snRNP protein expression enhances the formation of Cajal bodies containing p80-coilin and SMN

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SUMMARY

Splicing snRNPs (small nuclear ribonucleoproteins) are essential sub-units of the spliceosome. Here we report the establishment of stable cell lines expressing fluorescently tagged SmB, a core snRNP protein. Analysis of these stable cell lines has allowed us to characterize the nuclear pathway that leads to snRNP accumulation in nuclear speckles and has identified a limiting nucleolar step in the pathway that can be saturated by overexpression of Sm proteins. After nuclear import, newly assembled snRNPs accumulate first in a subset of Cajal bodies that contain both p80-coilin and the survival of motor neurons protein (SMN) and not in bodies that contain p80-coilin but lack SMN. Treatment of cells with leptomycin B (LMB) inhibits both the accumulation of snRNPs in nuclear bodies and their subsequent accumulation in speckles. The formation of Cajal bodies is enhanced by Sm protein expression and the assembly of new snRNPs. Formation of heterokaryons between HeLa cell lines expressing Sm proteins and primary cells that usually lack Cajal bodies results in the detection of Cajal bodies in primary cell nuclei. Transient over-expression of exogenous SmB alone is sufficient to induce correspondingly transient Cajal body formation in primary cells. These data indicate that the level of snRNP protein expression and snRNP assembly, rather than the expression levels of p80-coilin or SMN, may be a key trigger for Cajal body formation.

Key words: snRNPs, Cajal bodies, SMN, p80-coilin, leptomycin B

INTRODUCTION

Cajal bodies (also called coiled bodies) are nuclear structures of varying number and size found both in cultured cell lines and in vivo in plant and animal cells (Bohmann et al., 1995; Matera, 1999; Gall, 2000). The autoantigen p80-coilin is commonly used as a molecular marker for the Cajal body (Andrade et al., 1991). Nuclear antigens shown to colocalize with p80 coilin in Cajal bodies include basal transcription factors, cell cycle factors, splicing snRNPs and nucleolar factors including snoRNPs (Gall et al., 1999; Matera, 1999). There is also evidence that Cajal bodies can associate with specific gene loci, including histone and U2 snRNA gene clusters (Callan et al., 1991; Gao et al., 1997; Matera and Ward, 1993; Schul et al., 1999a; Schul et al., 1998; Smith et al., 1995a; Smith et al., 1995b). Association of Cajal bodies with a tandem array of U2 snRNA genes was shown to depend on U2 snRNA expression from this locus (Frey et al., 1999). Consequently, roles for the Cajal body in a number of nuclear processes have been proposed, including transport, regulation of gene expression and the assembly of macromolecular complexes (Gall, 2000; Gall et al., 1999; Matera, 1999; Schul et al., 1999b).

The Cajal body contains splicing snRNPs, which are subunits of the spliceosome. It does not contain non-snRNP protein splicing factors or nascent pre-mRNA, however, and the snRNP components appear to be specifically newly assembled particles (Carvalho et al., 1999; Narayanan et al., 1999; Sleeman and Lamond, 1999). Splicing snRNPs are RNA-protein complexes, comprising a central core of snRNA together with a number of common (Sm) proteins and additional snRNP-specific proteins such as U1A and U2B″. Four of the five spliceosomal snRNAs, (U1, U2, U4 and U5) are transcribed in the nucleus, then exported to the cytoplasm where the 5′ cap is hypermethylated to form a characteristic 2,2,7-tri-methyl guanosine (TMG) cap (Fischer et al., 1993; Hamm et al., 1990; Hermann et al., 1995; Lehmeier et al., 1994; Lerner and Steitz, 1979; Lührmann et al., 1990; Mattaj, 1986; Nagai and Mattaj, 1994; Raker et al., 1996). The Sm proteins also bind in the cytoplasm and trigger re-import of the snRNP into the nucleus (Kambach et al., 1999).

The bulk of splicing snRNPs accumulate in interchromatin granule clusters (IGCs or speckles) where other non-snRNP splicing factors such as ASF/SF2 and SC-35 are also found, and in a diffuse nucleoplasmic pool, which probably includes snRNPs actively involved in splicing of nascent pre-mRNA. In a small percentage of cells (~3% of HeLa cells), snRNPs are also found within the nucleolus (Lyon et al., 1997; Sleeman et al., 1998). Using Sm proteins fused to fluorescent protein tags to label newly assembled snRNPs, we have recently identified a temporal pathway of snRNP localization in the nucleus in which they accumulate first in Cajal bodies, then in nucleoli and only later accumulate in speckles (Sleeman and Lamond, 1999).

The survival of motor neurons protein (SMN), implicated in the pathology of the inherited neurodegenerative disease spinal muscular atrophy (SMA), is a component of Cajal bodies both in vivo and in cultured cells, although in some cell lines it is also found in separate bodies termed ‘gems’ (gemini of coiled bodies) (Liu and Dreyfuss, 1996; Matera and Frey, 1998;

Young et al., 2000). SMN directly interacts with Sm proteins and forms a complex in the cytoplasm that includes Sm and other proteins. SMN plays an important cytoplasmic role in the maturation of splicing snRNPs (Charroux et al., 1999; Charroux et al., 2000; Liu et al., 1997; Pellizzoni et al., 1999). In the nucleus, SMN has been proposed to have a direct role in splicing pre-mRNA, possibly involving recycling of splicing complexes (Fischer et al., 1997; Pellizzoni et al., 1998).

In this study, we have analyzed the nuclear localization of newly imported splicing snRNPs in mammalian cells using stable cell lines expressing Sm proteins tagged with either yellow or cyan fluorescent proteins (YFP or CFP). The results indicate that newly imported snRNPs selectively localize in a subset of Cajal bodies that contain the SMN protein and that their subsequent accumulation in speckles can be blocked by leptomycin B. Furthermore, transient expression of Sm proteins is sufficient to cause Cajal body formation in primary cells.

MATERIALS AND METHODS

Plasmid constructs

pEYFP-SmB and pEGFP-SmD1 have been described elsewhere (Sleeman and Lamond, 1999). The SMN cDNA was cloned from a HeLa cDNA library (Clontech) by PCR using the enzyme KlenTaq polymerase (Clontech), according to the manufacturers instructions. PCR primers were designed (24 mers) to the N- and C-termini of the proteins' cDNA sequence using a previously deposited sequence for this cDNA in the GenBank database (accession no. U18423). The N-terminal and C-terminal sequence primers contained *Bam*HI and *Eco*RI sites, respectively, added to their 5′ ends. The PCR products were purified on a Qiagen PCR purification column according to the manufacturers instructions. Purified PCR products were digested with the appropriate enzymes and cloned using standard methods into the compatible sites (*Bgl*II and *Eco*RI) of the vector pEGFP-C1 (Clontech). After testing for the presence of insert SMN cDNA by restriction digests of miniprep (Qiagen) plasmid cDNA clones, the DNA was subsequently sequenced on both strands to confirm the identity of the cloned gene. Plasmid DNA sequencing was performed on an ABI 377 automated DNA sequencer (Perkin Elmer) using the BIG dye terminator cycle sequencing method according to the manufacturer's instructions.

Cell culture and transfection assays

HeLa cells were obtained from ATCC. Strain HeLa (D) was a gift from Prof. David Lane. DFSF1 cells were a gift from Dr Sonia Lain. Cells were grown in Dulbecco's modified Eagles' medium supplemented with 10% fetal calf serum and 100U/ml penicillin and streptomycin (Life Technologies Ltd). For immunofluorescence assays, cells were grown on coverslips and transfected (if necessary) using Effectene transfection reagent (Qiagen) according to the manufacturers instructions. For the preparation of cell lysates, cells were grown in 10 cm diameter dishes.

Establishment of stable cell lines

HeLa cells were grown in 10 cm diameter dishes and transfected using Effectene transfection reagent (Qiagen). 24 hours after transfection, cells were put under selection with G418 at 200 μg/ml in DMEM supplemented with 10% FCS. After 2 weeks of selection, resistant colonies were picked and screened for expression of the fluorescently tagged proteins. Clones showing acceptable levels of expression were seeded at clonal density, grown under selection for a further 2 weeks, then resistant colonies picked. These were screened by fluorescence microscopy and colonies showing expression of the tagged proteins in all cells were identified for expansion as cell lines.

Preparation of cell lysates and immunoblotting

Cells were washed twice with ice-cold PBS and then lysed in 0.5 ml of ice-cold 50 mM Tris-HCl pH 7.5; 0.5 M NaCl; 1% (v/v) Nonidet P-40; 1% (w/v) sodium deoxycholate; 0.1% (w/v) SDS; 2 mM EDTA plus complete protease inhibitor cocktail (Roche, one tablet per 25 ml). The lysate was passed through a Qiashredder column (Qiagen) to break up the DNA and then cleared by centrifugation for 15 minutes at 4°C and 13,000 ***g***. Lysates were electrophoresed on an 8% SDS-polyacrylamide gel and transferred to nitro-cellulose membranes for immunoblotting. Primary antibodies used were anti-GFP mouse monoclonal (Roche), Y12 anti-Sm mouse monoclonal (Pettersson et al., 1984), rabbit serum 856 anti-U1A (Hamm et al., 1990), mouse monoclonal 4G3 anti-U2B″ (Habets et al., 1989), mouse monoclonal Ab-1 anti-TMG (Calbiochem) and mouse monoclonal MANSMA1 anti-SMN (Young et al., 2000).

Immunoprecipitation of snRNP complexes

Lysates from HeLa cells expressing EGFP-SmD1 were pre-cleared by incubation with agarose beads on a shaking platform for 1 hour, then incubated for 1 hour on a shaking platform with 20 μl agarose beads coupled to anti-TMG antibodies (Calbiochem) or control, agarose beads. The beads were washed with 20 mM Hepes pH 7.9; 420 mM NaCl; 1.5 mM $MgCl_2$; 0.2 mM EDTA; 10% glycerol. The beads and equal amounts of cleared input lysate and unbound lysate were separated on an 8% SDS-polyacrylamide gel and transferred to nitro-cellulose membranes for immunoblotting with anti-GFP and anti-Sm (Y12) antibodies.

