A novel nuclear structure containing the survival of motor neurons protein

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Spinal muscular atrophy (SMA) is a common, often fatal, autosomal recessive disease leading to progressive muscle wasting and paralysis as a result of degeneration of anterior horn cells of the spinal cord. A gene termed survival of motor neurons (SMN), at 5q13, has been identified as the determining gene of SMA (Lefebvre et al., 1995). The SMN gene is deleted in >98% of SMA patients, but the function of the SMN protein is unknown. In searching for hnRNP-interacting proteins we found that SMN interacts with the RGG box region of hnRNP U, with itself, with fibrillarin and with several novel proteins. We have produced monoclonal antibodies to the SMN protein, and we report here on its striking cellular localization pattern. Immuno-localization studies using SMN monoclonal antibodies show several intense dots in HeLa cell nuclei. These structures are similar in number (2–6) and size (0.1–1.0 µm) to coiled bodies, and frequently are found near or associated with coiled bodies. We term these prominent nuclear structures gems, for Gemini of coiled bodies.
Keywords: gems/hnRNP/spinal muscular atrophy/survival of motor neurons protein

Introduction

Spinal muscular atrophy (SMA) is a severe neuromuscular disorder which results in the loss of motor neurons in the spinal cord (Roberts et al., 1970; Pearn, 1973, 1978; Czeizel and Hamula, 1989). It is a common fatal autosomal recessive disease affecting 1 in 6000 newborns. Two recently discovered genes, the survival of motor neurons (SMN) gene (Lefebvre et al., 1995) and the neuronal apoptosis inhibitor protein (NAIP) gene (Roy et al., 1995), have been suggested as the gene responsible for SMA (Lewin, 1995). The SMN gene is localized to human chromosome 5q13 (Melki et al., 1994; Lefebvre et al., 1995). This region contains a 500 kb inverted repeat and a copy of the gene is found in each of these repeating units. The two copies are identical except for five silent base changes. The centromeric copy (°BCD541) can undergo alternative splicing of exon 7, resulting in a putative protein with a different C-terminus (Lefebvre et al., 1995). The telomeric SMN gene is deleted in >98% of SMA patients (Lefebvre et al., 1995). The cDNA of the SMN gene encodes a putative protein of 294 amino acids with a predicated mol. wt of ~32 kDa. The function

of the protein is unknown, and its sequence reveals no significant homology to any other proteins in the database. Studies of the SMN protein at the molecular and cellular level have not been described.

HnRNP proteins are a group of abundant nuclear proteins that bind pre-mRNAs and nuclear mRNAs and play important roles in the processing and transport of mRNAs (Dreyfuss et al., 1993). They contain several RNA binding motifs, including the RNP motif, KH domains and the RGG box (Burd and Dreyfuss, 1994), and appear to interact both with RNA directly and with each other. In the course of experiments to identify and clone proteins that interact with hnRNP U, we used the RGG box-containing region near the carboxyl-terminus of the hnRNP U protein (Piñol-Roma et al., 1988; Kiledjian and Dreyfuss, 1992; Dreyfuss et al., 1993) as bait in the yeast two-hybrid screen and have isolated several cDNAs encoding the SMN protein (Lefebvre et al., 1995). Subsequent experiments confirmed this interaction and further revealed interactions between SMN and additional proteins, including fibrillarin—the small nucleolar RNA binding protein (Lischwe et al., 1985; Aris and Blobel, 1988; Tollervey et al., 1991). Using antibodies that we have generated to the SMN protein, we have determined the cellular localization of this protein. Immunolocalization of the SMN protein revealed a novel nuclear structure. This structure, which we call gems, is located in close proximity to coiled bodies (for reviews, see Brasch and Ochs, 1992; Lamond and Carmo-Fonseca, 1993; Gall et al., 1995), often appearing to interact directly with them. In addition to its presence in the discrete dot-like gems, SMN is also found in the cytoplasm. Interestingly, the number, size and response to metabolic conditions that gems and coiled bodies exhibit are similar. Both of these structures also disassemble and reassemble during the cell cycle in a similar fashion. This suggests that gems have functions related to that of coiled bodies. Coiled bodies are conserved subnuclear structures found in both plant and animal cells which were first discovered in 1903 (Ramon y Cajal, 1903). Although the specific functions of coiled bodies are as yet unknown, their components suggest that they have roles in the metabolism of snRNPs and thus in pre-mRNA processing. The observations made in this report suggest that gems may also have a function in RNA metabolism. This, in turn, suggests that SMA may result from a defect in a nuclear post-transcriptional mechanism of RNA metabolism.

Results

The SMN protein interacts with the RGG region of hnRNP U, with itself and with fibrillarin

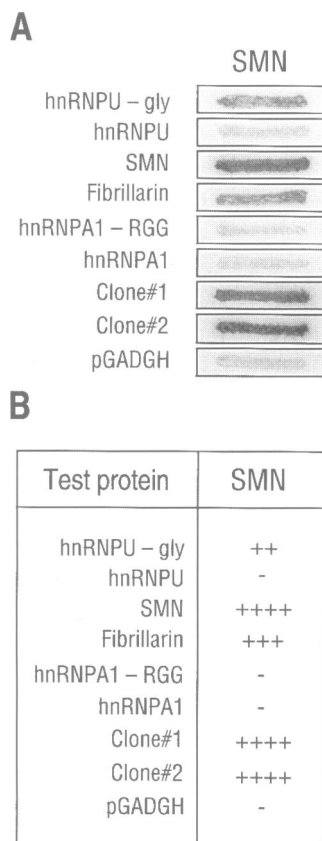In a search for proteins that interact with the hnRNP U protein, we screened a HeLa cDNA library using the

A

SMN

hnRNPU – gly	
hnRNPU	
SMN	
Fibrillarin	
hnRNPA1 – RGG	
hnRNPA1	
Clone#1	
Clone#2	
pGADGH	

B

Test protein	SMN
hnRNPU – gly	++
hnRNPU	–
SMN	++++
Fibrillarin	+++
hnRNPA1 – RGG	–
hnRNPA1	–
Clone#1	++++
Clone#2	++++
pGADGH	–

Fig. 1. SMN protein interacts with hnRNP U-Gly, fibrillarin and SMN itself. (**A**) β-Galactosidase assays in the yeast two-hybrid system showing interaction between SMN protein and test proteins. All interaction assays were performed with full-length SMN fused to the Gal4 DNA binding domain against test proteins fused to the Gal4 activation domain, except for hnRNP U-Gly (amino acids 687–806) where these sequences were fused to the Gal4 DNA binding domain against full-length SMN fused to the activation domain. (**B**) Summary table of the yeast two-hybrid result.

RGG box-containing carboxy-terminal region (amino acids H687 to Y806) of this protein (U-Gly) (Kiledjian and Dreyfuss, 1992; Piñol-Roma et al., 1988; Dreyfuss et al., 1993) as bait in the yeast two-hybrid system. Several human cDNA clones encoding U-Gly-interacting proteins were isolated. Three of these corresponded to the same mRNA, and all three cDNAs were identical to the full-length sequence of the telomeric copy of the SMN gene (Lefebvre et al., 1995). To understand this interaction further, one of these SMN clones was used as a bait to screen the HeLa library for SMN-interacting proteins. Several of the SMN-interacting proteins that were isolated include SMN itself, fibrillarin and two novel proteins, designated Clone #1 and #2, whose complete sequence and further characterization will be described elsewhere. Interestingly, of the five SMN clones isolated from this latter screen, three are the product of the telomeric SMN gene. The other two are the alternatively spliced centromeric ᶜBCD541 gene lacking exon 7. A summary of the yeast two-hybrid experiments is shown in Figure 1. SMN interacts with hnRNP U-Gly; however, it could not be demonstrated to interact with full-length hnRNP U. We, therefore, do not know presently if SMN protein and hnRNP U interact in vivo. The data demonstrate that SMN can interact with itself and with fibrillarin. Fibrillarin

is a 34 kDa RNA binding protein that is associated with several nucleolar small RNAs including U3, U8 and U13 (Tyc and Steiz, 1989; Baserga et al., 1991). It is an evolutionarily highly conserved protein that contains an RNP motif and an RGG box (Lischwe et al., 1985; Aris and Blobel, 1988; Tollervey et al., 1991; Burd and Dreyfuss, 1994), and it is found in the dense fibrillar compartment of the nucleolus and in coiled bodies (Raska et al., 1990). Genetic experiments in Saccharomyces cerevisiae indicate that fibrillarin is required for pre-rRNA processing and that it is essential for viability (Jansen et al., 1991; Tollervey et al., 1991), but its specific functions are not known. The hnRNP A1 RGG-containing region (Burd and Dreyfuss, 1994) and full-length A1 were also tested for interaction with SMN as controls and they showed no interaction in this assay.

