

Newly assembled snRNPs associate with coiled bodies before speckles, suggesting a nuclear snRNP maturation pathway

Judith E. Sleeman and Angus I. Lamond

Background: Small nuclear ribonucleoproteins (snRNPs), which are essential components of the mRNA splicing machinery, comprise small nuclear RNAs, each complexed with a set of proteins. An early event in the maturation of snRNPs is the binding of the core proteins – the Sm proteins – to snRNAs in the cytoplasm followed by nuclear import. Immunolabelling with antibodies against Sm proteins shows that splicing snRNPs have a complex steady-state localisation within the nucleus, the result of the association of snRNPs with several distinct subnuclear structures. These include speckles, coiled bodies and nucleoli, in addition to a diffuse nucleoplasmic compartment. The reasons for snRNP accumulation in these different structures are unclear.

Results: When mammalian cells were microinjected with plasmids encoding the Sm proteins B, D1 and E, each tagged with either the green fluorescent protein (GFP) or yellow-shifted GFP (YFP), a pulse of expression of the tagged proteins was observed. In each case, the newly synthesised GFP/YFP-labelled snRNPs accumulated first in coiled bodies and nucleoli, and later in nuclear speckles. Mature snRNPs localised immediately to speckles upon entering the nucleus after cell division.

Conclusions: The complex nuclear localisation of splicing snRNPs results, at least in part, from a specific pathway for newly assembled snRNPs. The data demonstrate that the distribution of snRNPs between coiled bodies and speckles is directed and not random.

Background

Within the nucleus, which houses the cell's chromosomes, many complex pathways need to be coordinated. Unlike the cytoplasm, the nucleus is not compartmentalised into membrane-bound organelles. Many nuclear antigens are, however, localised in different subnuclear structures, distinct from the surrounding nucleoplasm, that can be resolved by both light and electron microscopy (for recent reviews, see [1–4]). The most obvious structure in the interphase nucleus is the nucleolus, which contains the ribosomal gene clusters and is the site of ribosome production. It may also have other roles in RNA metabolism and transport [5]. A variety of other nuclear bodies and structures have also been described, including coiled bodies, 'gemini' of coiled bodies ('gems'), promyelocytic leukaemia (PML) bodies, interchromatin granule clusters, interchromatin-granule-associated zones and perichromatin fibrils [6–17].

Early events in the maturation of small nuclear ribonucleoproteins (snRNPs) are known to occur in the cytoplasm. Following their transcription in the nucleus, snRNAs are transported to the cytoplasm where the Sm proteins bind and the 5' end of the snRNA is modified to form the characteristic trimethylguanosine (TMG) cap (Figure 1). These events are essential for the re-import of newly made

Address: Department of Biochemistry, University of Dundee, Wellcome Trust Building, Dow Street, Dundee DD1 5EH, UK.

Correspondence: Angus I. Lamond
E-mail: a.i.lamond@dundee.ac.uk

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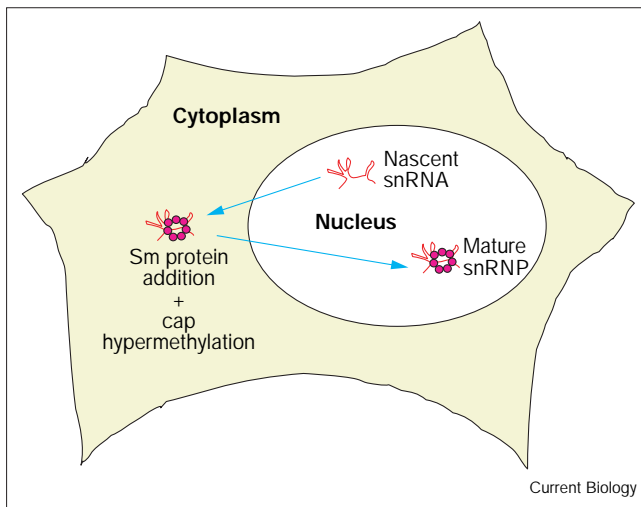
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snRNPs into the nucleus [18–21]. The Sm proteins [22] are a group of seven polypeptides (B/B', D1, D2, D3, E, F and G) [19,23] that form stable subcomplexes before their assembly onto snRNAs [24–26]. The Sm subcomplexes D1–D2 and E–F–G form a stable partial complex with snRNA, with the subsequent addition of D3–B/B' completing the core, seven-protein Sm snRNP complex [26]. It has recently been proposed that the Sm protein complex forms a seven-membered ring around the snRNA [27]. Additional events required for the production of a fully mature snRNP particle include the addition of snRNP-specific proteins and numerous base and sugar modifications of the snRNA itself [28]. The location of the snRNA modification reactions is unknown, although it has been demonstrated that a small nucleolar RNA (snoRNA) can act as a guide to direct 2'-O-methylation of U6 snRNA [29].

