and coilin proteins. In contrast, in early G1 cells (D), SMN foci persist in the cytoplasm (D, arrowhead), while Cajal bodies are already detected in the nucleus (D, arrows).

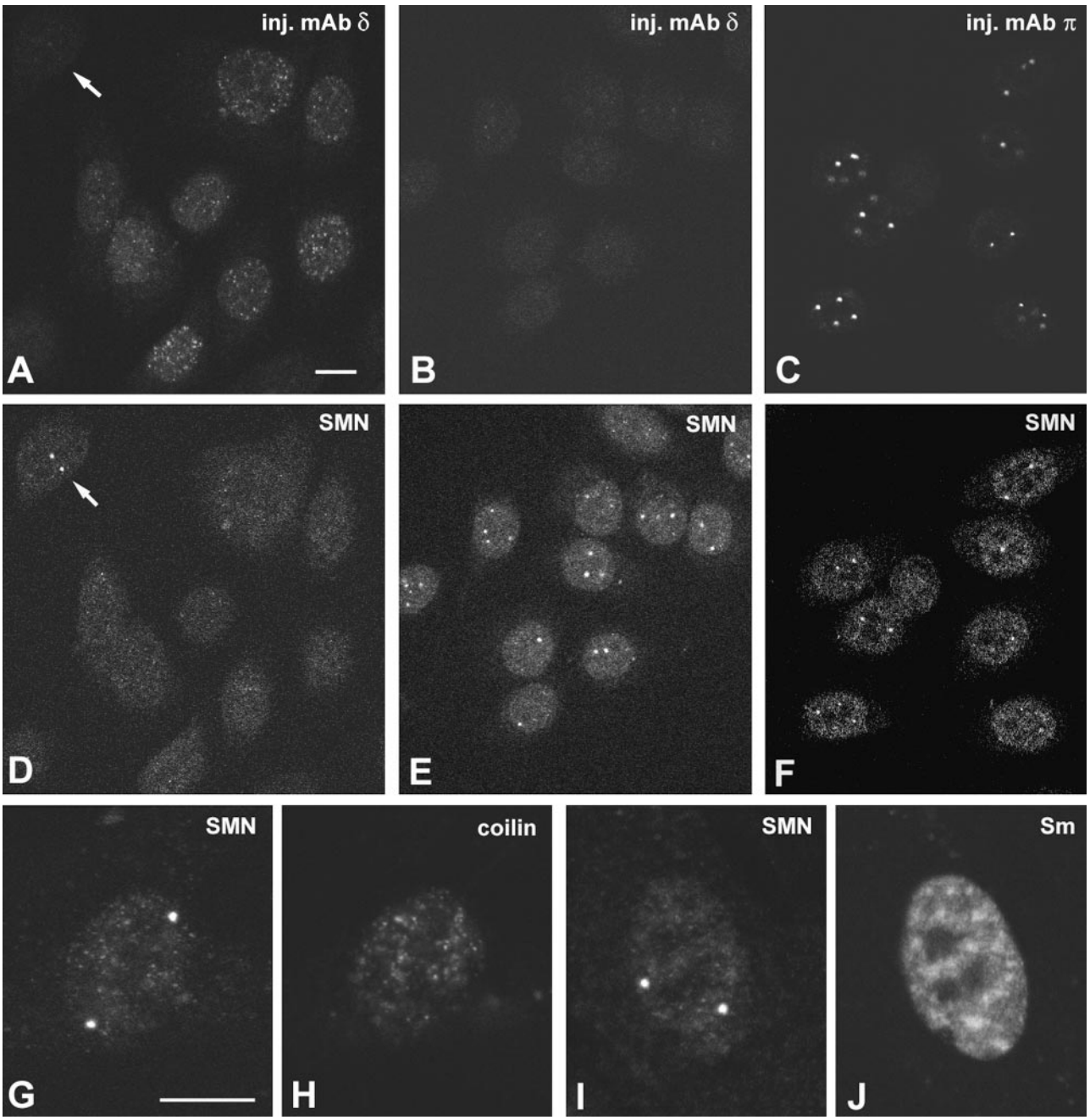


Figure 5. Cajal bodies represent a platform for colocalization of SMN and snRNPs. (A–F) HeLa cells were microinjected in the nucleus with purified anticoilin monoclonal antibodies and allowed to incubate for 24 h. (A) The cells were incubated with fluorescein-conjugated anti–mouse IgG to detect the injected antibody. The arrow points to a noninjected cell; note that all injected cells are devoid of Cajal bodies. (D) Double labeling with anti–SMN antibody does not reveal any intranuclear staining; however, the noninjected cell contains SMN localized in Cajal bodies (D, arrow). (B) A field of cells that were not injected. Note that most of these cells contain SMN in Cajal bodies (E). (C) Microinjection of monoclonal antibody 5P10-pi, which does not affect the structure of Cajal bodies. (F) In these cells, SMN localizes in Cajal bodies. (G–J) WI-38 cells were double labeled with either anti–SMN (G) and anticoilin (H) antibodies, or anti–SMN (I) and anti–Sm (J) antibodies. Note that gems are present in cells devoid of Cajal bodies (G and H) and that gems are not enriched in snRNPs (I and J). Bars, 10 μm.

cells with anti–SMN antibodies fails to reveal any intranuclear foci, in clear contrast with the bright staining of Cajal bodies observed in the nucleus of noninjected cells (Fig. 5, A and D, arrow, and B and E). As expected, Cajal bodies are brightly stained by anti–SMN antibody in the nucleus of cells microinjected with monoclonal antibody 5P10-pi (Fig. 5, C and F), which does not interfere with the Cajal body structure (Almeida et al., 1998).