Fixation and immunofluorescence

Cells were washed in PBS and fixed for 5 minutes in 3.7% (w/v) paraformaldehyde in PBS at room temperature. Permeabilization was performed with 1% Triton X-100 in PBS for 15 minutes at room temperature. Cells were incubated with primary antibodies diluted in PBS with 1% goat serum for 35 minutes to 1 hour, washed 3×10 minutes with PBS, incubated for 35 minutes to 1 hour with the appropriate secondary antibodies diluted in PBS with 1% goat serum and washed 3×10 minutes with PBS. Antibodies used were Y12 mAb anti-Sm (Pettersson et al., 1984) (dilution 1:500), MANSMA1 mAB anti-SMN, 4G3 mAb anti-U2B″ (Habets et al., 1989), Ab-1 mAb anti-TMG (Calbiochem), and 204,10 rabbit polyclonal anti-p80 coilin (Bohmann et al., 1995) (dilution 1:350). TRITC-conjugated goat anti-mouse and Cy5 conjugated goat anti-rabbit secondary antibodies were used (Jackson Immunochemicals).

Microscopy

Confocal fluorescence microscopy of fixed cells was carried out using a Zeiss LSM 410 confocal laser-scanning microscope. Excitation wavelengths of 488 nm (EGFP/EYFP), 543 nm (TRITC) and 633 nm (Cy5) were used. Deconvolved digital images of fixed and live cells were recorded using a Zeiss Delta Vision Restoration microscope (Applied Precision Inc.) equipped with a 3D motorized stage and a Photometrics CH350 camera as described previously (Platani et al., 2000).

Quantitation of fluorescence signals

Quantitation of the proportion of fluorescence in Cajal bodies was carried out using an intensity threshold on stacks of deconvolved z-sections. A segmentation algorithm was used to find 'objects' by applying a global threshold to each stack of z-sections. Objects were defined as sets of contiguous pixels above the specified threshold. Where Cajal bodies could not be distinguished from bright speckles automatically by the segmentation algorithm, objects determined by their shape to be speckles, rather than Cajal bodies, were removed manually. The same segmentation algorithm was used to identify and

quantify speckles as opposed to diffuse nucleoplasmic background. In each case, the total fluorescence contained in each compartment was expressed as a percentage of the total fluorescence within the stack of z-sections following a correction to allow for background fluorescence within the sample.

Microinjection of FP-Sm constructs

Plasmid DNA was diluted to 20 μg/ml with 100 mM glutamic acid pH 7.2 (with citric acid); 140 mM KOH; 1 mM $MgSO_4$ and 1 mM DTT prior to injection into living MCF-7 or HeLa cells using an Eppendorf 5242 microinjector.

Heterokaryon formation

Cells expressing fluorescent proteins were mixed in a ratio of 1:2 with the parental HeLa cell line in 6 cm diameter petri dishes containing coverslips and cultured until 80-90% confluent. They were then fused using 50% polyethylene glycol-1000 solution (PEG) (hybrimax, Sigma). The culture medium was drained and 1.5 ml of 50% PEG added. The dishes were rocked gently for 90 seconds, then the PEG was washed away thoroughly using several changes of fresh culture medium (a modification of Spector et al.) (Spector et al., 1997).

RESULTS

Establishment of stable cell lines expressing YFPSmB or CFPSmB

Stable cell lines that constitutively express either YFPSmB, or CFPSmB were selected after transfecting HeLa cells with either pECFPSmB or pEYFPSmB (see Materials and Methods). Immunostaining confirmed that both the tagged EYFP-SmB and ECFPSmB fusion proteins showed an identical sub-cellular localization to endogenous Sm proteins, both in interphase cells (Fig. 1iA,B; and data not shown) and during mitosis (Fig. 1iC,D; and data not shown). During interphase the EYFP-SmB and ECFPSmB proteins are specifically nuclear and show the characteristic punctate pattern including speckles (Fig. 1iB,D, arrows) and Cajal bodies (Fig. 1iB, arrowheads). During mitosis, the tagged proteins are seen in speckles (Fig. 1iD, arrows). Both stable cell lines undergo mitosis at a similar frequency and divide at a similar rate to the parental HeLa cell line (Fig. 1iC,D; and data not shown).

To analyze expression levels of the fusion proteins, cells from lines EYFPSmBE1 and ECFPSmBE8.8 were lysed, separated by SDS PAGE and the proteins transferred to nitrocellulose membrane and detected using anti-FP antibodies and anti-Sm antibody Y12 (Fig. 1ii). A single, FP-positive band at 58 kDa, consistent with the expected size of YFP or CFP-tagged SmB, was detected in both cell lines (Fig. 1ii, lanes 2,4), but is absent from the parental HeLa line (lane 1). Expression of YFP alone in the parental HeLa line yields a single band of ~30 kDa. The Y12 antibody detected doublet bands of endogenous Sm proteins at 28/29 kDa (lanes 5-8) and the FP-tagged SmB band specifically in the FP-Sm cell lines (lanes 6-8). The levels of YFPSmB and CFPSmB are both lower than endogenous SmB, indicating that the tagged proteins are not overexpressed.

Analysis of snRNP localzation using a heterokaryon assay

EYFPSmBE1 cells were mixed with parental HeLa cells and fused with 50% polyethylene glycol (PEG). In the resulting heterokaryons EYFPSmBE1 and parental HeLa nuclei shared a cytoplasm. Cells fixed at different times after fusion showed synchronous uptake of YFPSmB into parental nuclei. At 30 minutes after fusion the YFP-SmB signal in parental HeLa cells was nuclear with the only sites of accumulation being specifically in Cajal bodies (Fig. 2iA-G, arrows). YFP-SmB first showed accumulation in speckles 3-4 hours after fusion (Fig. 2iC-G, arrowheads). At 16 hours after fusion, YFP-SmB in parental nuclei was indistinguishable from EYFP-SmBE1 nuclei (Fig. 2iG). Nucleolar accumulation of YFPSmB-tagged snRNPs was seen in only 5-10% of parental nuclei at the 3-9 hour time points (data not shown). At early time points, a 20-fold increase (P<0.05%) in the percentage of nuclear YFPSmB (2-3% of total) found in Cajal bodies was seen relative to

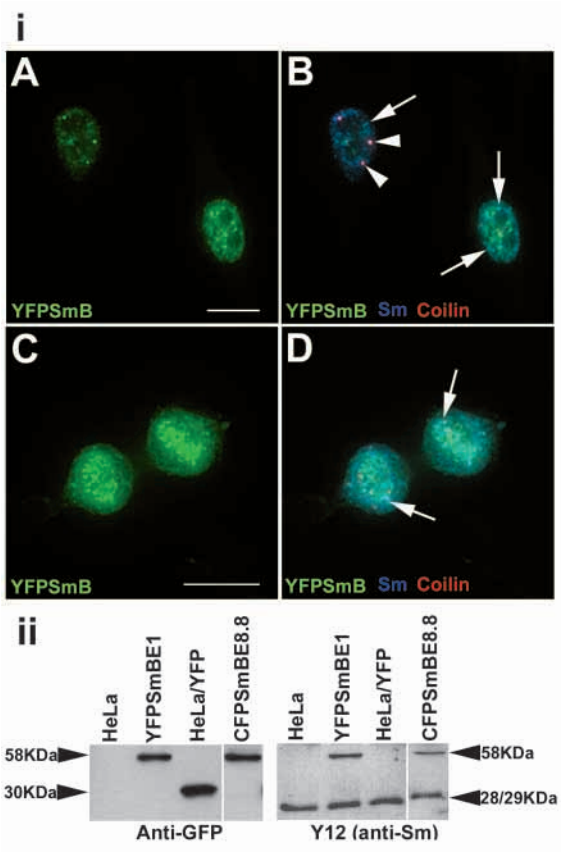

Fig. 1. (i) YFP-SmB colocalizes with endogenous Sm proteins in cells from line YFP-SmBE1. Maximum intensity projections of deconvolved serial sections through cells from line YFPSmBE1 in interphase (A,B) and telophase (C,D). The YFPSmB protein (A,C) colocalizes with endogenous Sm proteins (B,D) in Cajal bodies (arrowheads) and speckles (arrows). Bar, 10 μm. (ii) YFP and CFP-SmB are expressed as single fusion proteins at levels lower than endogenous SmB. SDS-PAGE analysis of whole cell lysates from lines YFPSmBE1, CFPSmBE8.8, parental HeLa cells and HeLa cells transiently transfected with a plasmid encoding YFP. An antibody to G/YFP (left-hand panel) showed a single band at 30 kDa in cells expressing YFP alone and single bands at the size predicted for YFPSmB or CFPSmB (58 kDa) in cells from lines YFPSmB E1 and CFPSmBE8.8, respectively. Antibody Y12 to endogenous Sm proteins (right-hand panel) gave a band at 28/29 kDa in all cell lines, representing endogenous SmB/SmB'. In cells from lines YFPSmBE1 and CFPSmBE8.8, additional, less intense bands were detected at 58 kDa, representing YFPSmB and CFPSmB, respectively.