Immunostaining of cells with antibodies to Sm proteins demonstrates that snRNPs have a complex nuclear localisation, comprising a punctate staining pattern with an additional diffuse component. The punctate pattern is formed by snRNPs interacting with different subnuclear structures, including mainly interchromatin granule cluster speckles, perichromatin fibrils and coiled bodies [14,15]. The diffuse nucleoplasmic snRNP staining most likely

Figure 1



Maturation of a splicing snRNP. Following its transcription in the nucleus, a nascent snRNA is exported to the cytoplasm where it forms a complex with the core, Sm, proteins. Hypermethylation to form the TMG cap structure then takes place. Only then is the partially mature snRNP particle re-imported into the nucleus.

includes snRNPs actively involved in splicing of nascent transcripts [30–34].

Here, by fusing Sm proteins to fluorescent protein tags based on the green fluorescent protein (GFP), we analysed the interaction of newly assembled snRNPs with nuclear structures following their initial import into the nucleus. Nascent snRNPs were seen to accumulate first in coiled bodies and, only at later times, in speckles. The data are consistent with a directed movement of snRNPs from coiled bodies to speckles during their maturation.

Results

Expression of fluorescent-tagged versions of Sm proteins as single fusion proteins in a variety of cell types

To analyse the kinetics of snRNP localisation, we tagged three separate Sm proteins (SmB, SmD1 and SmE) with either GFP or yellow-shifted GFP (YFP). The tagged Sm proteins were chosen to include members of each of the three Sm protein subcomplexes that assemble into the seven-protein Sm complex with snRNA [24–26]. We have recently described plasmid pHGFP–SmE which expresses, under the control of the cytomegalovirus (CMV) promoter, the S65T mutant of human GFP (hGFP-S65T) fused in frame at the amino terminus of full-length human SmE [35]. Two additional plasmids were constructed. One expresses full-length human SmD1 tagged at its amino terminus with enhanced GFP (pEGFP–SmD1, see Materials and methods). The second expresses full-length human SmB tagged at its amino terminus with enhanced YFP (pEYFP–SmB, see Materials and methods). These

plasmids also used the CMV promoter to drive expression of the fusion protein (Figure 2a).

To characterise the expression of the two new fusion proteins, whole cell lysates were prepared from HeLa and 293 cells following transient transfection with plasmids pEYFP–SmB and pEGFP–SmD1. Approximately 80% of cells expressed the tagged Sm protein at the time of lysis (data not shown). The lysates were separated by SDS–PAGE, transferred to nitrocellulose membrane and probed with an antibody that recognises GFP and YFP (Figure 2b). Each of the fusion proteins was detected as a single band of the expected size. We conclude that any GFP/YFP fluorescence seen in cells transfected with these plasmids results from the fusion proteins, rather than from free GFP. Nitrocellulose membranes prepared in the same way were probed with the anti-Sm antibody Y12 [36] (Figure 2b). Endogenous SmB protein was detected in all cell lysates, with a larger band corresponding to the EYFP–SmB fusion protein detected only in lysates of cells expressing pEYFP–SmB. The intensity of the EYFP–SmB band was significantly lower than that of the endogenous SmB protein, indicating that there was not a vast overexpression of the Sm proteins in transfected cells. The incorporation of the tagged Sm proteins into snRNP particles was confirmed by affinity purification of snRNPs from transfected HeLa cells using a monoclonal antibody that recognises the TMG cap of the snRNA (Figure 2c). EGFP–SmD1 and endogenous Sm proteins were found exclusively in the affinity purified snRNP fraction, with none detected in the unbound fraction. In contrast, EGFP expressed alone was found exclusively in the unbound fraction, with none copurifying with the snRNPs. Additional experiments using anti-TMG cap antibodies indicated that both the EYFP–SmB and hGFP–SmE fusion proteins were also assembled into snRNP particles (data not shown, see also [35]).