To further address the role of the Cajal body in the localization of SMN, primary fibroblasts were also examined because this type of cell is normally devoid of Cajal bodies (Spector et al., 1992; Carmo-Fonseca et al., 1993). As

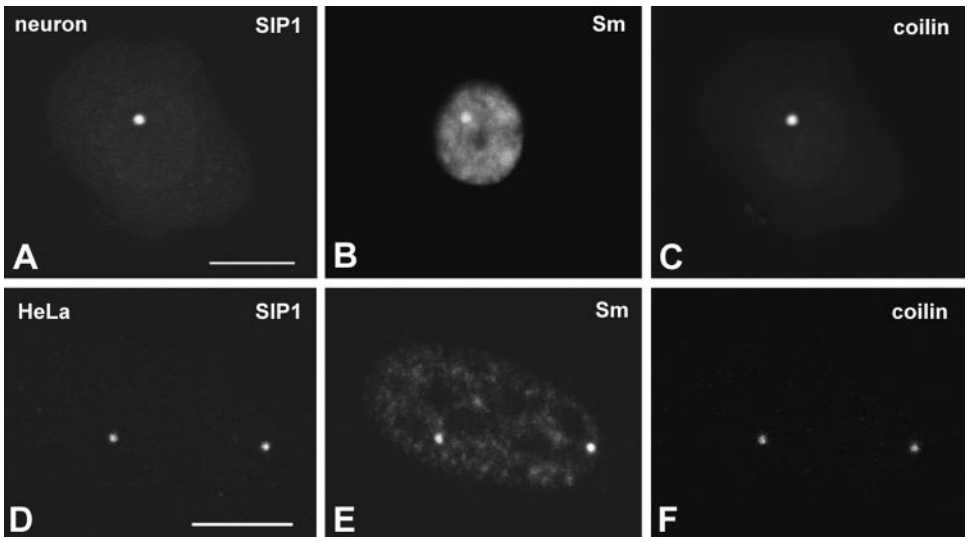


Figure 6. SIP1 colocalizes with snRNPs in the Cajal body. Squash preparations from rat trigeminal ganglia neurons (A–C) and HeLa cells (D–F) were triple labeled with anti–SIP1 (A and D), anti–Sm (B and E), and anticoilin (C and F) antibodies. These experiments were performed using mouse anti–SIP1 monoclonal antibody 2E17 (detected with Texas red), rabbit anticoilin serum 204.3 (detected with Cy5), and human anti–Sm autoimmune serum C45 (detected with FITC). Bars, 10 μm.

shown in Fig. 5, several fibroblasts contain SMN localized in nuclear foci (gems), despite the absence of Cajal bodies (Fig. 5, G and H). As observed in HeLa cells, gems in fibroblasts are not labeled by anti–Sm antibodies (Fig. 5, I and J).

Taken together, these results suggest that Cajal bodies provide a structural framework for the colocalization of SMN and snRNPs in the nucleus.

SIP1 Colocalizes with SMN and snRNPs in the Cajal Body

Next, we examined the distribution of SIP1, a novel protein that forms a complex with SMN (Liu et al., 1997). Immunolocalization studies were performed using anti–SIP1 monoclonal antibody 2E17 (Liu et al., 1997), anti–Sm, and anticoilin antibodies. As depicted in Fig. 6, both primary neurons and HeLa cells show a precise colocalization of SIP1 and snRNPs in the Cajal body. The presence of SIP1 in Cajal bodies was further confirmed at the electron microscopical level (data not shown). Thus, Cajal bodies represent unique intranuclear sites where snRNPs colocalize with SMN and SIP1.

Leptomycin B Affects the Intranuclear Distribution of snRNPs

To investigate whether snRNPs accumulated in the Cajal body are "in transit" from the cytoplasm, we treated HeLa cells with leptomycin B, a drug that inhibits U snRNA export to the cytoplasm (Fornerod et al., 1997) and, consequently, reimport of assembled snRNPs to the nucleus.

HeLa cells were incubated with 10, 20, or 30 nM leptomycin B for 1–10 h. Although these drug concentrations produced similar effects, at the lower doses the time of response was longer and more variable. We therefore decided to work with a concentration of 30 nM. For snRNP detection, the cells were labeled with antibodies directed against Sm proteins (Y12; Lerner et al., 1981), the U2 snRNP-specific B″ protein (4G3; Habets et al., 1989), and the m3G cap of snRNAs (R1131; Reuter et al., 1984); in addition, in situ hybridization was performed with a U2 snRNA-specific antisense probe (Carmo-Fonseca et al., 1991). In nontreated cells, snRNPs are highly enriched in Cajal bodies (foci in Fig. 7), whereas after 3 h of drug treatment, snRNPs are no longer concentrated in the Cajal body (Fig. 7). In fact, the U2 snRNP is normally approximately threefold more concentrated in Cajal bodies than in the nucleoplasm, but after leptomycin B treatment the average intensity of U2 snRNP labeling in the Cajal body is similar to that in the nucleoplasm (Fig. 8).

In addition to depleting snRNPs from the Cajal body, leptomycin B causes a decrease in the number of Cajal bodies per nucleus and a redistribution of coilin into the nucleolus (Fig. 9, A–C). Since Cajal bodies are very dynamic structures with a high turnover rate (Rebelo et al., 1996), leptomycin B could either stimulate the disassembly of preexisting Cajal bodies, or inhibit the assembly of new Cajal bodies. To specifically investigate the effect of leptomycin B on the assembly of new Cajal bodies, we examined the distribution of coilin in cells that have completed mitosis in the presence of the drug. In nontreated samples, Cajal bodies are visible in >80% of early G1 cells (these cells are identified by the persistence of the contractile ring connecting their cytoplasms; Fig. 9, D, E, and H). In contrast, the vast majority of early G1 cells incubated with leptomycin B are devoid of Cajal bodies (Fig. 9, F, G, and H). Instead of Cajal bodies, some of these cells contain perinucleolar patches of coilin reminiscent of what is observed in neurons under physiological conditions (see Fig. 3 B). As coilin appears to shuttle from the nucleolus to the Cajal body (Isaac et al., 1998; Lafarga et al., 1998), these results suggest that leptomycin B interferes with the efflux of coilin from the nucleolus. As a possible consequence, coilin would accumulate within the nucleolus (Fig. 9 A), becoming unavailable for assembly of new Cajal bodies. Due to their high turnover rate (Rebelo et al., 1996), this would lead to a progressive disappearance of Cajal bodies from the nucleoplasm.