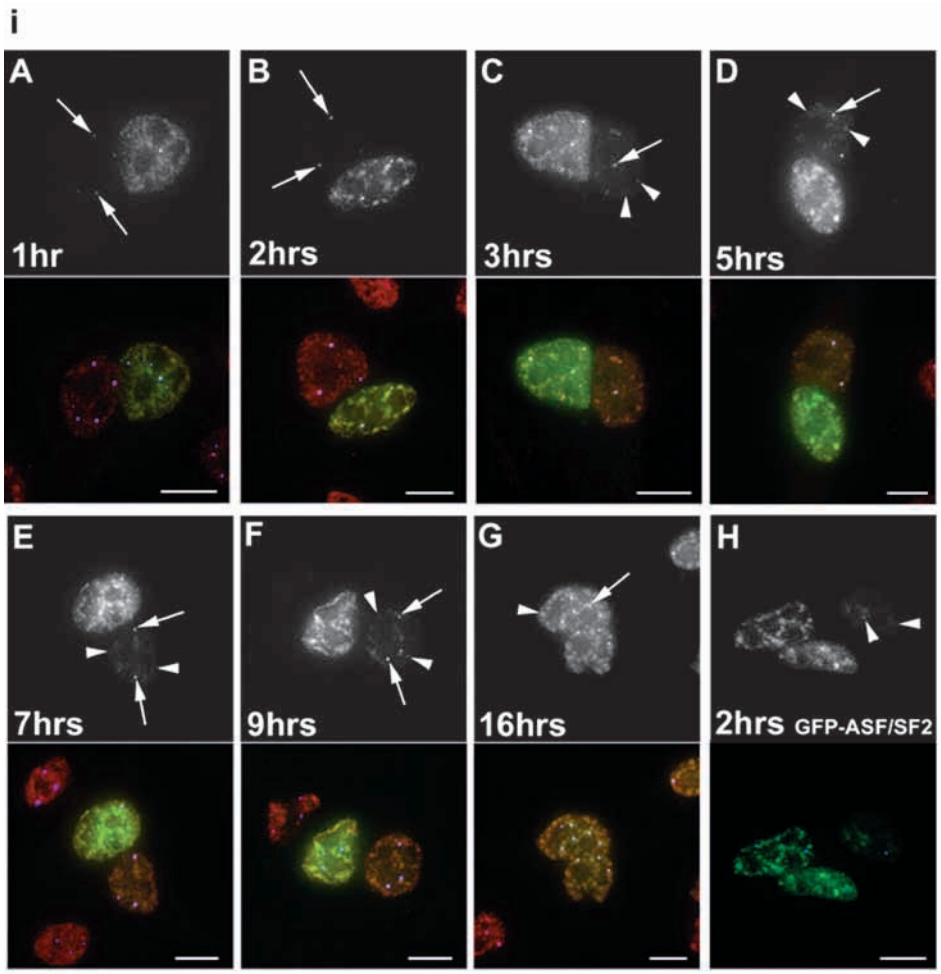

Fig. 2. (i) Three dimensional projections of parental HeLa cells fused to cells from line YFPSmBE1 fixed 1,2,3,5,7,9 and 16 hours after heterokaryon formation (A-G). YFPSmB is shown in grayscale in the top panels, the color panels show YFPSmB (green) overlain with Y12 (anti-Sm) staining (red) and anti-p80-colin staining (blue). YFPSmB is seen in the Cajal body by 1hour after fusion (arrows). Accumulation of YFPSmB in speckles is first seen 3 hours after fusion (arrowheads) and increases with time until, by 16 hours after fusion, cells from line YFPSmBE1 are indistinguishable from cells from the parental HeLa line (G). At the 16 hour timepoint, Cajal bodies are still clearly seen in all cells. Parental HeLa cells fused to HeLa cells expressing GFP-ASF/SF2 (H) show accumulation of GFP-ASF/SF2 in speckles (arrowheads) 2 hours after fusion, with no prior accumulation in Cajal bodies seen. Immunodetection of p80-coilin in these cells (color panel, blue channel) demonstrates that Cajal bodies are present in these cells (arrows). Bar, 10 μm. (ii) Quantification of the proportion of YFPSmB in different nuclear compartments over time. The proportion of YFPSmB signal detected in the Cajal body (red) and speckle (blue) compartments are expressed as a percentage of total nuclear fluorescence for early time points after fusion (1-2 hours), timepoints where speckles are beginning to be visible (4-5 hours), and in unfused cells from line EYFPSmBE1.

unfused cells from line EYFPSmBE1 (0.1% of total) (Fig. 2ii). Additionally, at 4-5 hour timepoints, ~0.7% of the total YFPSmB signal is seen in Cajal bodies, with approximately 14% of the total signal found in speckles. This compares with an average of ~30% of total YFPSmB signal found in speckles in cells from line EYFPSmB at steady state (P<0.01). Heterokaryon fusions between cells expressing an FP-tagged non-snRNP protein splicing factor, GFP-ASF/SF2, and non-expressing HeLa cells, demonstrated that GFP-ASF/SF2 localized rapidly to speckles, without first accumulating in Cajal bodies (Fig. 2H, arrows). Similar results were obtained with the FP-tagged U2AF65 and U2AF35 subunits (data not shown). These data reveal that newly assembled snRNPs show a preferential association with Cajal bodies compared with either mature snRNP or non-snRNP protein splicing factors.

We next tested whether the sequential accumulation of tagged snRNPs in Cajal bodies followed by speckles depended upon the expression level of FP-Sm proteins. Equal numbers

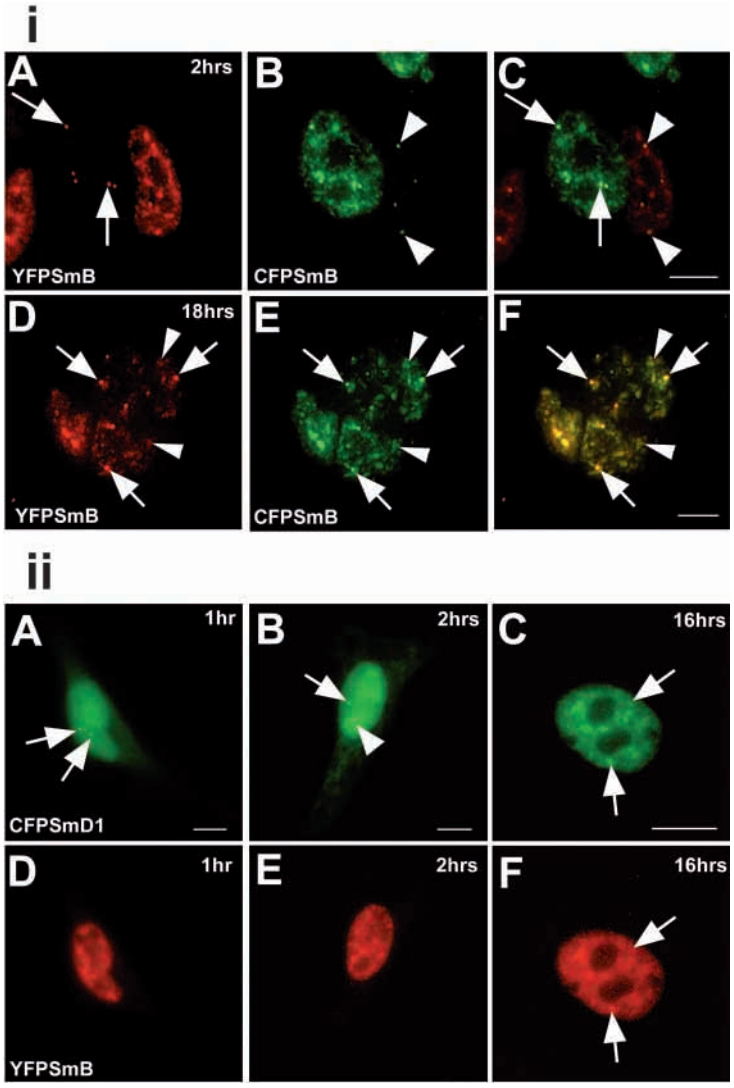

Fig. 3. (i) Three-dimensional projections of cells from line YFPSmBE1 fused to cells from line CFPSmBE8.8. A to C shows one cell from each line fused together, fixed two hours after fusion, YFPSmB has accumulated in the Cajal bodies of the nucleus from line CFPSmBE8.8 (arrows in A), while CFPSmB has accumulated in the Cajal bodies of the nucleus from line YFPSmBE1 (arrowheads in B). D to F shows three cells fused together, fixed 16 hours after fusion. Each of the nuclei shows a pattern of Cajal bodies (arrows) and speckles (arrowheads) for both YFPSmB (D) and CFPSmB (E). (ii) Conventional fluorescence images of cells from line YFPSmBE1 injected with the expression vector for ECFPSmD1, fixed after 1hour (A,D), 2 hours (B,E) and 16 hours (C,F). YFPSmB shows a speckled pattern in cells at all time points (D-F), while CFPSmD1 appears first in Cajal bodies after 1 hour (arrows in A), in nucleoli after 2 hours (arrowhead in B), finally showing a speckled pattern after 16 hours (arrows in C and F). Bar, 10 μm.

of EYFP-SmBE1 and ECFPSmBE8.8 cells were mixed and fused with 50% PEG, generating heterokaryons with varying levels of tagged Sm proteins (Fig. 3i). The heterokaryons were analyzed after either 2 hours, or overnight incubation at 37°C. In all cases examined, regardless of expression level, newly imported YFP- and CFP-tagged Sm proteins accumulated specifically in Cajal bodies 2 hours after fusion (Fig. 3iA-C, arrows and arrowheads), and in speckles (Fig. 3iD-F, arrowheads in) and Cajal bodies (Fig. 3iD-F, arrows) after overnight incubation at 37°C. At 2 hours post-fusion, reciprocal transfer of the YFP-SmB and CFP-SmB proteins was observed, with the transferred fusion proteins accumulated specifically in Cajal bodies, while the stably expressed SmB fusion proteins show a speckled pattern in the same nucleus (Fig. 3iA-C, Cajal bodies marked with arrows and arrowheads).