GFP/YFP-tagged Sm proteins show the same localisation as endogenous Sm proteins

To determine the distribution patterns of the tagged Sm proteins, HeLa and MCF-7 cells were fixed 24 hours after transient transfection with pEYFP–SmB, pEGFP–SmD1 or pHGFP–SmE and analysed by confocal fluorescence microscopy (Figure 3, see also [35]). We saw a pattern of speckles, coiled bodies and diffuse staining, consistent with previously published Sm protein localisation data [14,15]. We conclude that each of the tagged Sm fusion proteins is expressed as a full-length protein, incorporated into snRNP particles, imported into the nucleus and localised in an identical pattern to endogenous snRNPs.

Newly made snRNPs accumulate in coiled bodies before speckles

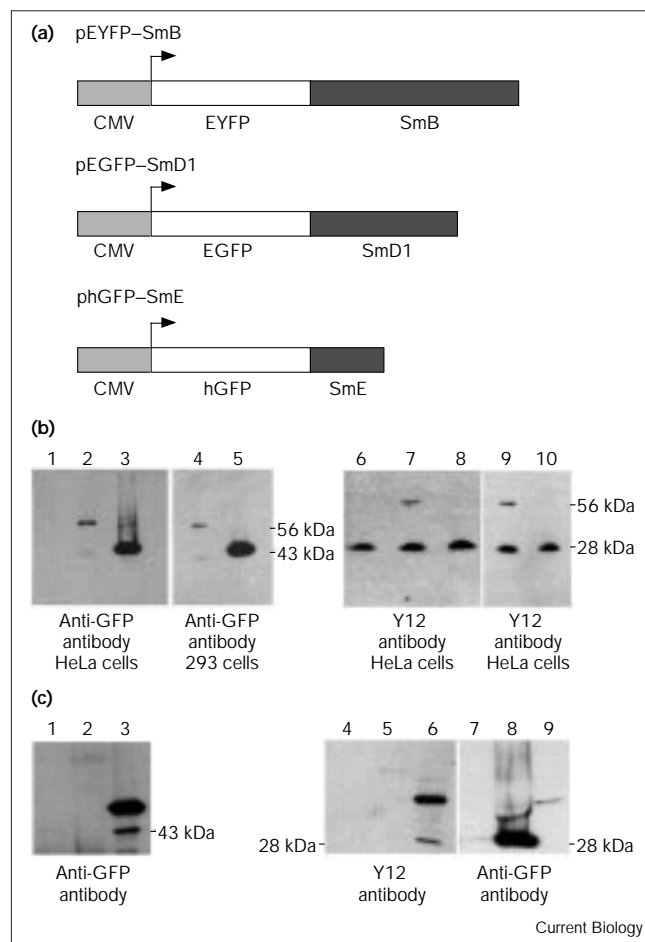
To specifically detect newly assembled snRNPs, we made use of the fact that Sm proteins must be assembled onto

Figure 2

GFP/YFP-tagged Sm proteins are expressed as single fusion proteins and assembled into snRNPs. (a) Schematic illustration of the expression plasmids used in this study. See the Materials and methods for details on how pEYFP-SmB and pEGFP-SmD1 were constructed. Plasmid pHGFP-SmE has been described previously [35].