Staining of leptomycin-treated cells with the 2B1 and 2E17 monoclonal antibodies reveals a progressive increase in the proportion of cells containing gems (Fig. 10, A and

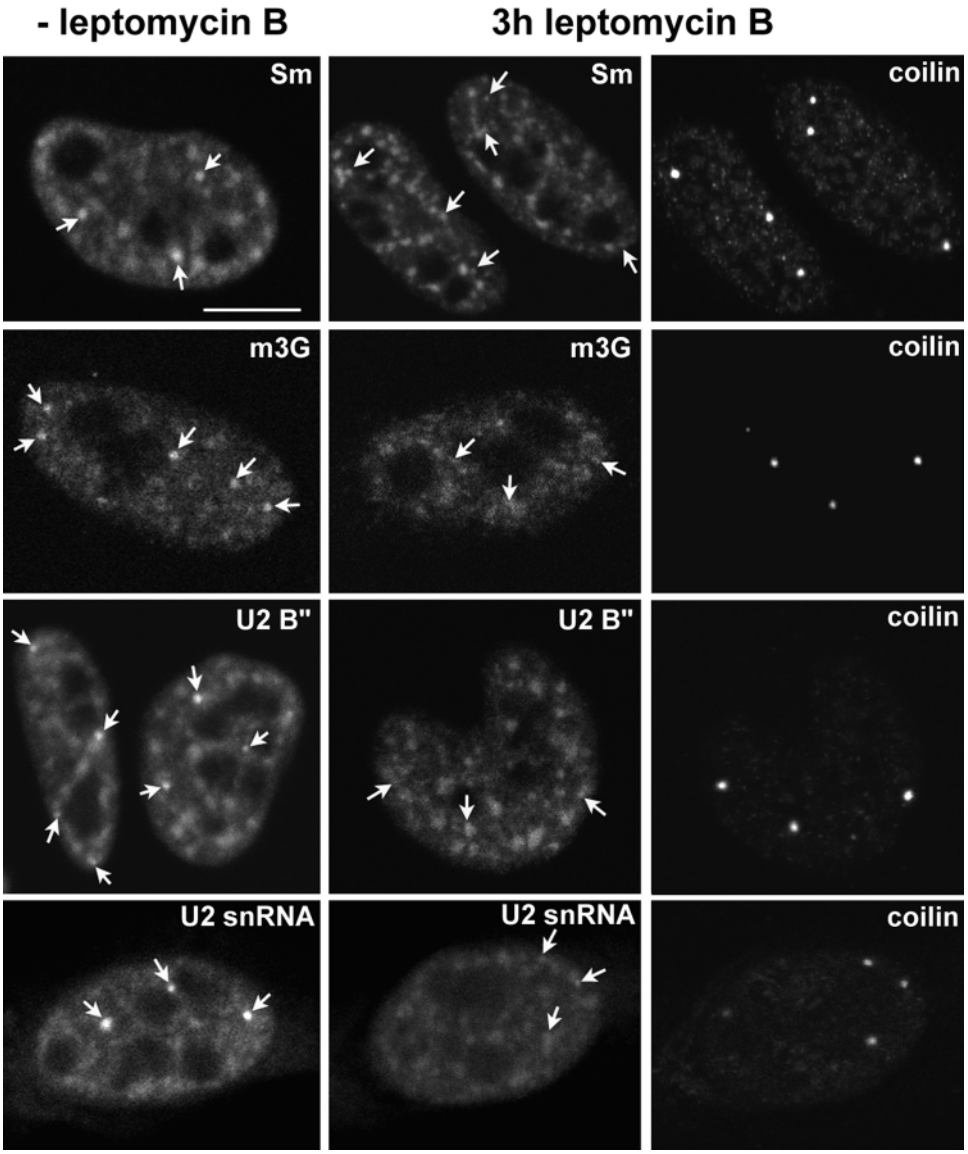


Figure 7. Leptomycin B alters the intranuclear distribution of snRNPs. HeLa cells were either untreated (-leptomycin B) or incubated with 30 nM leptomycin B for 3 h (3h leptomycin B). Untreated cells were labeled for snRNPs using Y12 monoclonal antibody (which recognizes the B, B′, and D peptides of the Sm family), antibody R1131 (which recognizes the m3G cap of the U snRNAs), 4G3 monoclonal antibody (which recognizes the U2 snRNP-specific protein B″), and an antisense riboprobe complementary to U2 snRNA. Arrows denote high concentration of snRNPs in the Cajal body. After 3 h of leptomycin B treatment, the cells were double labeled for snRNPs and coilin. Arrows indicate the position of Cajal bodies. Note that snRNPs are no longer highly enriched in the Cajal body. Bar, 10 μm.

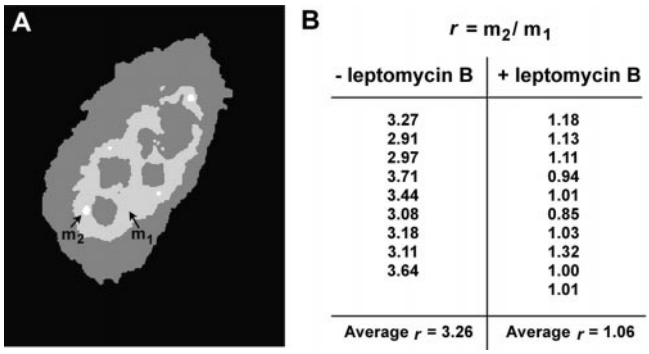
B), most probably due to an effect on the normal trafficking dynamics of SMN-SIP1 proteins in the nucleus. However, both SMN and SIP1 are still highly enriched in Cajal bodies (Fig. 10 A, and data not shown), suggesting that SMN-SIP1 complexes remain associated with Cajal bodies in the absence of newly imported snRNPs from the cytoplasm. This implies that if SMN, SIP1, and snRNPs are part of a common complex in the Cajal body, there must be a mechanism that triggers the release of snRNPs from this structure.

Discussion

This work reports two novel protein components of the Cajal (coiled) body, the Survival Motor Neuron gene product and its associated protein, SIP1. As SMN and SIP1 play an important role in snRNP biogenesis and recycling (Fischer et al., 1997; Liu et al., 1997; Pellizzoni et al., 1998), this finding provides a direct link between the Cajal body and snRNP metabolism.

The SMN and SIP1 proteins were previously immunolocalized in HeLa cells to discrete nuclear foci termed gemini of coiled bodies or gems (Liu and Dreyfuss, 1996). The data presented here indicate that gems are observed in a variable (but small) proportion of cells in culture. In the vast majority of cells studied, including primary neurons directly removed from organisms, SMN and SIP1 are present exclusively in the Cajal body and gems were never identified. Notably, the presence of SMN in Cajal bodies was also detected by other groups (see Matera and Frey, 1998; Matera, 1999; Sleeman and Lamond, 1999a).