Monoclonal antibodies 2B1 and 18I6 are specific for the SMN protein

To examine the interaction of these RNA binding proteins with SMN and to characterize SMN further, we generated monoclonal antibodies to the SMN protein by immunizing mice with purified recombinant His6-tagged SMN protein. Two hybridomas, 2B1 and 18I6, were selected for the following experiments. Several lines of evidence demonstrated that the monoclonal antibodies obtained from these hybridomas indeed recognize the SMN protein specifically. First, both 2B1 and 18I6 efficiently immunoprecipitated the SMN protein which was produced by in vitro transcription and translation from the SMN cDNA (Figure 2A). In contrast, neither 2B1 nor 18I6 immunoprecipitated proteins produced similarly from the mRNAs of hnRNP A1 or hnRNP C1, two of the most abundant nuclear RNA binding proteins. Furthermore, control antibodies such as SP2/0 did not immunoprecipitate the SMN protein. Second, the monoclonal antibody 2B1 reacts efficiently with purified His6–SMN but not with another similarly produced and purified His6-tagged RNA binding protein, ROX2, on an immunoblot (Figure 2B). Third, immunoprecipitations from total [^{35}S]methionine-labeled HeLa material using 2B1 and 18I6 revealed a single specifically immunoprecipitated protein whose size corresponds to the mobility of in vitro translated SMN (Figure 2C). Finally, immunoblotting of total HeLa material with 2B1 and 18I6 also shows a single band whose size is similar to that seen in the above mentioned experiments. In addition, the immunoblotting experiments revealed cross-reacting bands of similar mobility to SMN by SDS–PAGE in mouse and Xenopus laevis (Figure 2D). We, therefore, conclude that both 2B1 and 18I6 are specific for the SMN protein, and that likely homologs are found in other vertebrate organisms.

Immunolocalization of the SMN protein reveals a novel nuclear structure

Use of the anti-SMN monoclonal antibodies 2B1 and 18I6 in immunofluorescence microscopy experiments to determine the cellular localization of SMN produced a striking result. In HeLa cells, a human cervical carcinoma epithelial cell line, the 2B1 antibody stained several prominent dot-like structures in the nucleus, and there was a weak but significant staining throughout the cytoplasm (Figure 3A and B). Other anti-SMN monoclonal antibodies
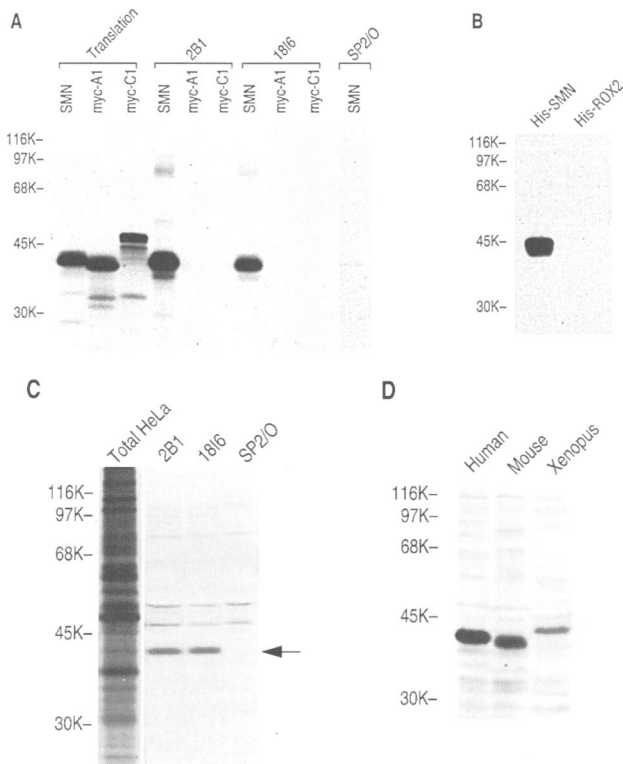
Fig. 2. Monoclonal antibodies 2B1 and 18I6 are specific for SMN protein. (**A**) Immunoprecipitation using monoclonal antibody 2B1 and 18I6 of the ^{35}S-labeled *in vitro* translated protein product of SMN, hnRNP A1 and hnRNP C1. Non-immune antibody SP2/0 does not precipitate SMN protein under the same conditions. (**B**) Immunoblotting using monoclonal antibody 2B1 on purified His–SMN and His–ROX2. (**C**) Immunoprecipitation using 2B1 and 18I6 from total HeLa extract. Both monoclonal antibodies immunoprecipitate a specific band of ~40 kDa corresponding to SMN protein as compared with immunoprecipitation with non-immune antibody SP2/0. (**D**) Immunoblotting using monoclonal antibody 2B1 on total HeLa extract, total mouse 3T3 cell extract and *Xenopus* cell line CCL102 extract demonstrates that SMN protein is conserved in vertebrates.

all stained the same pattern. A small percentage of cells (≤10%) was observed which did not show SMN staining of nuclear structures, but the vast majority of the cells stained nuclear dots. In mitotic cells, the staining was mostly diffuse throughout the cytoplasm, except very rarely where some small remnants of the dots were observed (Figure 3A, indicated by 'm'). In newly divided daughter cells, the antibody showed only diffuse cytoplasmic staining but no nuclear dot structures (data not shown), suggesting that there is a lag period for the nuclear dots to reassemble after mitosis. Double immunofluorescence experiments using the monoclonal antibody 2B12 to the hnRNP C proteins and the monoclonal antibody 2B1 to SMN showed that there was no significant staining of hnRNP C protein in the SMN-containing structures (Figure 3C). In many of the cells, the immunological staining of the SMN-containing nuclear structures corresponded to phase-dense bodies that could be seen by phase microscopy alone and also by differential interference contrast (DIC) light microscopy (Figure 3B and D, indicated by arrows), suggesting that the immunofluorescence staining visualizes *bona fide* nuclear structures. The photography in Figure 3A slightly exaggerates the cytoplasmic SMN signal, whereas the confocal micro-

graphy in this and subsequent figures slightly underestimates the cytoplasmic signal.

The SMN-containing nuclear structures are related to but different from coiled bodies

In their size and number per nucleus, these SMN-containing structures appeared to us to be reminiscent of coiled bodies (Ramon Y Cajal, 1903; reviewed in Lamond and Carmo-Fonseca, 1993). To determine whether the SMN protein in fact co-localizes with coiled bodies, we used two coiled body markers, monoclonal antibody Pδ against p80-coilin (Andrade *et al.*, 1991; M.Carmo-Fonseca, personal communication) and human auto-immune antibody against fibrillarin (J.Craft, personal communication), in indirect double immunofluorescence experiments with anti-SMN. For these experiments, we used laser confocal microscopy to monitor the fluorescence labeling of each antibody, as well as the DIC light microscopy image to show the outline of the cells and identify nuclear structures. In all experiments, 6–14 horizontal optical sections of each field were scanned from top to bottom, and in many of the experiments the results of stacked images of all these scanned sections are presented. All figures present the computer-superimposed image of fluorescein (FITC)- and Texas red-coupled secondary antibody labeling of different primary antibodies. Examples of indirect double label immunofluorescence microscopy with anti-SMN (2B1) and anti-coilin monoclonal antibody (Pδ), using isotype-specific secondary antibodies, are shown in Figure 4. These experiments demonstrate that the SMN-staining nuclear bodies are often found adjacent to coiled bodies but, importantly, they are not identical to coiled bodies. Interestingly, the number and size of SMN-staining bodies and coiled bodies are very similar and, in most cases in HeLa cells, they appear to partially overlap, perhaps contacting each other, or to be positioned very close to each other. Because these features make the SMN-staining bodies appear like twins of coiled bodies we refer to them as 'gems', for Gemini of coiled bodies. The size of gems varies; in most cases in HeLa cells they are somewhat smaller than coiled bodies and in some cases they are about the same size. Therefore, based on the size estimates of coiled bodies, i.e. 0.1–1.0 µm (Lamond and Carmo-Fonseca, 1993; Roth, 1995), the size of gems can be estimated to be in about the same range, with a slightly smaller average size in HeLa cells. Some cells do not show staining of coiled bodies (Carmo-Fonseca *et al.*, 1993) and some do not show staining of gems, but all HeLa cells showed cytoplasmic staining of SMN. Identical results to those described here were obtained with rabbit polyclonal anti-coilin antibodies in place of the anti-coilin monoclonal antibody (data not shown).