(b) SDS-PAGE analysis of whole cell lysates from cells transiently transfected with pEYFP-SmB or pEGFP-SmD1. Lanes 1–3 and 6–8, lysates from HeLa cells transfected without DNA (lanes 1,6), or with pEYFP-SmB (lanes 2,7) or pEGFP-SmD1 (lanes 3,8); lanes 4,5,9,10, lysates from 293 cells transfected with pEYFP-SmB (lanes 4,9) or pEGFP-SmD1 (lanes 5,10). Lanes 1–5 were probed with a mixture of monoclonal antibodies to GFP (Roche); single bands were detected at the sizes predicted for EYFP-SmB (56 kDa) and EGFP-SmD1 (42 kDa). No bands were detected in control HeLa cells (lane 1) or 293 cells (data not shown) exposed to the transfection reagent in the absence of DNA. A duplicate blot (lanes 6–10) probed with the anti-Sm antibody Y12 allowed detection of the EYFP-SmB fusion protein in HeLa cells (lane 7) and 293 cells (lane 9). Cells transfected without DNA (lane 6) or with pEGFP-SmD1 (lanes 8,10) showed a doublet of bands at 28/29 kDa, representing endogenous SmB and SmB'.

(c) Affinity purification of snRNPs, using antibodies against the TMG cap, from HeLa cells transfected with pEGFP-SmD1 (lanes 1–6) or plasmid expressing EGFP alone (pEGFP-C1; lanes 7–9). EGFP-SmD1 was detected in the purified snRNP fraction (lane 3) using an anti-GFP monoclonal antibody, but was absent from the unbound fraction (lane 2) and from cell lysate incubated with control beads without anti-TMG antibody (lane 1). A duplicate blot stained with antibody Y12 confirmed that endogenous Sm proteins were also present specifically in the purified snRNP fraction (lane 6) but not in the unbound fraction (lane 5) or the control bead fraction (lane 4). Conversely, in a lysate from cells transfected with pEGFP-C1, EGFP was found only in the unbound fraction (lane 8) and was absent from the purified snRNP fraction (lane 9) and the control beads (lane 7). Additional bands in the snRNP fraction (lanes 3,6,9) result from cross-reaction of the anti-mouse secondary antibodies with the anti-TMG monoclonal antibody bound to the beads.

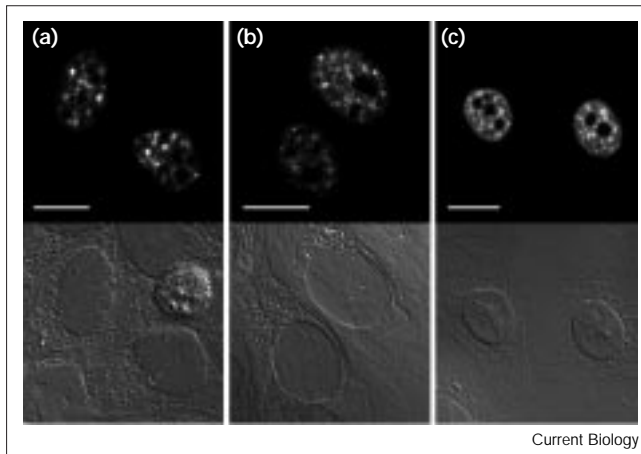


the newly synthesised snRNA in the cytoplasm before the nascent snRNP particle can be re-imported into the nucleus. Cells were analysed by confocal fluorescence microscopy at different times following micro-injection with plasmids encoding the respective tagged Sm fusion protein. Following injection, only a short burst of expression of the fusion protein was observed, most likely the result of rapid degradation of the naked plasmid DNA inside the cells. The observed pulse of expression facilitated kinetic studies. Newly assembled snRNPs, made fluorescent with the respective tagged Sm fusion protein, each showed similar, time-dependent, expression patterns both in fixed cells (Figure 4) and in live cells. The 'mature' punctate pattern, including speckles and coiled bodies (the pattern seen using antibodies to endogenous Sm proteins), was observed only at longer time points following microinjection (Figure 4d,i,n). Shortly after microinjection (1–4 hours), a weak diffuse signal was seen throughout the whole cell, including the cytoplasm (Figure 4a,f,k). Next, an accumulation in coiled bodies was evident (Figure 4b,g,l). This was confirmed by counterstaining with anti-p80 coilin antibodies (data not shown; see also below). Quantitation of the fluorescence indicated that