A similar result is obtained following microinjection of an expression vector encoding CFP-SmD1, resulting in transient overexpression of CFP-SmD1 in the YFP-SmBE1 HeLa cell line (Fig. 3ii). In this case a larger pool of diffuse nucleoplasmic signal from CFP-SmD1 is seen at the 1 hour and 2 hour time points, in addition to the clear concentration in Cajal bodies (Fig. 3iiA,B, arrows). A transient accumulation in nucleoli is also seen at the 2 hour time point (Fig. 3iiB, arrowheads). At 16 hours, the CFP-SmD1 snRNP signal no longer concentrates in nucleoli and a speckled pattern is seen (Fig. 3iiC, arrows). By contrast, the stably expressed YFP-SmB fusion protein shows a similar speckled pattern at each time point (Fig. 3iiD-F).

In summary, heterokaryon analyses demonstrate that newly imported Sm proteins show an accumulation in Cajal bodies prior to accumulating in speckles, independent of expression level. However, higher Sm protein expression enhances transient accumulation of the newly imported Sm proteins in nucleoli.

Newly imported snRNPs co-localize with SMN in Cajal bodies

In most cases Cajal bodies contain both p80-coilin and SMN proteins, although in a few cell lines, such as HeLa(D), some nuclear bodies contain coilin without SMN and vice versa. Fig. 4i shows a HeLa(D) nucleus containing large Cajal bodies labelled both with α-p80 coilin (Fig. 4iA, arrows) and α-SMN (Fig. 4iB, arrows). The same nucleus also contains both small bodies containing only p80-coilin (Fig. 4iA, arrowhead) and small bodies containing only SMN (Fig. 4iB, arrowheads). To determine which nuclear bodies accumulate new snRNP, HeLa(D) cells were transfected with a vector encoding YFPSmB and fixed after most transfected cells showed YFP-SmB in nuclear bodies (Fig. 4ii). Immunofluorescence analyses using antibodies to p80-coilin and SMN showed that newly imported snRNPs labeled with YFP-SmB only accumulated in nuclear bodies containing both SMN and coilin (Fig. 4iiA-D, arrows). Additional nuclear bodies containing coilin, but neither SMN nor new snRNP, were detected in transfected cells (Fig. 4ii, arrowhead in A and insert in A-D). This is unlikely to be due to an inability to detect SMN or snRNP in theses bodies because of their smaller size, as both SMN and snRNP can be detected in bodies of similar size in cells from line HeLa (D) and EYFPSmBE1 (Fig. 4iB,C; data not shown). Further experiments using PEG fusion to generate heterokaryons between cell line EYFP-SmBE1 and HeLa(D) cells also revealed this selective targeting of newly imported snRNPs to nuclear Cajal bodies containing both SMN and p80-coilin (Fig. 4iiE-H, arrows). Numerous nuclear bodies

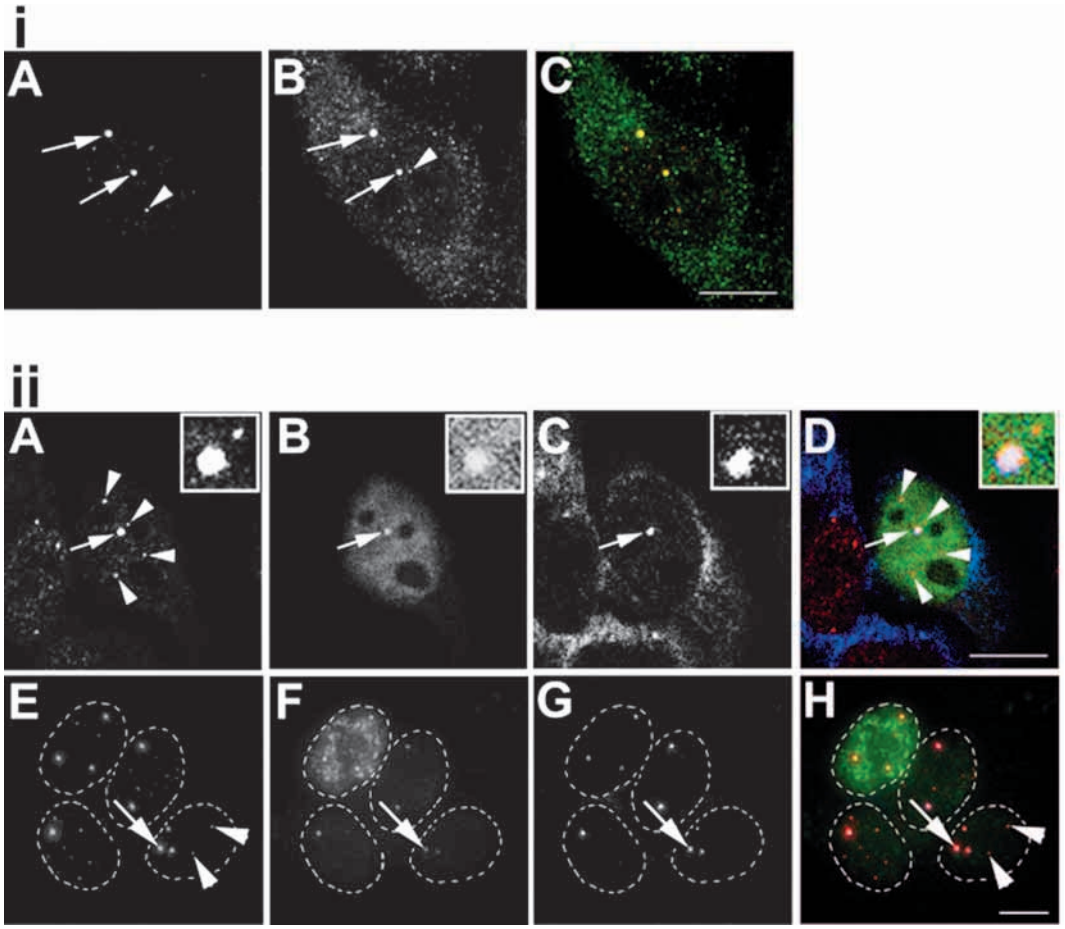

Fig. 4. (i) Maximum intensity projections of deconvolved serial sections through a cell from line HeLa (D). Immunodetection with antibodies to p80-coilin (A) and SMN (B) demonstrate that, in addition to large Cajal bodies containing both p80-coilin and SMN (arrows in A and B), these cells also contain smaller nuclear bodies containing p80-coilin in the absence of SMN (arrowhead in A) and containing SMN in the absence of p80-coilin (arrowhead in B). Bar, 10 μm. (ii) Single confocal sections of a cell from line HeLa (D) transfected with an expression plasmid encoding YFPSmB and fixed after 16 hours (A-D). The prominent Cajal body containing newly imported snRNP (arrow in B) also contains endogenous p80-coilin (arrow in A) and SMN (arrow in C). Additional bodies containing p80-coilin, but not new snRNP nor SMN, are also seen (arrowheads in A,D, see also inset magnified view in A-D). Bar, 10 μm. Maximum intensity projections of deconvolved serial sections through heterokaryons of cells from line HeLa (D) fused to a cell from line YFPSmBE1 fixed 1 hour after fusion (E-H). YFPSmB has accumulated in Cajal bodies of nuclei from line HeLa (D) (arrows in F). These bodies also contain endogenous p80-coilin (arrow in E) and SMN (arrow in G). Numerous additional bodies containing p80-coilin but neither new snRNP nor SMN are also seen (arrowheads in E,H). Bar, 10 μm.

containing p80-coilin, but neither SMN nor new snRNP, were seen in nuclei derived from the HeLa(D) cell line in these fusion experiments (Fig. 4iiE,H, arrowheads). This suggests that SMN and coilin may both be important for the accumulation of newly imported snRNP in Cajal bodies. Although HeLa(D) cells usually show nuclear bodies that contain SMN but not p80-coilin (Fig. 4i), it is interesting that no such bodies are observed following either heterokaryon formation, or after transient expression of FP-tagged SmB proteins, suggesting that both SMN and p80-coilin are required in Cajal bodies that accumulate new snRNP. Additionally, these data suggest that coilin-positive bodies lacking SMN, which are still seen following heterokaryon formation but which do not accumulate new snRNP, may have other roles in the nucleus that do not involve accumulating newly made snRNP.

Fusion of primary cells to HeLa cells leads to the formation of detectable Cajal bodies

To investigate where newly imported snRNPs localize in cells lacking Cajal bodies, heterokaryons were made between YFPSmBE1 cells and human primary foreskin fibroblasts, DFSF1 (Lain et al., 1999), in which nuclear Cajal bodies are not detected (Fig. 5iA-D; Fig. 6A-D). Heterokaryons were fixed either 2 hours or 7 hours after fusion and analyzed using anti-p80-coilin and anti-SMN antibodies. In the mixed culture of YFPSmBE1 and DFSF1 cells, prior to fusion, Cajal bodies containing p80-coilin (Fig. 5iA), YFPSmB (Fig. 5iB) and SMN (Fig. 5iC) were detected in YFPSmBE1 nuclei (Fig. 5iA-D, left hand outlined nucleus, arrows). By contrast, DFSF1 nuclei (Fig. 5iA-D,right hand outlined nucleus) showed little or no staining with anti-p80-coilin (Fig. 5iA) or anti-SMN (Fig. 5iC). Very weak, fine punctate nuclear staining can be detected