Coiled bodies can also be stained with anti-fibrillarin antibodies, which also stain the dense fibrillar component of nucleoli (Raska *et al.*, 1990). Indirect double label immunofluorescence staining with anti-SMN (2B1) and anti-fibrillarin human autoimmune antibody, using non-cross-reacting second antibodies, also demonstrated that coiled bodies and gems are distinct and separate structures (Figure 4C and D). As expected, double labeling with anti-p80-coilin and anti-fibrillarin showed complete co-localization in coiled bodies (Raska *et al.*, 1990; Figure
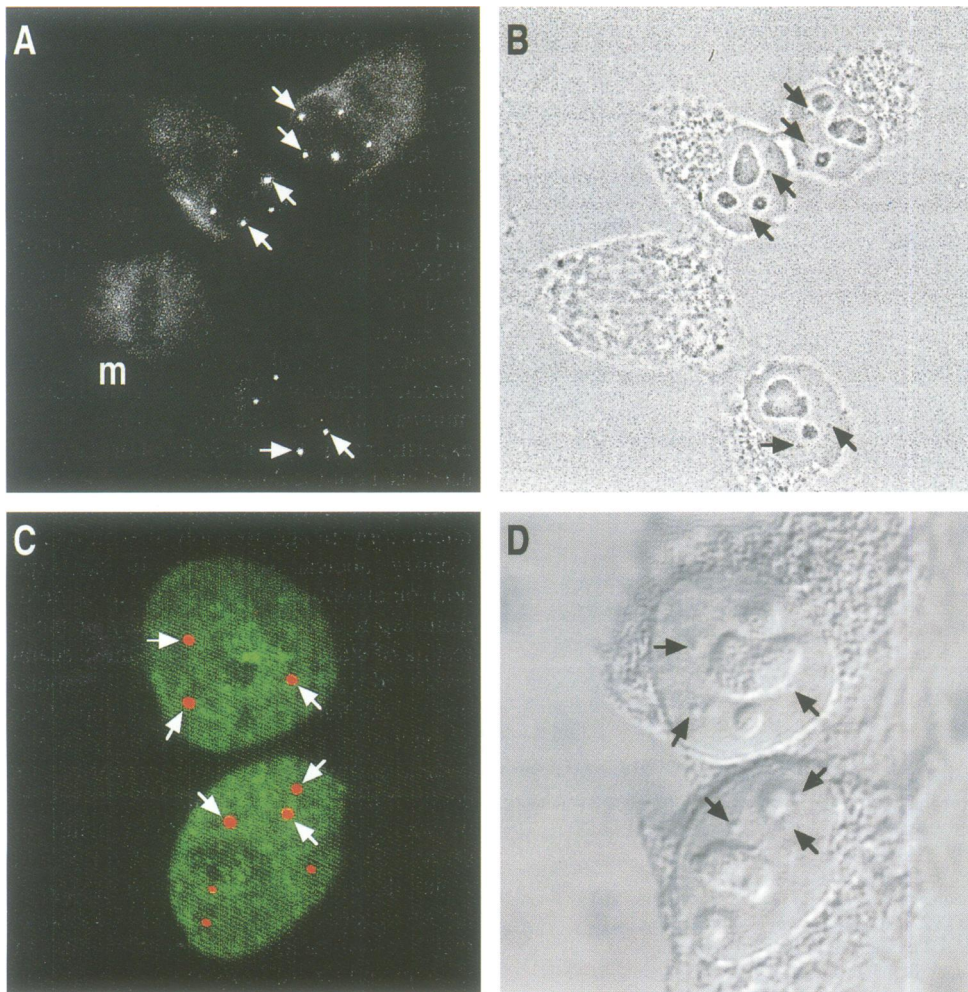
Fig. 3. SMN protein is found in discrete nuclear structures and in the cytoplasm. (**A**) The localization patterns of SMN proteins were determined by immunofluorescence analysis in a Zeiss light microscope with 63× objective using monoclonal antibody 2B1. The letter 'm' indicates mitotic cells. The result is the same using monoclonal antibody 18I6 (data not shown). (**B**) Phase contrast microscopy showing gems are light dense structures under the light microscope (arrow heads). (**C**) The computer overlay image of double labeling with anti-hnRNP C antibody 2B12 (green) and anti-SMN monoclonal antibody 2B1 (red). The corresponding DIC image is shown in (**D**).

4E and F). Thus, two coiled body markers, coilin and fibrillarin, are not found in gems, and SMN is not found in coiled bodies. However, these two structures often overlap and possibly contact each other. Once located by immunofluorescence with anti-SMN antibodies, we noted that gems are obviously visible by DIC alone. Using a computer-merged image of immunofluorescence and DIC we can readily identify gems on the DIC images, and some of these are indicated by arrows in Figure 4B and D.

Several antibodies to nuclear proteins have been described which stain discrete nuclear structures. These include PML bodies which contain ~20–30 small nuclear dots (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994), hnRNP I and the Ro antigens which react, in addition to general nucleoplasmic staining, with a discrete perinucleolar domain (Ghetti *et al.*, 1992; Matera *et al.*, 1995), and hnRNP L which stains, in addition to general nucleoplasmic staining, discrete nuclear loci possibly corresponding to amphibian lampbrush chromosomes giant loops (Piñol-Roma *et al.*, 1989). We have used antibodies to all of these proteins and found that none of their staining patterns resemble the staining pattern seen for gems (Q.Liu and G.Dreyfuss, unpublished results).

Gems and coiled bodies show similar responses to different metabolic conditions

Coiled bodies have been reported to become larger when cells are incubated at 32°C (Carmo-Fonseca *et al.*, 1993). To examine whether these conditions also affect the appearance of gems, we carried out immunofluorescence staining of HeLa cells after a 24 h incubation at 32°C, compared with control cells maintained at 37°C. The lower temperature incubation had a similar enlargement effect on gems as it had on coiled bodies (Figure 5A and C). Moreover, in contrast to 37°C when most gems localize very close to coiled bodies, at 32°C, in the majority of the cells, gems separate completely from coiled bodies (Figure 5).