approximately 2% of the signal due to tagged Sm protein in the nucleus was concentrated in coiled bodies at this stage. This result is similar to that obtained previously using anti-sense probes to detect endogenous U2 snRNA [37].

The next pattern seen included localisation of the tagged Sm protein within nucleoli and in coiled bodies (Figure 4c,h,m). At these early stages (1–6 hours after microinjection), little or no accumulation of the labelled snRNP was detected in speckles, although a diffuse nuclear compartment was clearly visible. At 6–15 hours after microinjection, labelled snRNPs showed the 'steady state' punctate Sm protein distribution that colocalised with endogenous Sm proteins (Figure 4d,i,n and data not shown). This pattern included diffuse nucleoplasmic staining, speckles and coiled bodies. Later still, the association of labelled snRNPs with coiled bodies decreased (Figure 4e,j,o; see also below). At later time points, the cytoplasmic signal was also absent, indicating that expression of the tagged Sm fusion protein had ceased and that the existing fusion proteins had all been chased into the nucleus. These data show that newly made snRNPs accumulate in the coiled body and nucleolus before accumulation in speckles. The loss of fluorescent Sm signal from coiled bodies at later time points, when the cytoplasmic

Figure 3

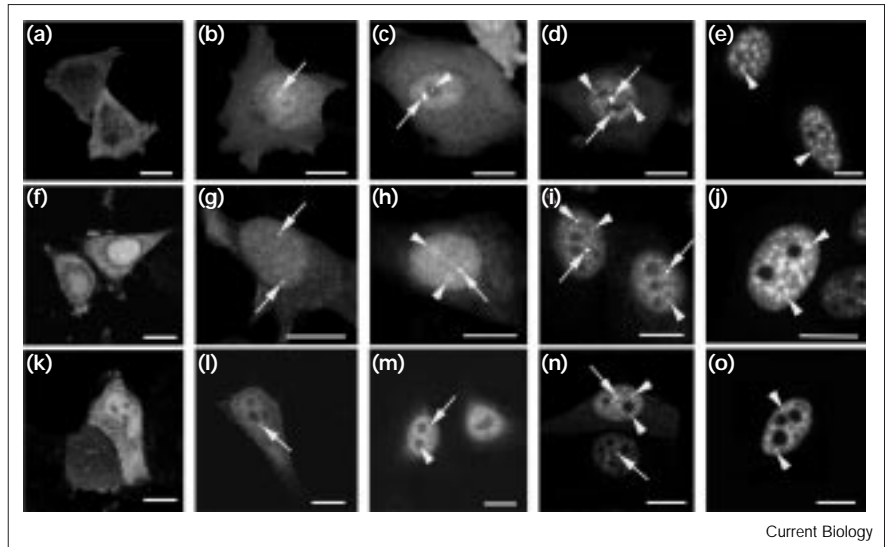


Sm proteins tagged with GFP or YFP show nuclear localisations identical to endogenous Sm proteins. Confocal fluorescence micrographs (upper panels) and corresponding Nomarski images (lower panels) of (a,b) MCF-7 cells and (c) HeLa cells transiently transfected with (a) pEYFP-SmB, (b) pEGFP-SmD1, or (c) pHGFP-SmE, and fixed 24 h after transfection. The bars represent 10 μ m.

signal was also no longer seen, suggests that this initial interaction with the coiled body is a transitory one.