A major difference between Cajal bodies and gems concerns the presence or absence of snRNPs. The SMN and SIP1 proteins colocalize with snRNPs in the Cajal body, whereas gems do not concentrate snRNPs. This makes it likely that Cajal bodies contain the SMN-SIP1–snRNP complexes detected in the nuclear fraction of somatic cells (Liu et al., 1997). As SMN and SIP1 associate with snRNPs in the cytoplasm, one possibility is that the SMN-SIP1–snRNP complex enters the nucleus and is targeted to



- leptomycin B	+ leptomycin B
3.27	1.18
2.91	1.13
2.97	1.11
3.71	0.94
3.44	1.01
3.08	0.85
3.18	1.03
3.11	1.32
3.64	1.00
	1.01
Average r = 3.26	Average r = 1.06

Figure 8. Leptomycin B depletes snRNPs from the Cajal body. Untreated and leptomycin B–treated cells were randomly selected for image segmentation and quantification. The ICM algorithm (Fwu and Djuriç, 1996) was applied to segment images into four classes (A). The average U2 snRNA labeling intensity of Cajal bodies (m_2) and nucleoplasm (m_1) was calculated for each image and their ratio ($r = m_2/m_1$) was estimated (B).

the Cajal body. The Cajal body may then represent an assembly platform where snRNPs in transit from the cytoplasm accumulate transiently, until an as yet unidentified stage of their maturation process is completed. Taking into account that 2′-*O*-methylation and pseudouridylation of U2 snRNA take place in the nucleus and are required for the final assembly of snRNP-specific proteins (Yu et al., 1998), it will be important to clarify whether snRNP complexes enriched in the Cajal body contain modified snRNAs. In this regard, it is important to note that the U2-specific proteins collectively termed SF3a/SF3b (Krämer, 1996), which assemble into the splicing-competent U2 snRNP 17S particle, are not detected in Cajal bodies (D. Nesic and A. Krämer, personal communication). This observation further supports the view that snRNPs concentrated in the Cajal body are not yet fully mature.

Assuming that both snRNPs and SMN complexes are constantly in motion throughout the cell and accumulate transiently in either Cajal bodies or gems, differences in their trafficking dynamics could account for the differences observed in their steady state nuclear distribution. In fact, expression of a dominant-negative amino-terminal deletion mutant of SMN (SMNΔN27), which is thought to block nuclear snRNP recycling, causes an accumulation of SMN in extremely enlarged Cajal bodies (Pellizzoni et al., 1998). Moreover, SMN contains a self-oligomerization domain that overlaps with the Sm-interacting domain (Lorson et al., 1998). This implies that SMN may either form a complex with snRNPs (most probably localized in the Cajal body) or self-oligomerize, giving rise to gems. A prediction from this model is that gems should become more

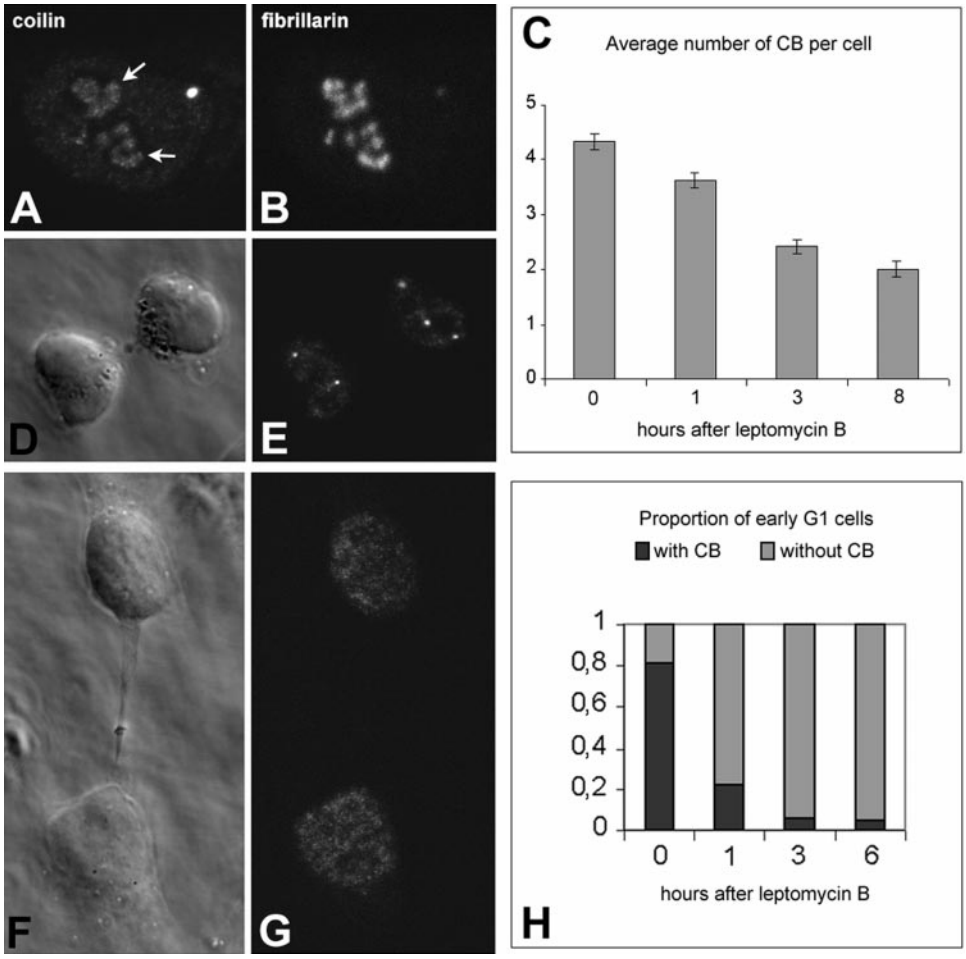


Figure 9. Leptomycin B affects the dynamics of the Cajal body. HeLa cells were treated with 30 nM leptomycin B for 3 h and double labeled using anticoilin (A) and antifibrillarin (B) antibodies. Note that coilin colocalizes with fibrillarin in intranucleolar foci (A, arrows). The graph depicted in C illustrates the effect of leptomycin B on the average number of Cajal bodies per cell nucleus (∼150 cells were counted at each time point; means ± SEM are indicated). (E and G) Early G1 cells stained with anticoilin antibodies; (D and F) corresponding phase contrast images. The cells depicted in D and E were untreated, whereas the cells in F and G were treated with leptomycin B for 3 h. In the absence of leptomycin B, Cajal bodies are seen in >80% of early G1 cells (H). In contrast, >95% of the cells that complete mitosis in the presence of leptomycin B fail to assemble new Cajal bodies (H).