The morphology of coiled bodies changes after actinomycin D treatment and appears clustered around nucleolar remnants (Raska *et al.*, 1990; Carmo-Fonseca *et al.*, 1993). We examined the effect of the same treatment on gems. Interestingly, at the lower level (0.04 µg/ml) of actinomycin D which inhibits RNA polymerase I transcription, the majority of the gems are not affected; only some of them co-localize with coiled bodies to form perinucleolar caps (data not shown). At higher actinomycin D concentrations that also inhibit RNA polymerase II transcription
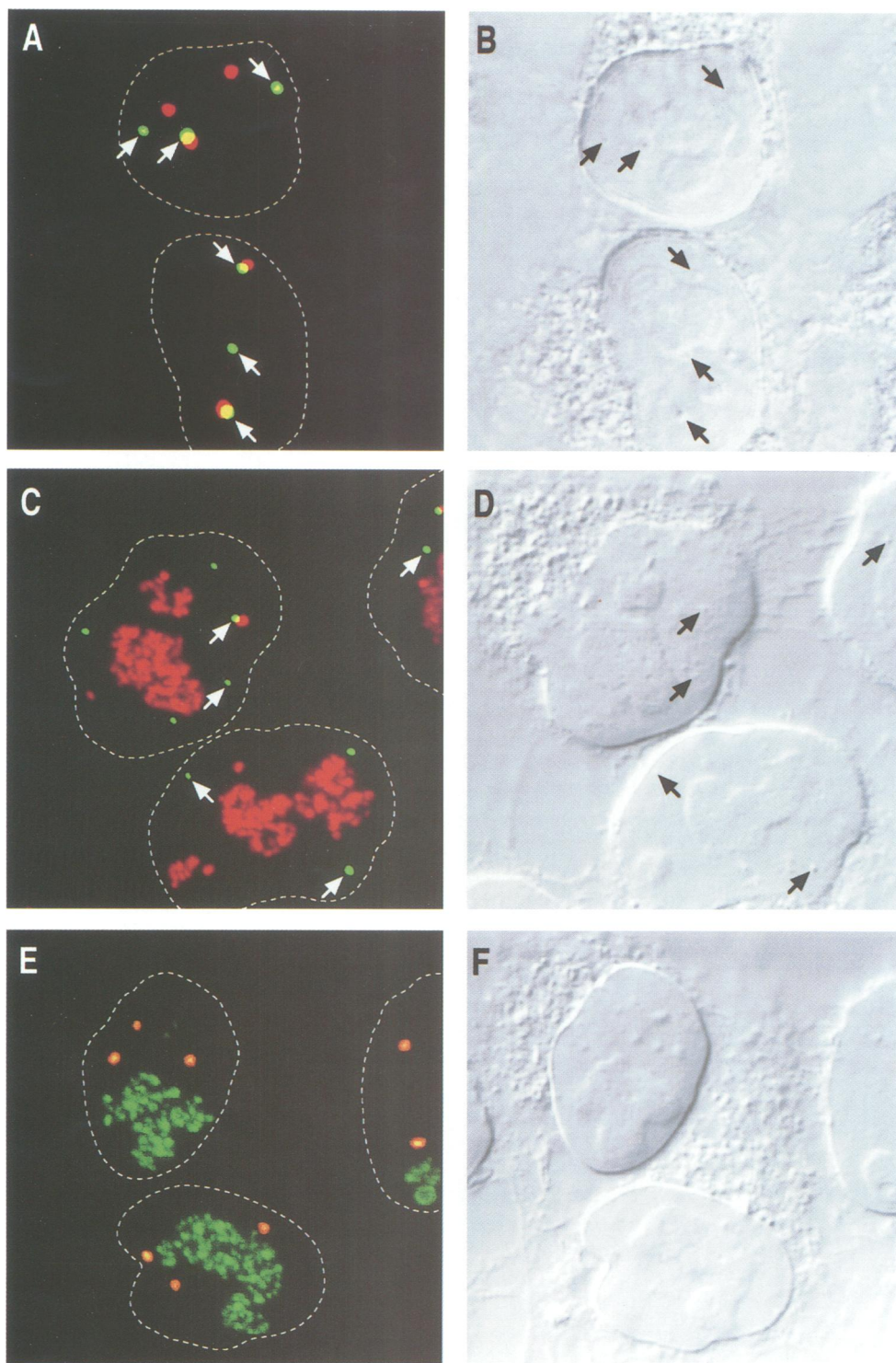
Fig. 4. Gems are a novel nuclear structure related to coiled bodies. The computer-superimposed confocal images of indirect double immunofluoresence experiments using HeLa cells with anti-SMN antibody 2B1 (green) and anti-p80-coilin antibody Pδ (red) in (**A**), anti-SMN antibody 2B1 (green) and anti-fibrillarin antibody 1881 (red) in (**C**) and anti-p80-coilin antibody R288 (red) and anti-fibrillarin antibody 1881 (green) in (**E**). The co-localization of red and green labeling results in a yellow color. The corresponding DIC images of (A), (C) and (E) are shown in (**B**), (**D**) and (**F**) respectively. Gems are indicated by arrows.

(5 μg/ml), the majority of gems disassembled and only small remnants can be seen (Figure 5E and F). However, the cytoplasmic SMN staining remained. These findings suggest that gems, like coiled bodies, are dynamic structures whose size and organization relative to that of other nuclear structures changes as the metabolic state of the cell changes, particularly in response to changes in RNA polymerase II activity.

Gems do not contain spliceosomal snRNPs

Coiled bodies contain, among other components, a high concentration of spliceosomal snRNPs (Fakan *et al.*, 1984;
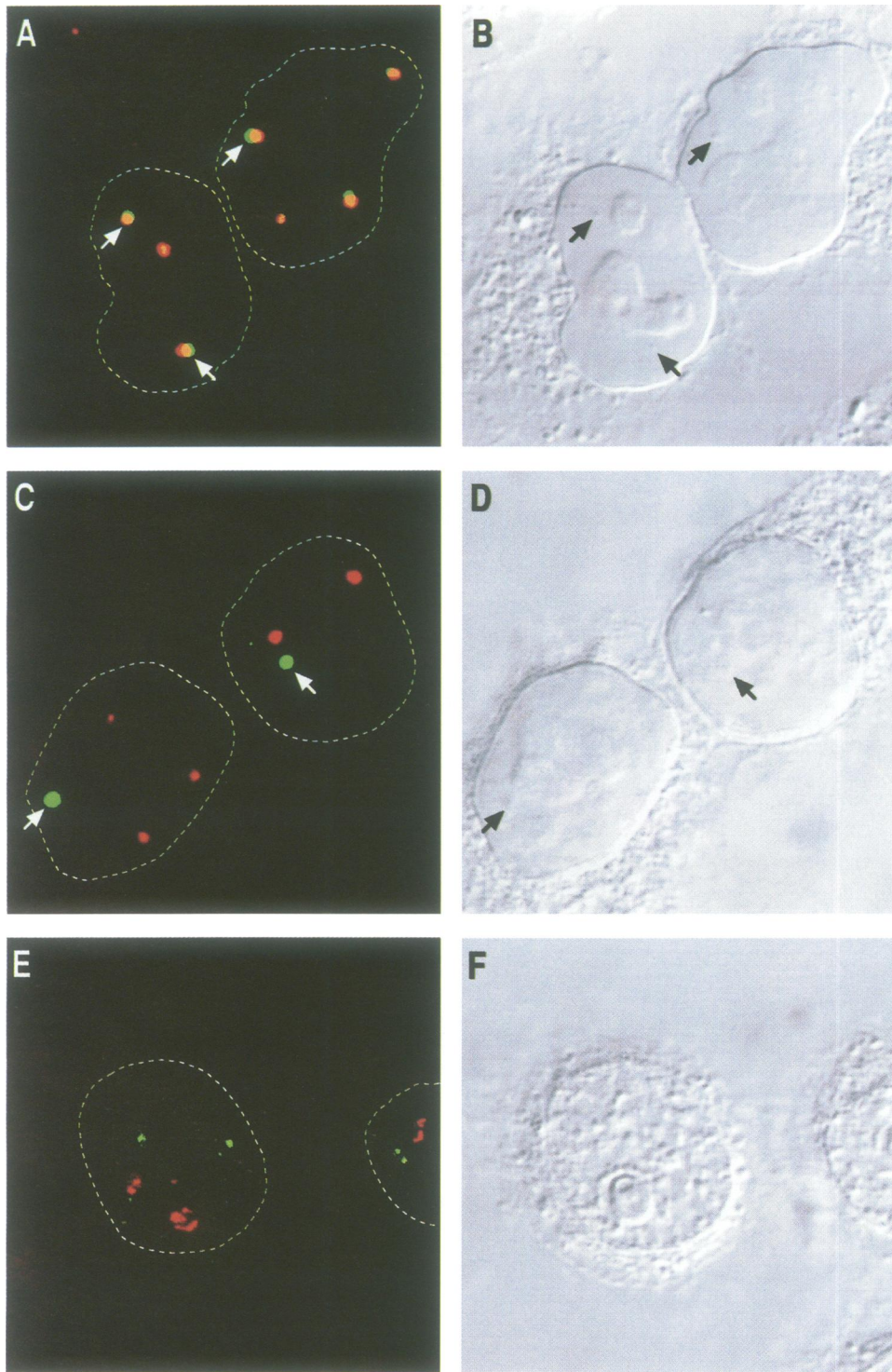
Fig. 5. Gems and coiled bodies respond similarly to different metabolic conditions. The computer-superimposed confocal images of indirect double immunofluorescence experiments using HeLa cells with anti-SMN antibody 2B1 (green) and anti-p80-coilin antibody Pδ (red) at 37°C (**A**), after incubation at 32°C for 24 h (**C**) and after treatment with 5 μg/ml actinomycin D for 3 h (**E**). The corresponding DIC images are shown in (**B**), (**D**) and (**F**) respectively. Gems are indicated by arrows.