Figure 4

(a–e) Three-dimensional projections of serial confocal sections through MCF-7 cells expressing pEYFP-SmB, fixed 1, 2, 4, 7 and 16 h, respectively, after microinjection. (a) After 1 h, EYFP-SmB was present throughout the cytoplasm and nucleus, with no preferential localisation to nuclear structures, but (b) 2 h after injection, there was some concentration in the coiled body (arrow). (c) After 4 h, many cells showed accumulation of EYFP-SmB in coiled bodies (arrow) and nucleoli (arrowhead) in addition to the diffuse signal throughout the cell. (d) By 7 h, most cells showed EYFP-SmB localisation to speckles (arrowheads) and the coiled body (arrows). The cytoplasmic signal was still seen. (e) By 16 h, most cells showed EYFP-SmB in nuclear speckles (arrowheads), with no pronounced accumulation in coiled bodies. The cytoplasmic signal was no longer seen. (f–j) Single confocal sections of MCF-7 cells expressing pEGFP-SmD1, fixed 1, 2, 4, 9 and 24 h, respectively, after injection. (f) EGFP-SmD1 was found throughout the cytoplasm and nucleus 1 h after injection, but (g) was concentrated in the coiled body (arrows) 2 h after injection. (h) Accumulation in coiled bodies (arrow) and nucleoli (arrowheads) was seen after 4 h. (i) After 9 h, EGFP-SmD1 localised to speckles (arrowheads) and coiled bodies (arrows).



(j) By 24 h, most cells showed EGFP-SmD1 in nuclear speckles (arrowheads), with no pronounced accumulation in coiled bodies. The cytoplasmic signal was no longer seen. (k–o) Single confocal sections of MCF-7 cells expressing pHGFP-SmE, fixed 4, 5, 6, 8 and 16 h, respectively, after injection. (k) After 4 h, hGFP-SmE was diffuse throughout the cytoplasm and nucleus (top cell), but (l) showed concentration in the coiled body

(arrow) 5 h after injection. (m) After 6 h, hGFP-SmE showed accumulation in coiled bodies (arrow) and nucleoli (arrowhead), and (n) after 8 h, localisation to speckles (arrowheads) and coiled bodies (arrows). (o) After 16 h, hGFP-SmE was seen in nuclear speckles (arrowheads), with no pronounced accumulation in coiled bodies. The cytoplasmic signal was no longer seen. The bars represent 10 μ m.

Indeed, preliminary evidence from fluorescence recovery after photobleaching (FRAP) experiments indicated that snRNPs move rapidly into coiled bodies and must, therefore, also leave coiled bodies rapidly, because the fluorescence signal did not continue to increase with time (our unpublished observations).

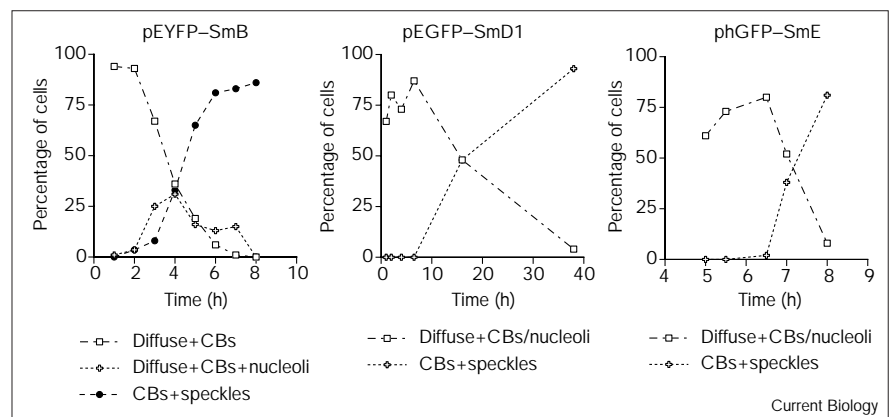
When the percentage of cells showing each localisation pattern is plotted against time, the early accumulation of labelled snRNPs in coiled bodies and nucleoli and later localisation to speckles is clear (Figure 5). The changing temporal localisation patterns are not caused by attaching the fluorescent-protein tag onto the Sm proteins, because we observed a similar time-dependent change in localisation pattern following micro-injection of a plasmid vector that expresses SmE tagged at its amino terminus with a Myc epitope tag (data not shown).