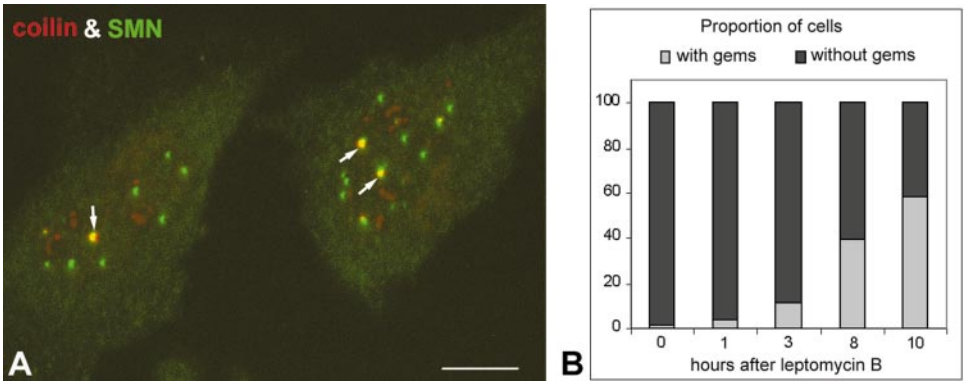


Figure 10. Leptomycin B affects the dynamics of gems. (A) Huh7 cells were treated with 30 nM leptomycin B for 3 h and double labeled using anticoilin (red staining) and anti–SMN (green staining) antibodies. A depicts a superimposition of eight optical sections through the cell nucleus (consecutive sections are separated by 0.5 μm). (B) HeLa cells were either untreated or incubated with 30 nM leptomycin B for 1, 3, 8, or 10 h. The cells were double labeled with anticoilin and anti–SMN antibodies. At each time point, ~100 cells containing Cajal bodies were randomly selected. The graph depicts the proportion of these cells that contained gems. Note that absence of SMN from Cajal bodies was very rarely observed (<1%). Bar, 10 μm.

prominent after depletion of snRNPs from the Cajal body, as indeed was observed after treatment of cells with leptomycin B.

The observed depletion of snRNPs from Cajal bodies caused by leptomycin B strongly suggests that spliceosomal snRNPs newly imported from the cytoplasm travel through the Cajal body. Leptomycin B is a cytotoxin that interacts directly with the nuclear export receptor CRM1, inhibiting its binding to nuclear export signals and Ran-GTP (Fornerod et al., 1997). As a consequence, this drug blocks the export of substrates recognized by CRM1, including Rev (Wolff et al., 1997) and proteins containing Rev-like leucine-rich nuclear export signals (Fornerod et al., 1997; Fukuda et al., 1997; Ossareh-Nazari et al., 1997). Although CRM1 mutations in yeast interfere with mRNA export (Stade et al., 1997), to date leptomycin B has been found to inhibit export of only one class of RNA in vertebrates, the U snRNAs (Fornerod et al., 1997). As shown in Figs. 7 and 8, 3 h after drug treatment, Cajal bodies are still present but are no longer brightly stained by antibodies directed against Sm proteins, the U2 snRNP-specific B″ protein, and the m3G cap of snRNAs, or by an antisense probe specific for the U2 snRNA. Thus, after exposure to leptomycin B the Cajal body fails to concentrate snRNPs. Since leptomycin B inhibits nuclear export of U snRNAs, the assembly of new snRNP particles in the cytoplasm and their subsequent transport to the nucleus is also blocked. The observed redistribution of snRNPs in the nucleus can therefore be explained if mature snRNPs present in the Cajal body leave the structure while new particles are prevented from reaching it. Direct visual evidence that snRNPs flow through the Cajal body has been recently obtained by microinjection of fluorescently tagged U snRNAs into the cytoplasm of *Xenopus* oocytes (Gall et al., 1999; Narayanan et al., 1999) and expression of green fluorescent protein–tagged Sm proteins in HeLa cells (Sleeman and Lamond, 1999b). These studies revealed that both snRNAs and snRNP proteins are first detected in Cajal bodies before they start to accumulate at other nuclear locations.

In addition to interfering with the concentration of snRNPs in the Cajal body, treatment of cells with leptomycin B also affects the distribution of coilin in the nucleus. Shortly after exposure to the drug, coilin is detected inside the nucleolus while the number of Cajal bodies decreases. Within the nucleolus, coilin colocalizes with fibrillarin in foci that correspond to the dense fibrillar component of the nucleolus. A similar staining pattern was previously observed in HeLa cells labeled with an anticoilin monoclonal antibody (mAb-ϕ) (Almeida et al., 1998). Remarkably, this antibody reacts with an epitope adjacent to a critical serine residue (serine 202) that, when mutated to aspartate, induces the formation of Cajal body-like structures inside the nucleolus (Lyon et al., 1997). The presence of Cajal bodies inside the nucleolus has also been reported in certain rare cases such as breast carcinoma cells, brown adipocytes, and hepatocytes of hibernating dormice (Malatesta et al., 1994; Ochs et al., 1994). Much more frequently, Cajal bodies are located in the vicinity of nucleoli, even physically attached to them, seemingly emerging from the dense fibrillar component (Ferreira and Carmo-Fonseca, 1995; Lafarga et al., 1998). Supporting this morphological evidence, several molecules are shared by both the Cajal body and the dense fibrillar component of the nucleolus, namely, fibrillarin (Raska et al., 1990, 1991), NAP57 and Nopp140 (Meier and Blobel, 1994). In particular, Nopp140 interacts with coilin and has been proposed to chaperone the transport of molecules from the nucleolus to the Cajal body (Isaac et al., 1998). It therefore appears that leptomycin B inhibits the flow of molecules between the nucleolus and the Cajal body, causing a retention of coilin in the dense fibrillar component and, consequently, a decrease in the number of Cajal bodies.