Carmo-Fonseca *et al.*, 1992, 1993). Antibodies to proteins of spliceosomal snRNPs such as Y12 (Petterson *et al.*, 1984), which reacts with the Sm epitope found on many of these proteins, stain coiled bodies intensely and also exhibit a speckled nuclear pattern that corresponds to interchromatin granules (Spector, 1993). Double immuno-fluorescence microscopy with anti-SMN (2B1) and the

anti-snRNP monoclonal antibody Y12 showed that gems are found near, or associated with, snRNP-containing coiled bodies (Figure 6A). However, at 32°C, gems and coiled bodies become clearly separated and, importantly, no staining of snRNPs could be detected in gems (Figure 6B). As expected, double labeling with Y12 and anti-p80-coilin showed complete co-localization at both 32 and
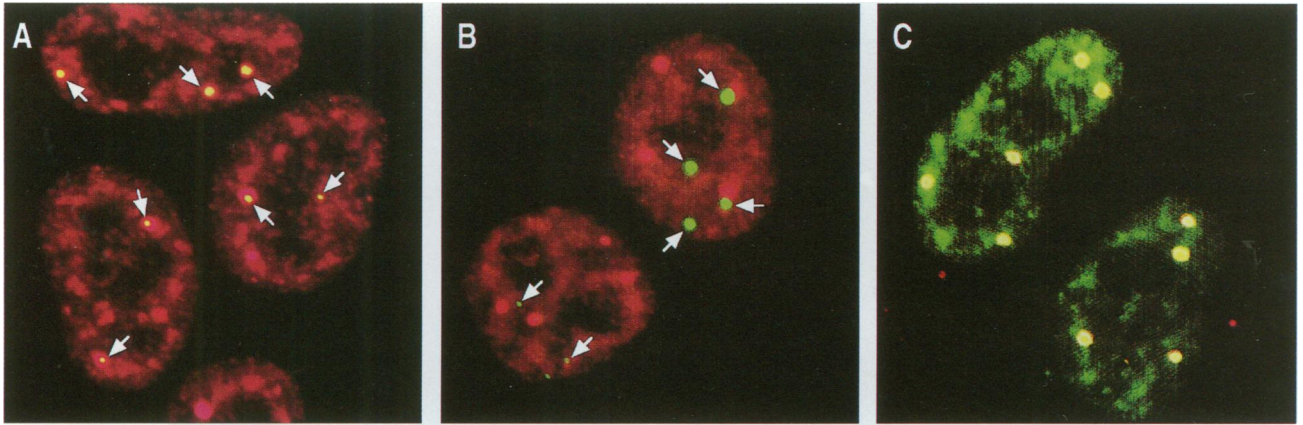
Fig. 6. Gems do not contain snRNPs. The same overlaid images using anti-SMN antibody 2B1 (green) and anti-Sm antibody Y12 (red) at 37°C (**A**) and 32°C (**B**). Gems separate completely from snRNP containing coiled bodies at 32°C. Labeling with anti-Sm antibody Y12 (green) and anti-p80-coilin antibody Pδ results in yellow foci at both 37°C (**C**) and 32°C (data not shown).

37°C (Carmo-Fonseca *et al.*, 1993, Figure 6C). We, therefore, conclude that although gems appear to be closely associated with coiled bodies and possibly interact with them, gems do not contain snRNPs, one of the hallmarks of coiled bodies.

Gems are a common nuclear structure

To determine whether gems are unique to HeLa cells or whether they are a common and general nuclear structure, we stained mouse fibroblast 3T3 cells with the anti-SMN antibody, 2B1, anti-p80-coilin and anti-fibrillarin (Figure 7). These experiments showed that 3T3 cells contain prominent gems, similar in number and size to those found in HeLa cells. The same cells, however, showed only an occasional coiled body, and these were not in close association with gems as is usually the case for HeLa cells. The cytoplasmic SMN staining was much more intense in 3T3 cells than seen in HeLa cells. In addition to the transformed cell lines, gems were also detected in primary human fibroblasts, although they were less prominent than in HeLa cells (data not shown). This observation is similar to that described for coiled bodies (Spector *et al.*, 1992; Carmo-Fonseca *et al.*, 1993). SMN mRNAs have been detected in all human tissues tested (Lefebvre *et al.*, 1995), and our findings here demonstrate that gems are likely to be a structure common to many, if not all, cell types in eukaryotes.

Discussion

Although it has been recognized for a long time that the cytoplasm of eukaryotic cells is a well-organized compartment containing many well-defined organelles, knowledge of the organization of the nucleus has been considerably lagging. Only very few structures of specific morphology and composition have been defined in the nucleus. The most prominent among the nuclear structures, the nucleoli, were first described >150 years ago (reviewed in Franke, 1988; Warner, 1990). Nucleoli, perhaps the only nuclear structure for which some functions are known, are the site of transcription and processing of pre-rRNAs, and the assembly of ribosomes also takes place within them (for review, see Gerbi *et al.*, 1990). Coiled bodies

were first described >90 years ago as 'nucleolar accessory bodies' by Ramon y Cajal (1903). They were later identified by electron microscopy as 'coiled bodies' (Hardin *et al.*, 1969; Monneron and Bernhard, 1969), but their function remains unknown. Several other smaller structures have been visualized in the nucleus by use of specific antibodies and, in some cases, also by electron microscopy (Brasch and Ochs, 1992). These include the numerous interchromatin granules (Fakan *et al.*, 1984; Spector *et al.*, 1992; Lamond and Carmo-Fonseca, 1993) which contain spliceosomal snRNP and several splicing factors, a perinucleolar compartment which contains Ro antigens, several snRNAs and hnRNP I (Ghetti *et al.*, 1992; Matera *et al.*, 1995) and PML bodies, also referred to as PODs or PML-oncogenic domains, which may contain several transcription factors (Dyck *et al.*, 1994; Koken *et al.*, 1994; Weis *et al.*, 1994). Discrete and characteristic nuclear staining patterns have also been observed by immunofluorescence microscopy for hnRNP L (Piñol-Roma *et al.*, 1989) and for hnRNP K (Siomi *et al.*, 1993). Little is known about these structures or about their functions, and in many cases it is not clear whether these are authentic discrete organelles or whether they represent merely transient aggregates. Here we describe a novel nuclear structure, termed gems. Gems are similar in number and size to coiled bodies and these two nuclear bodies are often found in close proximity to each other. Their size and discrete appearance, and the fact that they are visible by light microscopy, make gems one of the most prominent structures of the nucleus.