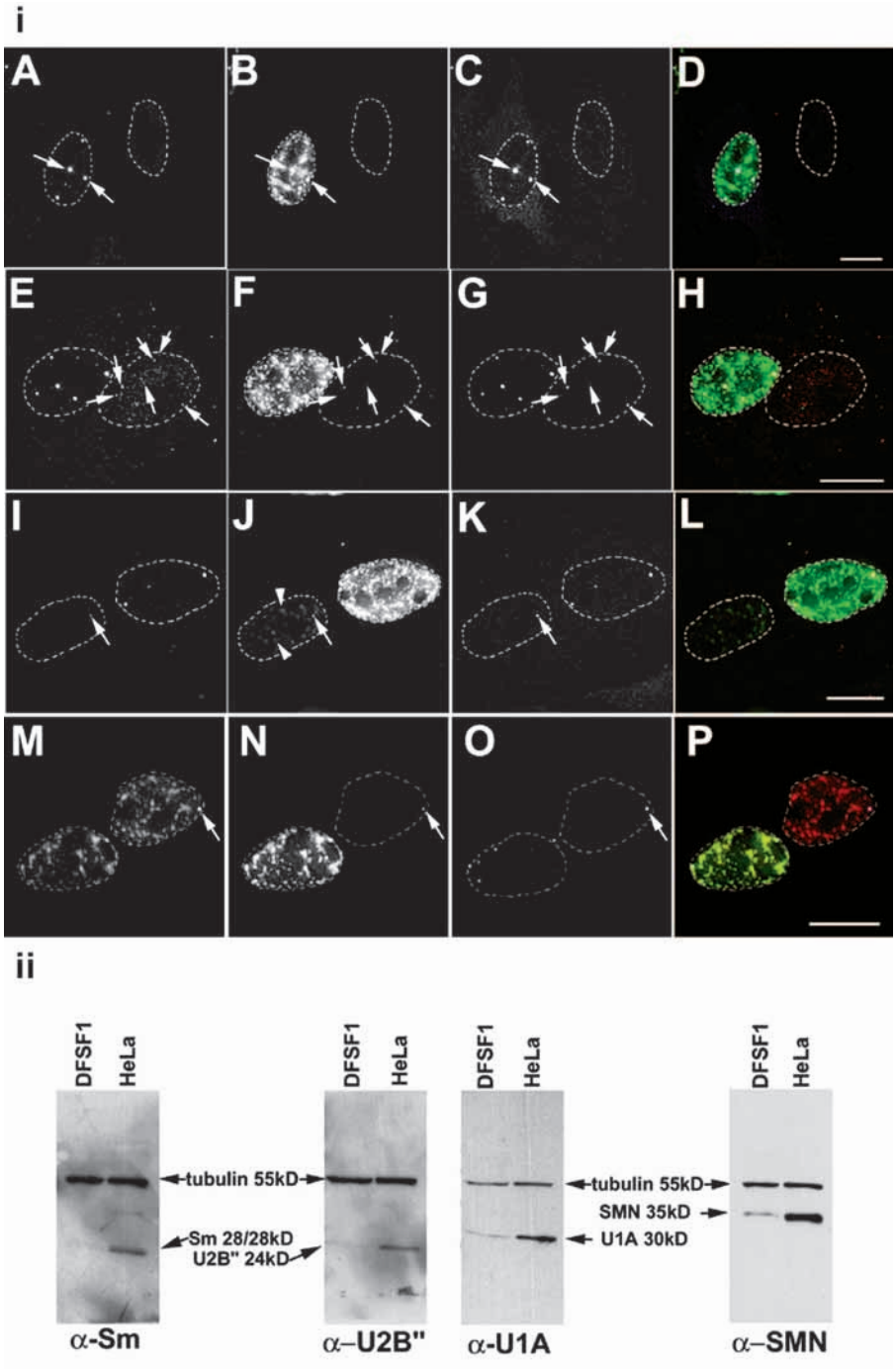

Fig. 5. Maximum intensity projections of deconvolved serial sections through heterokaryons of cells from line YFPSmBE1 and primary human fibroblasts (DFSF1), stained with antibodies to p80-coilin and SMN. Cells co-cultured without fusion show prominent Cajal bodies in nuclei from line YFPSmBE1 (arrows in A-C), containing p80-coilin (A), YFPSmB (B) and SMN (C). By contrast, DFSF1 nuclei show fine punctate staining with p80-coilin (A) and SMN (C), with no co-localization of the two into obvious Cajal bodies (overlay in D). In cells fixed 2 hours after PEG fusion (E-H), Cajal bodies are seen in nuclei from both cell types. YFPSmB is seen in nuclear bodies in DFSF1 nuclei (arrows in F). These bodies also contain both p80-coilin (arrows in E) and SMN (arrows in G). In cells fixed 16 hours after fusion, YFPSmB is seen in speckles in DFSF1 nuclei fused to YFPSmBE1 nuclei (arrowheads in J). Cajal bodies (arrows in I-K) containing YFPSmB (J), p80-coilin (I) and SMN (K) are also present in DFSF1 nuclei at this time point (overlay in L). Labeling of heterokaryons fixed 2 hours after PEG fusion with antibodies to the trimethyl cap of snRNAs (M) confirms that the nuclear bodies seen in DFSF1 cell nuclei at this time point contain snRNA (arrow in M) in addition to YFPSmB (arrow in N) and p80-coilin (arrow in O). Bar, 10 μm. (ii) Western blot analysis of total cell lysates from DFSF1 cells and HeLa cells. The intensity of signals seen for α-tubulin confirms that proteins from similar numbers of cells have been loaded in each lane. The core snRNP, Sm proteins, the U2 snRNP-specific protein U2B″, the U1 snRNP-specific proteinU1A, and the SMN protein are both much more abundant in HeLa cells than in DFSF1 cells.

in DFSF1 cells using antibodies to p80-coilin (Fig. 5iA; Fig. 6A) and SMN (Fig. 5iC; Fig. 6B). However, these two antigens do not co-localize in Cajal bodies (Fig. 6D) and neither antigen co-localizes with Sm proteins (data not shown). Two hours after fusion, YFPSmB was detected in small nuclear bodies in DFSF1 nuclei (Fig. 5iF, arrows). These bodies also contained p80-coilin (Fig. 5iE, arrows) and SMN (Fig. 5iG, arrows), showing a co-localization between these three antigens characteristic of Cajal bodies in HeLa cell nuclei. In cells analyzed 7 hours after fusion, YFPSmB was seen in speckles in DFSF1 nuclei (Fig. 5iJ, arrowheads) as well as in Cajal bodies containing p80-coilin (Fig. 5iI,J, arrow) and SMN (Fig. 5iK, arrow). Labeling with an antibody to the tri-methyl cap of snRNA (Fig. 5iM,P) confirms that the nuclear bodies seen in DFSF1 cells fused to HeLa cells contain snRNA in addition to Sm proteins (Fig. 5iM-P, arrow). These data indicate that the introduction of proteins or other factors from HeLa cells expressing YFPSmB into DFSF1 cells is sufficient to induce the formation of Cajal bodies containing new snRNP, SMN and p80-coilin in the DFSF1 cell nuclei. We conclude that the nuclei of DFSF1 primary cells are capable of forming Cajal bodies if the required factors and/or signals are present.

A comparison of snRNP protein levels present in DFSF1 cells and HeLa cells was made by separating whole cell lysates from each cell line by SDS-PAGE using a 10% gel, blotting onto nitrocellulose and detecting with antibodies to the core

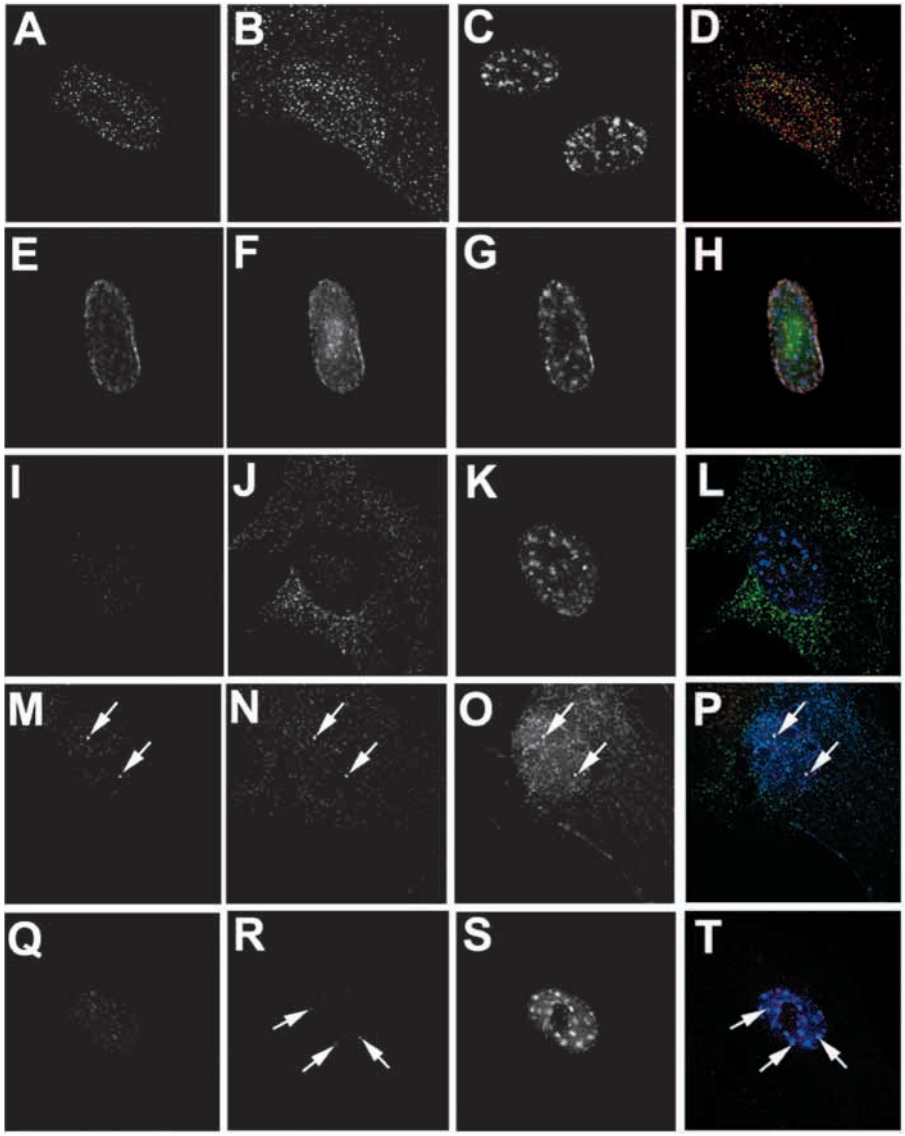

Fig. 6. Maximum intensity projections of deconvolved serial sections through control DFSF1 cells (A-D) and DFSF1 cells injected with expression vectors encoding GFPCoilin (E-H), GFPSMN (I-L) or YFPSmB (M-T). Control cells show weak, fine punctate staining with anti-p80-coilin (A) and anti-SMN (B) with no co-localization between the two (overlay in D). Endogenous Sm proteins show a speckled localisation (C). Over-expression of GFPCoilin leads to an increase in nucleoplasmic coilin (E, anti-p80 coilin; F, GFP-coilin; H, overlay), but not to formation of Cajal bodies. Endogenous snRNP proteins, detected with Y12, show a speckled nuclear localization (G). Overexpression of GFPSMN leads to fine punctate localization of GFPSMN, predominantly in the cytoplasm (J). No co-localization with p80-coilin (I) or endogenous Sm proteins (K) is seen. Over-expression of YFPSmB for 2 hours results in the formation of nuclear Cajal bodies (arrows in M-O) containing p80-coilin (M), SMN (N) and newly imported snRNP (O). In cells expressing YFPSmB for 16 hours, p80-coilin shows a fine punctate pattern (Q). SMN is seen in small nuclear bodies (arrows in R,T), but no longer shows co-localization with YFPSmB (S,T).