In summary, the major conclusions from this study are, first, that two proteins essential for snRNP biogenesis, SMN and SIP1, are localized in the Cajal body, and, second, that the nuclear export inhibitor leptomycin B causes a depletion of snRNPs from the Cajal body. The first observation provides a link between Cajal bodies and snRNP biogenesis, whereas the second indicates that the accumulation of snRNPs in the Cajal body is transient. Taken together, these results suggest that snRNPs flow through the Cajal body along their biogenesis pathway.

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References

Abbott, J., W.F. Marzluff, and J.G. Gall. 1999. The stem loop binding protein (SLBP1) is present in coiled bodies of the *Xenopus* germinal vesicle. *Mol. Biol. Cell.* 10:487–499.

Almeida, F., R. Saffrich, W. Ansorge, and M. Carmo-Fonseca. 1998. Microinjection of anti-coilin antibodies affects the structure of coiled bodies. *J. Cell Biol.* 142:899–912.

Bauer, D.W., C. Murphy, Z. Wu, C.-H.H. Wu, and J.G. Gall. 1994. In vitro assembly of coiled bodies in *Xenopus* egg extracts. *Mol. Biol. Cell.* 5:633–644.

Bellini, M., and J.G. Gall. 1998. Coilin can form a complex with the U7 small nuclear ribonucleoprotein. *Mol. Biol. Cell.* 9:2987–3001.

Bertrandy, S., P. Burlet, O. Clermont, C. Huber, C. Fondrat, D. Thierry-Mieg, A. Munnich, and S. Lefebvre. 1999. The RNA-binding properties of SMN: deletion analysis of the zebrafish orthologue defines domains conserved in evolution. *Hum. Mol. Genet.* 8:775–782.

Branlant, C., A. Krol, J.P. Ebel, E. Lazar, B. Haendler, and M. Jacob. 1982. U2 RNA shares a structural domain with U1, U4 and U5 RNAs. *EMBO (Eur. Mol. Biol. Organ.) J.* 1:1259–1265.

Brockes, J.P., K.L. Fields, and M.C. Raff. 1979. Studies on cultured rat Schwann cells: I. Establishment of purified population from cultures of peripheral nerve. *Brain Res.* 165:105–118.

Burge, C.B., T.H. Tuschl, and P.A. Sharp. 1999. Splicing of precursors to mRNAs by the spliceosomes. *In* The RNA World. R.F. Gesteland, T.R. Cech, and J.F. Atkins, editors. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 525–560.

Carmo-Fonseca, M., D. Tollervey, R. Pepperkok, S.M.L. Barabino, A. Merdes, C. Brunner, P.D. Zamore, M.R. Green, E.C. Hurt, and A.I. Lamond. 1991. Mammalian nuclei contain foci which are highly enriched in components of the pre-mRNA splicing machinery. *EMBO (Eur. Mol. Biol. Organ.) J.* 10: 195–206.

Carmo-Fonseca, M., R. Pepperkok, M.T. Carvalho, and A.I. Lamond. 1992. Transcription-dependent colocalization of the U1, U2, U4/U6 and U5 snRNPs in coiled bodies. *J. Cell Biol.* 117:1–14.

Carmo-Fonseca, M., J. Ferreira, and A.I. Lamond. 1993. Assembly of snRNP-containing coiled bodies is regulated in interphase and mitosis: evidence that the coiled body is a kinetic nuclear structure. *J. Cell Biol.* 120:841–852.

Dietz, H. 1998. Polishing the cutting edge of gems. *Nat. Genet.* 20:321–322.

Ferreira, J., M. Carmo-Fonseca, and A.I. Lamond. 1994. Differential interaction of splicing snRNPs with coiled bodies and interchromatin granules during mitosis and assembly of daughter nuclei. *J. Cell Biol.* 126:11–23.

Ferreira, J., and M. Carmo-Fonseca. 1995. The biogenesis of the coiled body during early mouse embryogenesis. *Development (Camb.).* 121:601–612.

Fey, E.G., G. Krochmalnic, and S. Penman. 1986. The nonchromatin substructures of the nucleus: the ribonucleoprotein (RNP)-containing and RNP-depleted matrices analyzed by sequential fractionation and resinless section electron microscopy. *J. Cell Biol.* 102:1654–1665.

Fischer, U., E. Darzynkiewicz, S.M. Tahara, N.A. Dathan, R. Lührmann, and I.W. Mattaj. 1991. Diversity in the signals required for nuclear accumulation of U snRNPs and variety in the pathways of nuclear transport. *J. Cell Biol.* 113:705–714.

Fischer, U., Q. Liu, and G. Dreyfuss. 1997. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell.* 90:1023–1029.

Fornerod, M., M. Ohno, M. Yoshida, and I.W. Mattaj. 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell.* 90:1051–1060.

Frey, M.R., and A.G. Matera. 1995. Coiled bodies contain U7 small nuclear RNA and associate with specific DNA sequences in interphase human cells. *Proc. Natl. Acad. Sci. USA.* 92:5915–5919.

Frey, M.R., A.D. Bailey, A.M. Weiner, and A.G. Matera. 1999. Association of snRNA genes with coiled bodies is mediated by nascent snRNA transcripts. *Curr. Biol.* 9:126–135.

Fukuda, M., S. Asano, T. Nakamura, M. Adachi, M. Yoshida, M. Yanagida, and B. Nishida. 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature.* 390:308–311.

Fwu, J.-K., and P.M. Djuriç. 1996. Unsupervised vector image segmentation by a tree structure-ICM algorithm. *IEEE (Inst. Electr. Electron Eng.) Trans. Biomed. Eng.* 15:871–880.

Gall, J.G., E.C. Stephenson, H.P. Erba, M.O. Diaz, and G. Barsacchi-Pilone. 1981. Histone genes are located at the sphere loci of newt lampbrush chromosomes. *Chromosoma.* 84:159–171.

Gall, J., M. Bellini, Z. Wu, and C. Murphy. 1999. Assembly of the nuclear transcription and processing machinery: Cajal bodies (coiled bodies) and transcriptosomes. *Mol. Biol. Cell.* In press.