Coiled bodies and gems are frequently seen in close proximity, possibly in direct contact with each other (Figure 4). Both structures show a similar response to low temperature incubation and to transcriptional inhibitors (Figure 5). Coiled bodies are dynamic structures and their formation is regulated in interphase and mitosis (Andrade *et al.*, 1993; Carmo-Fonseca *et al.*, 1993). Our data suggest that gems also disassemble and reassemble during the cell cycle. These observations suggest a functional relationship of coiled bodies and gems. Additional studies, including electron microscopy, will be required to address these issues, and such studies are now under way. Although the exact functions of coiled bodies are not known,
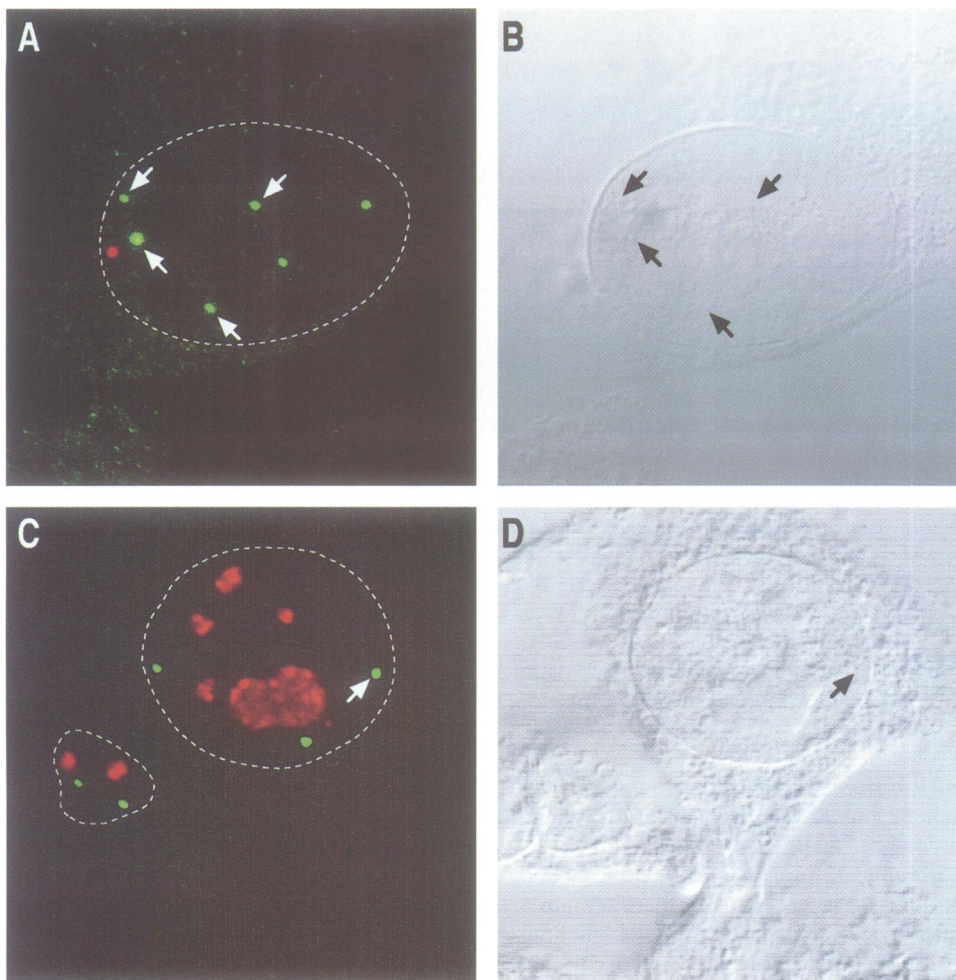
Fig. 7. Detection of gems in mouse NIH 3T3 cells. Double labeling with anti-SMN antibody 2B1 (green) and anti-coilin antibody Pδ (red) (**A**), and with 2B1 (green) and anti-fibrillarin antibody (red) (**C**) in mouse NIH 3T3 cells. The corresponding DIC images are shown in (**B**) and (**D**).

considerable information is available about their components and about their morphology, and these allow some speculations to be made about their likely roles. Coiled bodies are highly conserved in evolution and are found in both animal and plant cells (Ramon y Cajal, 1903). They were described originally as nucleolar accessory bodies because they are often observed at the periphery of nucleoli, especially in neurons. Consistent with a functional relationship between coiled bodies and nucleoli, they share several common constituents, including fibrillarin (Fakan *et al.*, 1984), Nopp140 (Meier and Blobel, 1992, 1994) and U3 snRNA (Raska and Dundr, 1993; Bauer *et al.*, 1994; Jimenez-Garcia *et al.*, 1994). However, rRNAs have not been detected in coiled bodies (Carmo-Fonseca *et al.*, 1993; Jimenez-Garcia *et al.*, 1994). In addition, coiled bodies have been reported to contain major spliceosomal snRNPs (U1, U2, U4/U6 and U5) and the splicing factor U2AF, but not the splicing factor SC-35 or pre-mRNAs (Carmo-Fonseca *et al.*, 1992, 1993). These findings suggest that coiled bodies are not likely to be involved directly in either pre-rRNA or pre-mRNA processing, but it is possible that they have roles in the metabolism of small nuclear RNAs which are, in turn, essential for these processes. The possible involvement of gems in RNA metabolism is underscored by the response of gems to transcriptional inhibitors. The interaction of

SMN with fibrillarin, a component of coiled bodies, detected by the yeast two-hybrid protein–protein interaction assay, suggests that gems may interact directly with coiled bodies through fibrillarin. As fibrillarin is also a component of the nucleolus and as coiled bodies are likely to interact with nucleoli (Raska *et al.*, 1990, 1991), it is also possible that gems have some functional relationship with nucleoli, although we did not consistently observe such an association in our studies so far.

A nuclear structure found in amphibian oocyte nuclei appears to be the counterpart of coiled bodies. This structure, the sphere organelle (Wu *et al.*, 1993; Gall *et al.*, 1995; Roth, 1995), contains the p80-coilin homolog SphI (Tuma *et al.*, 1993), as well as some components found in mammalian coiled bodies such as spliceosomal snRNPs and U7 snRNA (Wu and Gall, 1993; Frey and Matera, 1995). It has been suggested that the sphere organelle serves as a site of maturation of snRNPs (Wu *et al.*, 1993; Gall *et al.*, 1995). The cross-reactivity of the anti-SMN monoclonal antibody 2B1 with a polypeptide of similar size to SMN in *Xenopus* should make possible studies of gems in the oocyte of this organism and may help in understanding the function of this novel structure.

The marker that allowed us to find gems is the SMN protein (Lefebvre *et al.*, 1995). SMN is so far the only component of gems we know of, but it can be anticipated

that additional components of gems will soon be identified, and this may provide the necessary clues to understanding the functions of gems.

SMA is a common, often fatal, autosomal recessive disease (the second most common after cystic fibrosis), characterized by progressive degeneration of the anterior horn cells leading to symmetrical muscle weakness and progressive paralysis with muscular atrophy (Roberts *et al.*, 1970; Pearn, 1973, 1978; Czeizel and Hamula, 1989). It is a motor neuron disorder, and three types of SMA are recognized, based on age of onset and severity of muscle wasting and survivorship: type I, Werdnig–Hoffman disease; type II, an intermediate form; and type III, Kugelberg–Welander disease (Munsat, 1991). All three types of SMA map to chromosome region 5q11.2–q13.3. Two candidate genes for SMA have been determined within this region (Lefebvre *et al.*, 1995; Lewin, 1995; Roy *et al.*, 1995). One, named *SMN* (Lefebvre *et al.*, 1995) and the other, named *NAIP* (Roy *et al.*, 1995). The *SMN* gene has been mapped to 5q13, and detailed mapping of this region reveals that it is highly unstable and contains an inverted repeat of 500 kb. There is a copy of the gene in both the telomeric and centromeric repeating unit, and the copies are virtually identical except for five point mutations, none of which affect the coding of the protein sequence. Although both copies are expressed, it is unclear whether both are translated into functional proteins. In addition, the centromeric copy (*^{c}BCD541*) has an alternatively spliced isoform where exon 7 is deleted resulting in a putative protein with a different C-terminal end. Deletion of the telomeric *SMN* gene has now been reported in 98.6% of both juvenile and adult patients (Lefebvre *et al.*, 1995; Rodrigues *et al.*, 1995; Van der Steege *et al.*, 1995). Thus, there is a strong correlation between deletion of the *SMN* gene and SMA, and this deletion has been suggested to be the most reliable means of diagnosis of this disease (Lefebvre *et al.*, 1995; Rodrigues *et al.*, 1995; Wirth *et al.*, 1995). Interestingly, three out of five clones isolated in our yeast two-hybrid screen using *SMN* as a bait are the telomeric *SMN* product. The other two are the truncated version of centromeric *^{c}BCD541*. Experiments are under way to determine whether both copies are expressed and, if they are, whether the centromeric and telomeric isoforms localize differently in the cell.