snRNP, Sm, proteins, the U2 snRNP-specific protein U2B″ and the U1 snRNP-specific protein U1A (Fig. 5ii). The amount of total protein loaded was adjusted to obtain an equal signal for α-tubulin in each cell line. To achieve this, 25 μg of HeLa total protein was used and 100 μg of DFSF1 total protein. Amounts of all of these snRNP proteins are very low in DFSF1 cells compared with HeLa cells. Additionally, levels of SMN protein are lower than in HeLa cells (Fig. 5ii). This suggests that snRNP levels are lower in DFSF1 cells than in HeLa cells.

Transient expression of YFPSmB in primary cells enhances formation of Cajal bodies

DFSF1 cells were injected with expression vectors encoding fluorescent protein-tagged p80-coilin, SMN or SmB, fixed either 2 hours or 16 hours after injection and analyzed using antibodies to p80-coilin, SMN and Sm proteins (Fig. 6). In non-injected cells, p80-coilin (A) and SMN (B) both show weak, fine punctate nuclear staining, with no colocalisation, while SMN, but not coilin, also shows fine punctate cytoplasmic staining (Fig. 6D, overlay). Endogenous Sm proteins show a speckled localisation (Fig. 6C). After 2 hours, GFP-coilin was detected throughout the nucleus and nucleolus, with no accumulation in Cajal bodies (Fig. 6E,F,H). In the same nuclei, endogenous Sm proteins showed a speckled pattern, but no Cajal bodies (Fig. 6G). After 2 hours of transient expression, GFPSMN showed a punctate pattern, primarily in the cytoplasm (Fig. 6J). In cells expressing GFPSMN, endogenous p80-coilin showed a widespread fine punctate nuclear distribution (Fig. 6I) and endogenous Sm proteins showed a speckled nuclear distribution (Fig. 6K). By contrast, DFSF1 cells transiently expressing YFPSmB, fixed after 2 hours, showed nuclear Cajal bodies containing endogenous p80-coilin (Fig. 6M,P, arrows) and SMN (Fig. 6N,P, arrows) as well as the exogenous YFPSmB (Fig. 6O,P, arrows). Cells expressing YFPSmB fixed after 16 hours showed YFPSmB in a speckled nuclear pattern (Fig. 6S). Small nuclear bodies containing endogenous SMN are seen (Fig. 6R,T, arrows), but endogenous p80-coilin is not detected in these bodies (Fig. 6Q). We conclude that transient expression of exogenous Sm proteins is sufficient to induce Cajal body formation in DFSF1 cells, but only for a limited time.

LMB can prevent accumulation of snRNPs in Cajal bodies and speckles

Leptomycin B (LMB) blocks export of snRNA and, consequently, the re-import of TMG-snRNA assembled with Sm proteins (Fornerod et al., 1997; Carvalho et al., 1999). HeLa cells were injected with a plasmid encoding YFPSmB either in the presence or absence of medium containing LMB, cultured for 8 hours, and analyzed with antibodies to p80 coilin

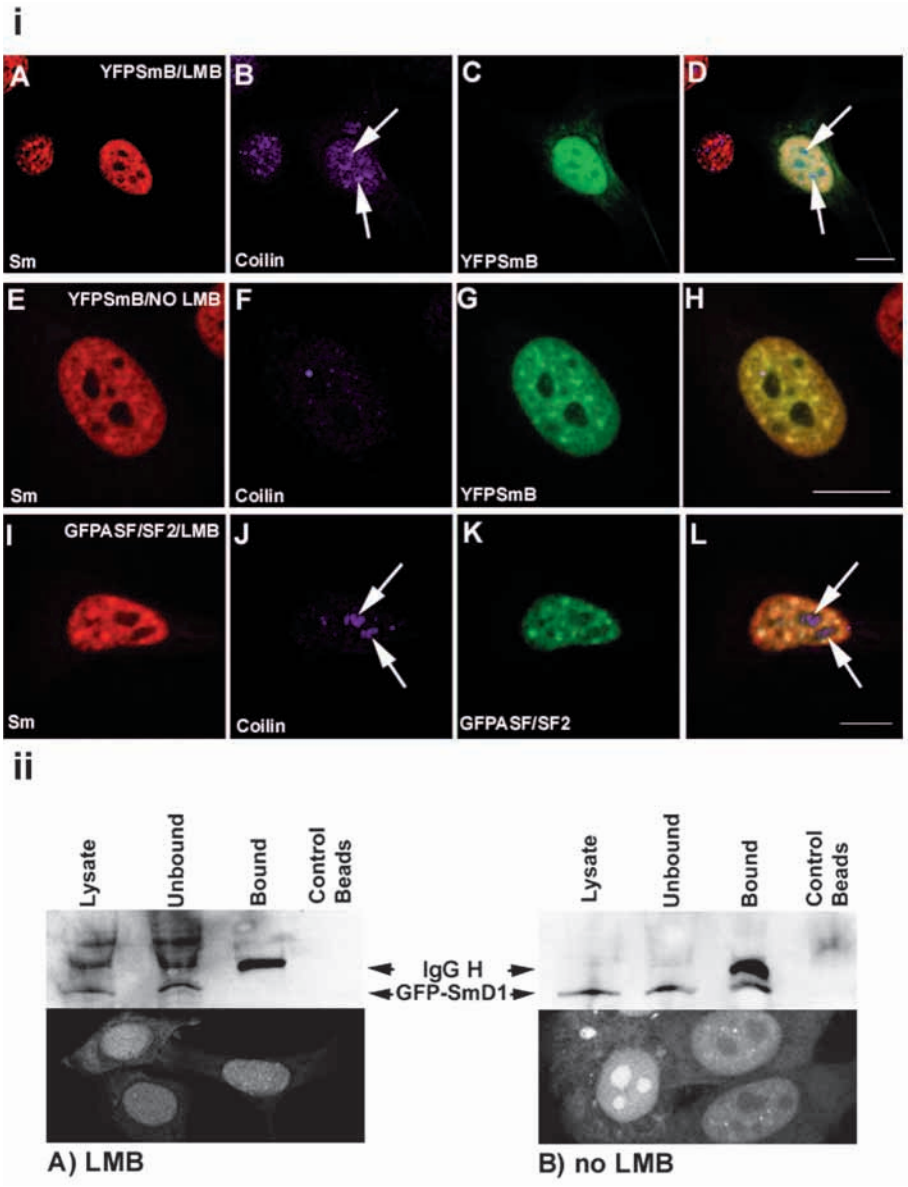

Fig. 7. (i) Confocal sections of HeLa cells injected with expression vectors encoding YFPSmB or GFPASF/SF2 in the presence or absence of LMB and incubated for 8 hours. In cells injected with YFPSmB in the presence of LMB (A-D), expressed YFPSmB does not accumulate in nuclear bodies or speckles (C). Nuclear speckles are still seen using antibodies to endogenous snRNPs (A). p80-coilin is found predominantly in the nucleolus (arrows in B). Control cells, injected with pYFPSmB in the absence of LMB (E-H) show a speckled pattern of YFPSmB (G), with endogenous Sm proteins in speckles and Cajal bodies (E) and p80-coilin in Cajal bodies (F). Cells injected with pGFPASF/SF2 in the presence of LMB (I-L) show the uptake of GFPASF/SF2 into speckles (K). p80-coilin is seen in the nucleoli (arrows in J,L). Bar, 10 μm. (ii) Immonoprecipitation of snRNPs from cells transfected in the presence (A) or absence (B) of LMB using anti-TMG antibodies, detected using anti-GFP antibodies (top panels). In the presence of LMB, GFP-SmD1 is not incorporated into snRNPs (compare lower band in bound versus unbound fraction). In the absence of LMB, GFP-SmD1 is seen in the bound, snRNP, fraction. The lower panels show 3D projections of serial confocal sections through cells treated with LMB (A) and control cells (B). In LMB-treated cells, GFP-SmD1 signal is diffuse, whereas in control cells (B), GFPSmD1 is seen in Cajal bodies and nucleoli.

and endogenous Sm proteins (Fig. 7i). Cells treated with LMB show a diffuse signal from YFPSmB throughout the nucleus (Fig. 7iC), with little or no accumulation in Cajal bodies, nucleoli or speckles. A lower level of diffuse cytoplasmic signal is also seen. Endogenous p80-coilin is mostly relocalized to the nucleolus in LMB treated cells (Fig. 7iB,D, arrows). Endogenous Sm proteins are no longer enriched in nuclear bodies (Fig. 7iA,D). Control cells injected with pYFP-SmB and cultured without LMB showed normal kinetics of Sm protein accumulation inspeckles (Fig. 7iE-H).