Gao, L., M.R. Frey, and A.G. Matera. 1997. Human genes encoding U3 snRNA associate with coiled bodies in interphase cells and are clustered on chromosome 17p11.2 in a complex inverted repeat structure. *Nucleic Acids Res.* 25: 4740–4747.

Habets, W.J., M.H. Hoet, B.A.W. DeJong, A. VanDerKemp, and W. VanVenrooij. 1989. Mapping of B cell epitopes on small nuclear ribonucleoproteins that react with human autoantibodies as well as with experimentally-induced mouse monoclonal antibodies. *J. Immunol.* 143:2560–2566.

Hardin, J.W., S.S. Spicer, and W.B. Green. 1969. The paranucleolar structure, accessory body of Cajal, sex chromatin, and related structures in nuclei of rat trigeminal neurons: a cytochemical and ultrastructural study. *Anat. Rec.* 164: 403–432.

Hervás, J.P., J. Villegas, D. Crespo, and M. Lafarga. 1980. Coiled bodies in supraoptic nucleus of the rat hypothalamus during the postnatal period. *Am. J. Anat.* 159:447–454.

Huang, S., and D.L. Spector. 1992. U1 and U2 small nuclear RNAs are present in nuclear speckles. *Proc. Natl. Acad. Sci. USA.* 89:305–308.

Huber, J., U. Cronshagen, M. Kadokura, C. Marshallsay, T. Wada, M. Sekine, and R. Lührmann. 1998. Snurportin1, an m3G-cap–specific nuclear import receptor with a novel domain structure. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:4114–4126.

Isaac, C., Y. Yang, and U.T. Meier. 1998. Nopp140 functions as a molecular link between the nucleolus and the coiled bodies. *J. Cell Biol.* 142:319–329.

Izaurralde, E., and I.W. Mattaj. 1992. Transport of RNA between nucleus and cytoplasm. *Semin. Cell Biol.* 3:279–288.

Izaurralde, E., A. Jarmolowski, C. Beisel, I.W. Mattaj, G. Dreyfuss, and U. Fischer. 1997. A role for the M9 transport signal of hnRNP A1 in mRNA nuclear export. *J. Cell Biol.* 137:27–35.

Jiménez-Garcia, L.F., M.L. Segura-Valdez, R.L. Ochs, L.I. Rothblum, R. Hannan, and D.L. Spector. 1994. Nucleogenesis: U3 snRNA-containing prenucleolar bodies move to sites of active pre-rRNA transcription after mitosis. *Mol. Biol. Cell.* 5:755–966.

Krämer, A. 1996. The structure and function of proteins involved in mammalian pre-mRNA splicing. *Annu. Rev. Biochem.* 65:367–409.

Lafarga, M., J.P. Hervas, M.C. Santa-Cruz, J. Villegas, and D. Crespo. 1983. The accessory body of Cajal in the neuronal nucleus. A light and electron microscopic approach. *Anat. Embryol.* 166:19–30.

Lafarga, M., M.T. Berciano, L.M. Garcia-Seguro, M.A. Andres, and M. Carmo-Fonseca. 1998. Acute osmotic/stress stimuli induce a transient decrease of transcriptional activity in the neurosecretory neurons of supraoptic nuclei. *J. Neurocytol.* 27:205–217.

Lamond, A.I., and W.C. Earnshaw. 1998. Structure and function in the nucleus. *Science.* 280:547–553.

Lefebvre, S., L. Bürglen, S. Reboullet, O. Clermont, P. Burlet, L. Viollet, B. Benichou, C. Cruaud, P. Millasseau, M. Zeviani, et al. 1995. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell.* 89:155–165.

Lefebvre, S., P. Burlet, Q. Liu, S. Bertrandy, O. Clermont, A. Munnich, G. Dreyfuss, and J. Melki. 1997. Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat. Genet.* 16:265–269.

Lefebvre, S., L. Bürglen, J. Frézal, A. Munnich, and J. Melki. 1998. The role of the SMN gene in proximal spinal muscular atrophy. 1998. *Hum. Mol. Genet.* 7:1531–1536.

Lerner, E.A., M.R. Lerner, L.A. Janeway, and J.A. Steitz. 1981. Monoclonal antibodies to nucleic acid containing cellular constituents: probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA.* 78:2737–2741.

Liu, Q., and G. Dreyfuss. 1996. A novel nuclear structure containing the survival of motor neurons protein. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:3555–3565.

Liu, Q., U. Fischer, F. Wang, and G. Dreyfuss. 1997. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell.* 90:1013–1021.

Lorson, C., and E.J. Androphy. 1998. The domain encoded by exon 2 of the survival motor neuron protein mediates nucleic acid binding. *Hum. Mol. Genet.* 7:1269–1275.

Lorson, C., J. Strasswimmer, J.M. Yao, J.D. Baleja, E. Hahnen, B. Wirth, L. Thanh, A.H.M. Burghes, and E.J. Androphy. 1998. SMN oligomerization defect correlates with spinal muscular atrophy. *Nat. Genet.* 19:63–66.

Lührmann, R. 1988. snRNP proteins. *In* Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. M. Birnstiel, editor. Springer Verlag, New York, NY. 71–99.

Lührmann, R., B. Kastner, and M. Bach. 1990. Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochim. Biophys. Acta.* 1087: 265–292.

Lyon, C.E., K. Bohmann, J. Sleeman, and A.I. Lamond. 1997. Inhibition of protein dephosphorylation results in the accumulation of splicing snRNPs and coiled bodies within the nucleolus. *Exp. Cell Res.* 230:84–93.

Malatesta, M., C. Zancanaro, T.E. Martin, E.K.L. Chan, F. Almaric, R. Lührmann, P. Vogel, and S. Fakan. 1994. Cytochemical and immunocytochemical characterization of nuclear bodies during hibernation. *Eur. J. Cell Biol.* 65: 82–93.

Marshallsay, C., and R. Lührmann. 1994. In vitro nuclear import of snRNPs: cytosolic factors mediate m3G-cap dependence of U1 and U2 snRNP transport. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:222–231.