The monoclonal antibodies to SMN may provide an important aid in the diagnosis of SMAs. The amino acid sequence of SMN did not provide any clues as to its possible functions and no characterization of the protein has been obtained previously. Thus, the localization of SMN and the identification of interacting proteins that we report here provide the beginning of the molecular and cellular characterization of this protein. It will be of particular interest to examine gems in SMA patients' cells. The protein–protein interaction studies revealed an interaction of SMN with RNA binding proteins, and gems respond to changes in RNA polymerase II transcription. These findings, and the close association of gems with coiled bodies, suggest that gems may have a function in RNA metabolism. This, in turn, suggests that SMA may result from a defect in nuclear post-transcriptional RNA metabolism. Further characterization of the SMN protein and of the novel proteins with which it interacts may lead

to an understanding of both the molecular mechanism of SMA and of the function of gems.

Materials and methods

Yeast two-hybrid interaction screening
The human HeLa cDNA library, yeast strains and yeast plasmids pGBT9, pGADGH, pVA3 and pTD1 were from Clontech Inc. The manipulation of yeast and the library screening were carried out according to the conditions suggested by the manufacturer. Briefly, the coding region of interest was cloned into the pGBT9 vector. The *S.cerevisiae* HF7c strain was first transformed with the pGBT9 derived construct and subsequently with the HeLa cDNA library. Approximately 6×10^6 transformants were seeded on eight 150 mm plates containing synthetic medium lacking histidine, leucine and tryptophan. His$^+$ colonies were grown on synthetic medium plates lacking leucine and tryptophan and then assayed for β-galactosidase activity by filter assay as described by the manufacturer. Of 6 million transformants screened, for the U-Gly screen, 81 were His$^+$ LacZ$^+$ colonies; and for the SMN screen, 146 were positive. The library plasmid was recovered from these clones into *Escherichia coli* HB101 strain. True positive clones were confirmed by their ability to transactivate *HIS3* and *LacZ* reporters when co-transforming HF7c with pGBT containing U-Gly or SMN.

Production of monoclonal antibodies to SMN
Anti-SMN antibodies 2B1 and 18I6 were prepared by immunizing A/J mice with His6-tagged SMN protein purified from Ni$^+$ chelation chromatography using the Novagen His-Bind Buffer Kit. Hybridoma production and screening and ascites fluid production were performed as previously described (Choi and Dreyfuss, 1984).

Production of proteins in vitro
The ^{35}S-labeled proteins were produced by an *in vitro* transcription-translation reaction (Promega Biotech) in the presence of [^{35}S]methionine (Amersham). His6–SMN fusion protein and His6–ROX2 were expressed from a pET bacterial expression system in the *E.coli* strain BL21(DE3)-pLysS and purified using Ni$^+$ chelation chromatography using the same kit as described above.

Cell culture and treatments
HeLa cells and NIH 3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO BRL, Gaithersburg, MD) supplemented with 10% fetal calf serum (FCS; GIBCO BRL). Low temperature incubations were carried out as follows. HeLa cells were shifted to 32°C and incubated for 24 h prior to fixation and permeabilization for immunostaining. For actinomycin D treatment, HeLa cells were incubated with 5 μg/ml of actinomycin D for 3 h before fixation for immunostaining. For *in vivo* labeling with [^{35}S]methionine, 50% confluent HeLa cells growing in 100 mm plates were incubated with 10 μCi/ml [^{35}S]methionine in DMEM without methionine supplemented with 10% FCS overnight before homogenization for immunoprecipitation.

Immunoprecipitations and immunoblotting
Immunoprecipitations were carried out in the presence of 1% Empigen BB buffer as previously described (Choi *et al.*, 1984).

For immunoblotting, proteins were resolved on an SDS–polyacrylamide (12.5%) gel and transferred to a nitrocellulose membrane (Schelicher & Schuell, Inc., Keene, NH) using a BioTrans Model B Transblot apparatus (Gelman Science) according to the manufacturer's instructions. Filters were incubated in blotting solution [phosphate-buffered saline (PBS), 5% non-fat milk] for at least 1 h at room temperature, rinsed with cold PBS, and then incubated with primary antibody for at least 1 h at room temperature. Filters were washed three times in PBS containing 0.1% Tween 20 and bound antibodies were detected using the peroxidase-conjugated goat anti-mouse IgG + IgM (Jackson ImmunoResearch Laboratories). The protein bands were visualized by an ECL Western blotting detection kit (Amersham) after washing three times in PBS containing 0.1% Tween 20.

Immunofluorescence microscopy
Immunofluorescence microscopy was carried out essentially as previously described (Choi and Dreyfuss, 1984) with the following exceptions. Primary monoclonal antibodies 2B1 and 18I6 were diluted 1:1000 in PBS containing 3% bovine serum albumin. The incubations with the first and second antibody were at room temperature for 1 h. In double

immunofluorescence experiments, primary or secondary antibodies were incubated at the same time.

Laser confocal fluorescence microscopy was performed with a Leica TCS 4D (Gemany) confocal microscope.

Antibodies used for these experiments were as follows, antibody against p80-coilin: monoclonal antibody Pδ (M.Carmo-Fonseca, personal communication) and rabbit poly serum R288 (Andrade *et al.*, 1993), antibody against fibrillarin: human autoimmune antibody 1881 (J.Craft personal communication).

References

Andrade,L.E.C., Chan,E.K.L., Raska,I., Peebles,C.L., Roos,G. and Tan,E.M. (1991) Human autoantibody to a novel protein of the nuclear coiled body: immunological characteriaztion and cDNA cloning of p80-coilin. *J. Exp. Med.*, **173**, 1407–1419.

Andrade,L.E.C., Tan,E.M. and Chan,E.K. (1993) Immunocytochemical analysis of the coiled body in the cell cycle and during cell proliferation. *Proc. Natl Acad. Sci. USA*, **90**, 1947–1951.

Aris,J.P. and Blobel,G. (1988) Identification and characterization of a yeast nucleolar protein that is similar to a rat liver nucleolar protein. *J. Cell Biol.*, **107**, 17–31.

Baserga,S.J., Yang,X.W. and Steitz,J.A. (1991) An intact Box C sequence in the U3 snRNA is required for binding of fibrillarin, the protein common to the major family of nucleolar snRNPs. *EMBO J.*, **10**, 2645–2651.

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

Brasch,K. and Ochs,R. (1992) Nuclear bodies (Nbs): a newly 'rediscovered' organelle. *Exp. Cell Res.*, **202**, 211–223.

Burd,C.G. and Dreyfuss,G. (1994) Conserved structure and diversity of functions of RNA-binding proteins. *Science*, **265**, 615–621.

Carmo-Fonseca,M., Pepperkok,R., Carvalho,M.T. and Lamond,A.I. (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., Ferreira,J. and Lamond,A.I. (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.

Choi,Y.D. and Dreyfuss,G. (1984) Monoclonal antibody characterization of the C proteins of heterogeneous nuclear ribonucleoprotein complexes in vertebrate cells. *J. Cell. Biol.*, **99**, 1997–2004.

Czeizel,A. and Hamula,J. (1989) A Hungarian study on Werdnig–Hoffmann disease. *J. Med. Genet.*, **26**, 761–763.

Dreyfuss,G., Matunis,M.J., Piñol-Roma,S. and Burd,C.G. (1993) HnRNP proteins and the biogenesis of mRNA. *Annu. Rev. Biochem.*, **62**, 289–321.

Dyck,J.A., Maul,G.G., Miller,W.J., Chen,J.D., Kakizuka,A. and Evans,R.M. (1994) A novel macromolecular structure is a target of the promyelocte-retinoic acid receptor concoprotein. *Cell*, **76**, 333–343.