Exogenously expressed GFP-ASF/SF2 localizes to speckles in LMB treated cells, confirming that the uptake of splicing factors into speckles is not blocked in these cells (Fig. 7iI-L). The nucleolar accumulation of p80-coilin during GFP-ASF/SF2 expression confirms that LMB was active (Fig. 7iI-L, arrows).

The effect of LMB treatment on the assembly of tagged Sm protein into snRNP particles was analyzed in HeLa cells transfected with pEGFPSmD1 and incubated for 16 hours either in medium containing LMB or in control culture medium. A coverslip from each dish was fixed with paraformaldehyde (PFA) for localization analysis, the remaining cells were lysed and assembled snRNPs isolated by immunoprecipitation using anti-TMG cap antibodies. Cells treated with LMB showed no incorporation of GFPSmD1 into snRNPs (Fig. 7iiA, top). Serial confocal micrographs of these cells showed diffuse signal from EGFP-SmD1 throughout the cell with no specific accumulation in any cytoplasmic or nuclear structures (Fig. 7iiA, bottom). Control cells transfected at the same time and incubated for 16 hours without LMB treatment showed incorporation of GFPSmD1 into snRNPs, although some tagged protein remained unassembled (Fig. 7iiB, top). Analysis of these cells by microscopy showed two localization patterns, with ~50% of cells showing GFP-SmD1 in Cajal bodies, nucleoli and the cytoplasm and ~50% showing GFP-SmD1 in Cajal bodies, speckles and the cytoplasm (Fig. 7iiB, bottom). These results demonstrate that treatment with LMB at the time of transfection abolishes assembly of GFP-SmD1 into snRNPs.

LMB blocks accumulation of snRNP in speckles

Cells were transfected with plasmids encoding YFPSmB or GFPSmD1 and cultured overnight until the majority of transfected cells showed some accumulation of FP-Sm proteins

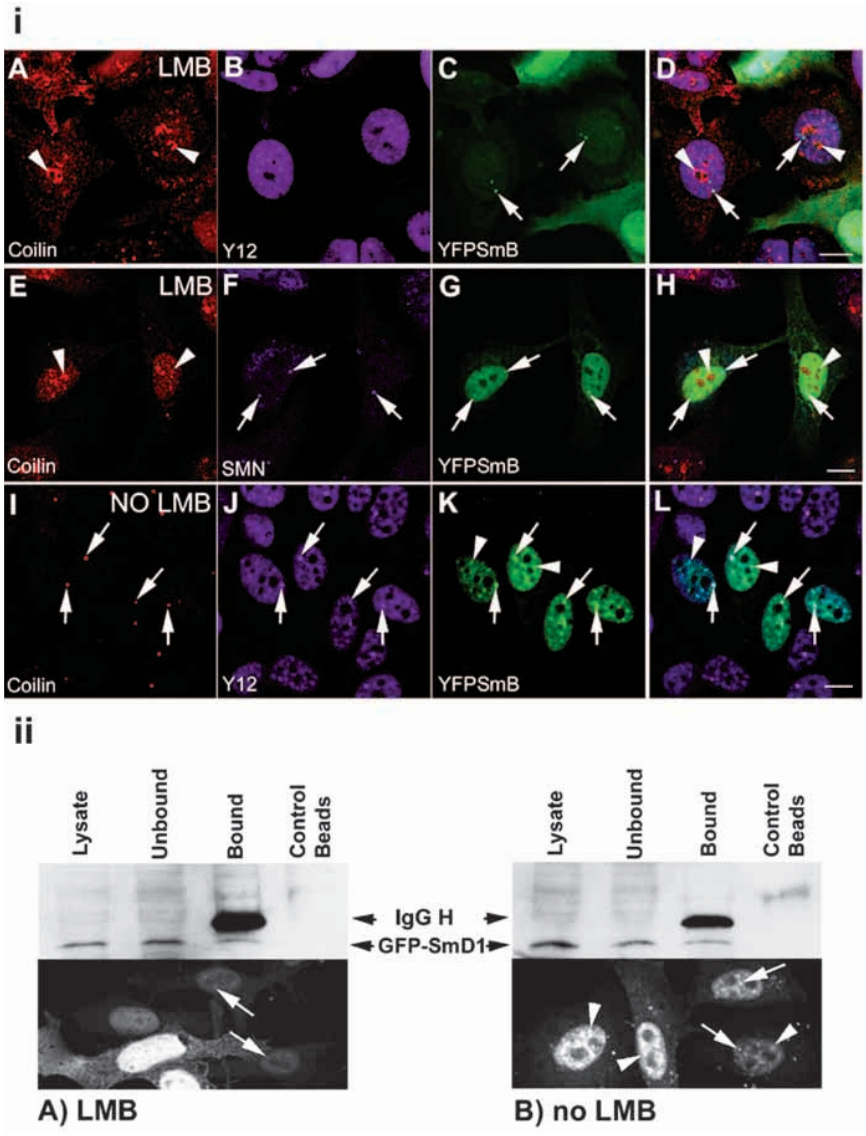

Fig. 8. (i) Confocal sections of HeLa cells transfected with YFPSmB and treated with LMB 16 hours after transfection (A-H) or cultured in the absence of LMB (I-L). YFPSmB is seen in nuclear bodies in cells treated with LMB (arrows in C,G), but not in speckles. These nuclear bodies contain SMN (arrows in F), but do not contain p80-coilin, which is seen in the nucleolus (arrowheads in A and E). In cells incubated in the absence of LMB, YFPSmB (K) is seen to co-localize with endogenous Sm proteins (J) in Cajal bodies (arrows in I-L) and speckles (arrowheads in I-L). Bar, 10 μm. (ii) Immonoprecipitation of snRNPs from cells transfected with GFPSmD1, incubated overnight, then incubated for 16 hours in the presence (A) or absence (B) of LMB using anti-TMG antibodies, detected using anti-GFP antibodies (top panels). GFPSmD1 (lower band) is seen in the bound fraction in LMB treated cells and in control cells. The lower panels show 3D projections of serial confocal sections through cells treated with LMB (A) and control cells (B). In LMB-treated cells, GFP-SmD1 signal shows localizations characteristic of early time points after transfection, with diffuse signal and accumulation in Cajal bodies but not speckles. In control cells (B), GFPSmD1 shows a speckled localization.

in Cajal bodies. They were then incubated with either control culture medium or with medium containing LMB for 8 hours, fixed and analyzed with antibodies to p80-coilin, SMN and Sm proteins (Fig. 8i). In LMB-treated cells YFP-SmB was detected in a diffuse nucleoplasmic pool and in nuclear bodies that also contained SMN (Fig. 8iC,F,G, arrows), but not p80 coilin, which instead localized predominantly to the nucleolus (Fig. 8iA,D,E,H, arrowheads). By contrast, control cells not exposed to LMB showed YFP-SmB in speckles (Fig. 8iK,L, arrowheads) as well as in Cajal bodies (Fig. 8iI-L, arrows). These Cajal bodies contained p80-coilin (Fig. 8iI) and SMN (data not shown). Similar results were obtained for GFPSmD1 (data not shown).

The effect of LMB treatment on the assembly of tagged Sm protein into snRNP particles was analyzed in HeLa cells transfected with pEGFPSmD1, incubated for 16 hours, then treated either with medium containing LMB, or with control culture medium, for a further 16 hours. A coverslip from each dish was fixed with PFA for localization analysis and the remaining cells lysed and snRNPs isolated by immunoprecipitation using anti-TMG cap antibodies. Cells treated with LMB 16 hours after transfection showed incorporation of GFP-SmD1 into snRNPs (Fig. 8iiA, top). Analysis of these cells by fluorescence microscopy showed that the majority of cells had diffuse nucleoplasmic GFPSmD1 and accumulation of GFP-SmD1 in Cajal bodies (Fig. 8iiA, bottom, arrows) and/or nucleoli (Fig. 8iiA, bottom, arrowheads). Control cells without LMB treatment fixed at the same time showed localization of GFP-SmD1 to Cajal bodies and speckles in all cells, with some residual cytoplasmic signal also seen (Fig. 8iiB, bottom, Cajal bodies arrowed). Immunoprecipitation with anti-TMG antibodies revealed that a similar proportion of GFP-SmD1 was assembled into snRNPs at this time-point as in the control cells without LMB (Fig. 8iiB, top). These results suggest that LMB can inhibit the normal accumulation of snRNPS in nuclear speckles.

DISCUSSION

In this study we have analyzed the nuclear snRNP pathway using stable cell lines that express fluorescent protein-tagged SmB. snRNPs labeled with tagged SmB protein first concentrate in Cajal bodies before accumulating in speckles following their initial entry into the nucleus. Analysis of cells expressing varying levels of snRNP proteins indicates that saturable steps in the snRNP transport pathway may occur both in the cytoplasm and in the nucleolus. We show that the nuclear bodies that accumulate newly imported snRNP always contain both p80 coilin and SMN. Both the initial concentration of snRNPs in Cajal bodies, and their subsequent relocation into

speckles, can be prevented by treating cells with the nuclear export inhibitor leptomycin B (LMB). Furthermore, we show that transient expression of exogenous SmB protein alone is sufficient to trigger the de novo formation of Cajal bodies in primary cells.