Matera, A.G., and D.C. Ward. 1993. Nucleoplasmic organization of small nuclear ribonucleoproteins in cultured human cells. *J. Cell Biol.* 121:715–727.

Matera, A.G., and M.R. Frey. 1998. Coiled bodies and gems: Janus or Gemini? *Am. J. Hum. Genet.* 63:317–321.

Matera, A.G. 1999. Nuclear bodies: multifaceted subdomains of the interchromatin space. *Trends Cell Biol.* 9:302–309.

Mattaj, I.W. 1986. Cap trimethylation of U snRNA is cytoplasmic and dependent on U snRNP protein binding. *Cell.* 46:905–911.

Mattaj, I.W. 1988. U snRNP assembly and transport. *In* Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. M. Birnstiel, editor. Springer Verlag, New York, NY. 100–114.

Meier, U.T., and G. Blobel. 1994. NAP57, a mammalian nucleolar protein with a putative homolog in yeast and bacteria. *J. Cell Biol.* 127:1505–1514.

Michaud, N., and D. Goldfarb. 1992. Microinjected U snRNAs are imported to oocyte nuclei via the nuclear pore complex by three distinguishable targeting pathways. *J. Cell Biol.* 116:851–861.

Monneron, A., and W. Bernhard. 1969. Fine structural organization of the interphase nucleus in some mammalian cells. *J. Ultrastruct. Res.* 27:266–288.

Narayanan, A., W. Speckmann, R. Terns, and M.P. Terns. 1999. Role of the box C/D motif in localization of small nucleolar RNAs to coiled bodies and nucleoli. *Mol. Biol. Cell.* 10:2131–2147.

Ochs, R.L., T.W. Stein, Jr., and E.M. Tan. 1994. Coiled bodies in the nucleolus of breast cancer cells. *J. Cell Sci.* 107:385–399.

Ossareh-Nazari, B., F. Bachelerie, and C. Dargemont. 1997. Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science.* 278:141–144.

Pellizzoni, L., N. Kataoka, B. Charroux, and G. Dreyfuss. 1998. A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell.* 95:615–624.

Ramón y Cajal, S.R. 1903. Un sencillo método de coloracion selectiva del reticulo protoplasmico y sus efectos en los diversos organos nerviosos de vertebrados e invertebrados. *Trab. Lab. Invest. Biol.* 2:129–221.

Raska, I., R.L. Ochs, L.E.C. Andrade, E.K.L. Chan, R. Burlingame, C. Peebles, D. Gruol, and E.M. Tan. 1990. Association between the nucleolus and the coiled body. *J. Struct. Biol.* 104:120–127.

Raska, I., L.E.C. Andrade, R.L. Ochs, E.K.L. Chan, C.-M. Chang, G. Roos, and E.M. Tan. 1991. Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. *Exp. Cell Res.* 195:27–37.

Rebelo, L., F. Almeida, C. Ramos, K. Bohmann, A.I. Lamond, and M. Carmo-Fonseca. 1996. The dynamics of coiled bodies in the nucleus of adenovirus-infected cells. *Mol. Biol. Cell.* 7:1137–1151.

Reddy, R., and H. Busch. 1988. Small nuclear RNAs: RNA sequences, structure and modifications. *In* Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles. M. Birnstiel, editor. Springer Verlag, New York, NY. 1–37.

Reuter, R., B. Appel, P. Bringmann, J. Rinke, and R. Lührmann. 1984. 5′-Terminal caps of snRNAs are reactive with antibodies specific for 2,2,7-trimethylguanosine in whole cells and nuclear matrices. *Exp. Cell Res.* 154:548–560.

Samtama, N., C.G. Doti, and A.I. Lamond. 1996. Neuronal differentiation in the rat hippocampus involves a stage-specific reorganization of subnuclear structure both in vivo and in vitro. *Eur. J. Neurosci.* 8:892–905.

Schrank, B., R. Gotz, J.M. Gunnersen, J.M. Ure, K.V. Toyka, A.G. Smith, and M. Sendtner. 1997. Inactivation of the survival motor neuron gene, a candidate for human spinal muscular atrophy, leads to massive cell death in early mouse embryos. *Proc. Natl. Acad. Sci. USA.* 94:9920–9925.

Sleeman, J.E., and A.I. Lamond. 1999a. Nuclear organization of pre-mRNA splicing factors. *Curr. Opin. Cell Biol.* 11:372–377.

Sleeman, J.E., and A.I. Lamond. 1999b. Newly assembled snRNPs associate with coiled bodies before speckles, suggesting a nuclear snRNP maturation pathway. *Curr. Biol.* 9:1065–1074.

Smith, K., K. Carter, C. Johnson, and J. Lawrence. 1995. U2 and U1 snRNA gene loci associate with coiled bodies. *J. Cell. Biochem.* 59:473–485.

Spector, D.L., G. Lark, and S. Huang. 1992. Differences in snRNP localization between transformed and nontransformed cells. *Mol. Biol. Cell.* 3:555–569.

Stade, K., C.S. Ford, C. Guthrie, and K. Weis. 1997. Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell.* 90:1041–1050.

Vankan, P., C. McGuigan, and I.W. Mattaj. 1990. Domains of U4 and U6 snRNAs required for snRNP assembly and splicing complementation in *Xenopus* oocytes. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1075–1081.

Wolff, B., J.-J. Sanglier, and Y. Wang. 1997. Leptomycin B is an inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. *Chem. Biol.* 4:139–147.

Wu, C.-H.H., and J.G. Gall. 1993. U7 small nuclear RNA in C snurposomes of the *Xenopus* germinal vesicle. *Proc. Natl. Acad. Sci. USA.* 90:6257–6259.

Wu, Z., C. Murphy, C-H.H. Wu, A. Tsvetkov, and J.G. Gall. 1993. Snurposomes and coiled bodies. *Cold Spring Harbor Symp. Quant. Biol.* 58:747–754.

Wu, C., C. Murphy, and J.G. Gall. 1996. The Sm binding site targets U7 snRNA to coiled bodies (spheres) of amphibian oocytes. *RNA (NY).* 2:811–823.

Yu, Y.-T., M.-D. Shu, and J.A. Steitz. 1998. Modifications of U2 snRNA are required for snRNP assembly and pre-mRNA splicing. *EMBO (Eur. Mol. Biol. Organ.) J.* 17:5783–5795.