Fakan,S., Leser,G. and Martin,T.E. (1984) Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. *J. Cell Biol.*, **98**, 358–363.

Franke,W.W. (1988) Matthias Jacob Schleiden and the definition of the cell nucleus. *Eur. J. Cell Biol.*, **47**, 145–156.

Frey,M.R. and Matera,A.G. (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.

Gall,J.G., Tsvetkov,A., Wu,Z.A. and Murphy,C. (1995) Is the sphere organelle coiled body a universal nuclear component. *Dev. Genet.*, **16**, 25–35.

Gerbi,S.A., Savino,R., Stebbino-Boaz,B., Jeppesen,C. and Rivera-Leon,R. (1990) A role for U3 small nucleolar ribonuclear protein in the nucleolus? In Hill,W.E., Dahlberh,A., Garrett,R.A., Moore,P.B., Schlessinger,D. and Warner,J.R. (eds), *The Ribosome—Structure, Function and Evolution*. American Society for Microbiology, Washington, DC, pp. 452–468.

Ghetti,A., Piñol-Roma,S., Michael,W.M., Morandi,C. and Dreyfuss,G. (1992) hnRNP I, the polypyrimidine tract-binding proteins: distinct nuclear localization and association with hnRNAs. *Nucleic Acids Res.*, **20**, 3671–3678.

Hardin,J.H., Spicerr,S.S. and Greene,W.B. (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.

Jansen,R.P., Hurt,E.C., Kern,H., Lehtonen,H., Carmo-Fonseca,M., Lapeyere,B. and Tollervey,D. (1991) Evolutionary conservation of the human nucleolar protein fibrillarin and its functional expression in yeast. *J. Cell Biol.*, **113**, 715–729.

Jimenez-Garcia,L.F., Segura-Valdez,M. de L., Ochs,R.L., Rothblum,L.I., Hannan,R. and Spector,D.L. (1994) Nucleologenesis: U3 snRNA-containing prenuclear bodies move to sites of active pre-rRNA transcription after mitosis. *Mol. Biol. Cell*, **5**, 955–966.

Kiledjian,M. and Dreyfuss,G. (1992) Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. *EMBO J.*, **11**, 2655–2664.

Koken,M.H. *et al.* (1994) The t(15;17) translocation alters a nuclear body in a retinoic acid-reversible fashion. *EMBO J.*, **13**, 1073–1083.

Lamond,A.I. and Carmo-Fonseca,M. (1993) *Trends Cell Biol.*, **3**, 198–204.

Lefebvre,S. *et al.* (1995) Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*, **80**, 155–165.

Lewin,B. (1995) Genes for SMA: multum in parvo. *Cell*, **80**, 1–5.

Lischwe,M.A., Ochs,R.L., Reddy,R., Cook,R.G., Yeoman,L.C., Tan,E.M., Reichlin,M. and Busch,H. (1985) Purification and partial characterization of a nucleolar scleroderma antigen. *J. Biol. Chem.*, **260**, 14304–14310.

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

Matera,A.G., Frey,M.R., Margelot,K. and Wolin,S.L. (1995) A perinucleolar compartment contains several RNA-polymerase III transcripts as well as the polypyrimidine tract-binding protein, hnRNP I. *J. Cell Biol.*, **129**, 1181–1193.

Meier,U.T. and Blobel,G. (1992) Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell*, **70**, 127–138.

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

Melki,J. *et al.* (1994) De novo and inherited deletions of the 5q13 region in spinal muscular atrophies. *Science*, **264**, 1474–1477.

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

Munsat,T.L. (1991) Workshop report: international SMA collaboration. *Neuromusc. Disord.*, **1**, 81.

Pearn,J. (1973) The gene frequency of acute Werdnig–Hoffmann disease (SMA type I): a total population survey in north-east England. *J. Med. Genet.*, **10**, 260–265.

Pearn,J. (1978) Incidence, prevalence and gene frequency studies of chronic childhood spinal muscular atrophy. *J. Med. Genet.*, **15**, 409–413.

Petterson,I., Hinterberger,M., Mimori,T., Gottlieb,E. and Steitz,J.A. (1984) The structure of mammalian small nuclear ribonucleoproteins. Identification of multiple protein components reactive with anti-(U1) ribonucleoprotein and anti-Sm autoantibodies. *J. Biol. Chem.*, **259**, 5907–5814.

Piñol-Roma,S., Choi,Y.D., Matunis,M.J. and Dreyfuss,G. (1988) Immunoprecipitation of heterogeneous nuclear ribonucleoprotein particles reveals an assortment of RNA-binding proteins. *Genes Dev.*, **2**, 215–227.

Piñol-Roma,S., Swanson,M.S., Gall,J.G. and Dreyfuss,G. (1989) A novel heterogeneous nuclear RNP protein with a unique distribution on nascent transcripts. *J. Cell Biol.*, **109**, 2575–2587.

Ramon y Cajal,S.R. (1903) Un sencillo metodo de coloracion seletiva del reticulo protoplasmico y sus efectos en los diversos organos nerviosos de vertebrados y invertebrados. *Trab. Lab. Invest. Biol.*, **2**, 129–221.

Raska,I. and Dundr,M. (1993) Compartmentalization of the cell nucleus: case of the nucleolus. *Chromosomes Today*, **11**, 101–119.

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

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

Roberts,D.G., Chavez,J. and Court,S.D.M. (1970) The genetic component in child mortality. *Arch. Dis. Child.*, **45**, 33–38.

Rodrigues,N.R., Owen,N., Talbot,K., Ignatius,J., Dubowitz,V. and Davies,K.E. (1995) Deletions in the survival motor neuron gene on 5q13 in autosomal recessive spinal muscular atrophy. *Hum. Mol. Genet.*, **4**, 631–634.

Roth,M.B. (1995) Spheres, coiled bodies and nuclear bodies. *Curr. Opin. Cell Biol.*, **7**, 325–328.

Roy,N. *et al.* (1995) The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell*, **80**, 167–178.

Siomi,H., Matunis,M.J., Michael,W.M. and Dreyfuss,G. (1993) The pre-mRNA binding K protein contains a novel evolutionarily conserved motif. *Nucleic Acids Res.*, **21**, 1193–1198.

Spector,D.L. (1993) Macromolecular domains within the cell nucleus. *Annu. Rev. Cell Biol.*, **9**, 265–315.

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

Tollervey,D., Lehtonen,H., Carmo-Fonseca,M. and Hurt,E.C. (1991) The small nucleolar RNP protein NOP1 (fibrillarin) is required for pre-rRNA processing in yeast. *EMBO J.*, **10**, 573–583.

Tuma,R.S., Stolk,J.A. and Roth,M.B. (1993) Identification and characterization of a sphere organelle protein. *J. Cell Biol.*, **122**, 767–773.

Tyc,K. and Steitz,J.A. (1989) U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus. *EMBO J.*, **8**, 3113–3119.

Van der Steege,G., Grootscholten,P.M., Van der Vlies,P., Draaijers,T.G., Osinga,J., Cobben,J.M., Scheffer,H. and Buys,C.H. (1995) PCR-based DNA test to confirm clinical diagnosis of autosomal recessive spinal muscular atrophy. *Lancet*, **345**, 985–986.

Warner,J.R. (1990) The nucleolus and ribosome formation. *Curr. Opin. Cell Biol.*, **2**, 521–527.

Weis,K., Rambaud,S., Lavau,C., Jansen,J., Carvalho,T., Carmo-Fonseca,M., Lamond,A. and Dejean,A. (1994) Retinoic acid regulates aberrant nuclear localization of PML–RAR α in acute promyelocytic leukemia cells. *Cell*, **76**, 345–356.

Wirth,B., Hahnen,E., Morgan,K., Didonato,C.J., Dadze,A., Rudnik-Schoneborn,S., Simard,L.R., Zerres,K. and Burghes,A.H.M. (1995) Allelic association and deletions in autosomal recessive proximal spinal muscular atrophy: association of marker genotype with disease severity and candidate cDNAs. *Hum. Mol. Genet.*, **4**, 1273–1284.

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

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