Expression of FP-SmB in stable cell lines

In stable HeLa cell lines that constitutively express the SmB core snRNP protein fused to either yellow or cyan fluorescent protein tags, protein analyses show that the tagged SmB is expressed at lower levels than the endogenous protein and adopts an identical nuclear localization pattern. Furthermore, the stable cell lines have an identical number of Cajal bodies and grow and divide at a similar rate to the parental HeLa cells, indicating that expression of the tagged SmB protein is not toxic. The stable cell lines have allowed us to demonstrate conclusively, using heterokaryon assays, that snRNPs accumulate in Cajal bodies prior to speckles upon their initial entry into the nucleus, with a ~20-fold increase in the proportion of newly imported YFPSmB in Cajal bodies relative to that seen for steady-state YFP-SmB. This is consistent with the results previously obtained using transient expression protocols to analyze snRNP localization (Sleeman and Lamond, 1999). However, these earlier studies were complicated by the inevitable transient overexpression of snRNP proteins. We note that the use of stable cell lines expressing fusion proteins in conjunction with heterokaryon assays can avoid the problem of protein over-expression when a novel factor is expressed in the nucleus, and may therefore be useful in future for analyzing localization pathways and other events for a wide range of cellular factors in addition to snRNPs. The pathway observed in our studies of splicing snRNPs shows similarities to that reported independently for small nucleolar ribonucleoproteins (snoRNPs), which also concentrate in Cajal bodies prior to their accumulation in nucleoli (Gall et al., 1999; Narayanan et al., 1999). Although the snoRNPs do not subsequently accumulate in speckles, their pathway is similar to that observed for early steps in splicing snRNP localization, where snRNPs transiently accumulate in Cajal bodies and nucleoli before they concentrate in speckles.

A saturable nucleolar step

The import of new snRNPs requires Sm protein binding and subsequent TMG cap formation (Fischer et al., 1993; Hamm et al., 1990; Hermann et al., 1995; Lehmeier et al., 1994; Lerner and Steitz, 1979; Lührmann et al., 1990; Mattaj, 1986; Nagai and Mattaj, 1994; Raker et al., 1996). Since we have demonstrated that FP-tagged Sm proteins are assembled into TMG-capped snRNP particles (Sleeman and Lamond, 1999), it is probable that the nuclear FP-Sm signal represents predominantly, if not exclusively, FP-Sm proteins assembled onto snRNA. Nucleolar accumulation of tagged snRNP in heterokaryon assays was less frequently observed than in over-expressing cells following microinjection of Sm-protein expression vectors (Sleeman and Lamond, 1999). Additionally, over-expression of CFP-SmD1 in a stable cell line expressing YFPSmB resulted in nuclear accumulation of CFP-SmD1 (Fig. 3ii). This accumulation is not seen when cells from a stable cell line expressing small amounts of CFPSmB are fused to cells from a stable cell line expressing small amounts of YFPSmB. These data implicate a nucleolar stage of snRNP maturation as a possible rate-limiting step. The nucleolus, which is the major site of rRNA modification, may also be a site of modification for Sm-containing snRNAs. Consistent with this idea, modification of U6 snRNA has been demonstrated to involve a small guide RNA, similar to the snoRNAs that guide modification of rRNA (Tycowski et al., 1998). Additionally, 2′O-methylation and psuedouridylation of U2 snRNA, necessary for the addition of snRNP-specific proteins, occurs in the nucleus rather than the cytoplasm (Yu et al., 1998). If some snRNAs are modified in the nucleolus, it is possible that nucleolar snRNA modification may be a rate-limiting step in snRNP maturation.

A possible nuclear role for SMN in snRNP maturation

In cell lines showing separate p80-coilin positive Cajal bodies without SMN and SMN-positive ‘gems’ that lack p80-coilin, new snRNP accumulated specifically in a sub-set of nuclear bodies containing both p80-coilin and SMN. A cytoplasmic role for SMN in snRNP maturation has previously been demonstrated, while SMN in the nucleus has been reported to play a direct role in splicing, possibly involving the re-cycling of splicing complexes (Fischer et al., 1997; Liu et al., 1997; Pellizzoni et al., 1999; Pellizzoni et al., 1998). Our data raise the possibility that the involvement of SMN in snRNP maturation may extend into the nucleus. The accumulation of SMN together with snRNPs in Cajal bodies has previously been suggested as a link between snRNP maturation and the Cajal body (Carvalho et al., 1999). The fact that, in this study, we observe nuclear bodies containing p80-coilin without either SMN or new snRNP indicates that more than one class of Cajal body can form and that different Cajal bodies may have distinct functions. For example, some, but not all, Cajal bodies have been shown to associate with specific gene loci (Callan et al., 1991; Gao et al., 1997; Matera and Ward, 1993; Schul et al., 1999a; Schul et al., 1998; Smith et al., 1995), while recent data on in vivo Cajal body dynamics indicate that they can be separated into different classes according to their size and rate of movement (Boudonck et al., 1999; Platani et al., 2000; Snaar et al., 2000). Nuclear gems containing SMN, but not p80-coilin, are absent from nuclei that are actively importing new snRNP following either microinjection or heterokaryon assays in cell lines where distinct gems are usually seen (see overlays in Fig. 4). This argues that different classes of nuclear bodies, including Cajal bodies and gems, may be dynamic, inter-related structures.

A leptomycin B-sensitive snRNP pathway

In mammalian cells, LMB blocks nuclear export including the export of snRNA into the cytoplasm and is therefore predicted to block the import of FP-Sm proteins into the nucleus, because Sm protein assembly onto snRNA is a cytoplasmic event occurring before re-import of the snRNP into the nucleus (Fornerod et al., 1997; Carvalho et al., 1999). Treatment of cells with LMB prevents both the accumulation of snRNP in nuclear bodies and the later accumulation of snRNPs in speckles. Depending on its time of addition, LMB can also lead to the accumulation of newly imported snRNPs in residual nuclear bodies containing SMN but depleted of p80-coilin. LMB is a specific inhibitor of CRM1, a receptor essential for nuclear export of factors with the leucine rich NES motif

(Ullman et al., 1997). The interaction of CRM1 with export signals is blocked by LMB binding directly to CRM1 (Kudo et al., 1998). It is not yet clear whether the effect of LMB on snRNP localization we observe involves CRM1 or a novel receptor that is also a target for LMB, although the specificity of LMB makes CRM1 the most likely candidate. The defect in snRNP uptake into speckles in the presence of LMB may be due to a deficiency in a factor(s) that requires CRM1 for its function. LMB may also interfere with nuclear transport events required for snRNP localization within the nucleus as distinct from export events. Nuclear export of proteins and RNPs could be viewed as a form of intranuclear transport in which the destination of the factors being transported is the nuclear pore complex (NPC). Subsequent movement through the NPC (i.e. export) requires Ran and GTP. There may be alternative nuclear destinations, distinct from the NPC, for factors transported by CRM1. For example, CRM1 could transport factors to Cajal bodies, nucleoli or speckles. The effect of LMB in blocking the accumulation of new snRNPs in speckles does not reflect a gross rearrangement of snRNPs in treated cells, as endogenous, mature snRNPs are still localized in speckles. Neither does it cause a general block in the ability of speckles to accumulate new components because newly expressed GFP-ASF/SF2 is rapidly accumulated in speckles in the presence of LMB. Biochemical analysis of snRNPs isolated from cells treated with LMB after 16 hours of GFP-SmD1 expression demonstrates that assembled, TMG-capped snRNPs containing FP-tagged Sm proteins are present in these cells. However, the nuclear FP-tagged snRNP is concentrated in residual nuclear bodies containing SMN but lacking p80-coilin. They do not accumulate in speckles, although a diffuse nucleoplasmic pool is seen. This suggests that Sm protein assembly on snRNA and TMG cap formation are not sufficient for the accumulation of new snRNPs into speckles. Mature splicing factors have been demonstrated to equilibrate rapidly between speckles and the nucloplasmic pool using FRAP experiments (Phair and Misteli, 2000). Although YFPSmB is present in a nucleoplasmic pool in LMB-treated cells, with a similar proportion assembled onto snRNA as seen in control cells without LMB it does not accumulate in speckles. This suggests that the accumulation of newly imported snRNP into speckles may require either additional factors or a pathway whose presence or activity is sensitive to LMB.

Expression of Sm proteins triggers Cajal body formation

If transient accumulation in Cajal bodies is important for snRNP maturation, it is surprising that some primary cells show few or no Cajal bodies when analyzed by immunofluorescence (Spector et al., 1992; Young et al., 2000). This could mean either that the pathway is non-essential, or that alternative pathways are used in different cell types, or that the detection of prominent Cajal bodies depends on the overall flux of maturing snRNPs through the system. Our analysis here of primary human foreskin fibroblasts (DFSF1 cells) suggests that, in at least some cases, the latter explanation may be correct. Although DFSF1 cells lack prominent Cajal bodies under normal growth conditions, we show here that Cajal bodies, containing p80-coilin, SMN and snRNP, will form rapidly in the nuclei of DFSF1 cells that are fused to HeLa cells. Importantly, it was observed that transient exogenous expression of SmB protein alone in DFSF1 cells is sufficient to trigger them to form Cajal bodies. By contrast, they do not form Cajal bodies upon transient overexpression of either p80-coilin or SMN proteins or simply as a result of the stress of microinjection. These data indicate that Cajal body formation may be linked to the level of snRNP expression. Consistent with this idea, we observe that transient expression of SmB causes a corresponding transient appearance of Cajal bodies. This is also consistent with previous observations that Cajal bodies are more common in rapidly dividing cells (Spector et al., 1992) and that Cajal bodies are kinetic nuclear structures (Carmo-Fonseca et al., 1993). A comparison of the total amount of snRNP proteins present in DFSF1 cells with HeLa cells demonstrates that DFSF1 cells contain lower levels of snRNP proteins. They also divide more slowly, with a doubling time of ~60 hours as opposed to ~16 hours for HeLa cells. Since a large amount of new snRNP must be made when a cell divides, a role for the Cajal body in snRNP maturation is fully in agreement with the increased visibility of Cajal bodies in rapidly dividing cells. However, these data need not imply that Cajal bodies must be detectable or functional in all cell types and we cannot exclude the existence of alternative or parallel snRNP maturation pathways. Nonetheless, the present data support the view that Cajal bodies may play an important role in snRNP maturation and/or transport events (Gall et al., 1999; Matera, 1999; Sleeman and Lamond, 1999).

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