Kinetics of snRNP localisation in living cells

Time-lapse fluorescence microscopy of living HeLa cells expressing EGFP-SmD1 confirmed that the newly made snRNPs, labelled with GFP/YFP-tagged Sm proteins, accumulated first in coiled bodies then in the nucleolus (Figure 6a,b). Cells showing EGFP-SmD1 accumulation in coiled bodies were imaged once every 10 minutes over a 240 minute time course. An increase in EGFP-SmD1

Figure 5

Time-dependent localisation of GFP/YFP-tagged Sm proteins. The percentages of cells showing different localisation patterns of GFP/YFP-tagged Sm protein were plotted against time. Each data set was obtained by injecting 150 cells for fixation at each time point and counting every cell expressing GFP/YFP-tagged Sm protein. The data shown are representative of three separate experiments for pEGFP-SmD1 and pHGFP-SmE and two experiments for pEYFP-SmB. For pEYFP-SmB, cells showing signal in the nucleolus and in coiled bodies (CBs) have been plotted separately from those showing accumulation of EYFP-SmB in coiled bodies alone to emphasise that the nucleolar signal is seen later than the coiled-body signal.



signal in the nucleolus with time was observed. The EGFP-SmD1-containing coiled bodies maintained relatively constant positions in the nucleus during this time period, both with respect to each other and to the nucleoli. In a separate experiment, MCF-7 cells expressing hGFP-SmE and showing nucleolar accumulation of labelled snRNP, together with a low level of snRNP accumulation in speckles, were imaged every 20 minutes over 100 minutes (Figure 6c). During this time, the intensity of the signal in the speckles increased and these structures became more distinct. Preliminary quantitative analysis suggested that the percentage of fluorescent signal in speckles increased approximately twofold during this time course. These data confirm that newly made snRNPs show a temporal variation in localisation in living cells. Coiled bodies are the first site of accumulation of nascent snRNPs, with subsequent accumulation in the nucleolus and finally in speckles. Diffuse nucleoplasmic snRNP staining was seen throughout.

GFP-tagged non-snRNP protein splicing factors do not localise in coiled bodies and nucleoli at early time points

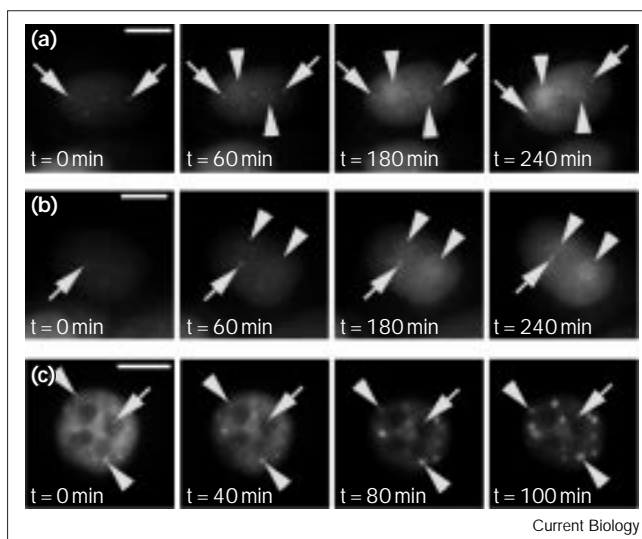
The initial targeting to coiled bodies and nucleoli appears to be a feature unique to Sm proteins, and is not seen with other non-snRNP protein splicing factors. For example, GFP-tagged splicing factors, such as ASF/SF2 (Figure 7), and GFP fusions to both subunits of U2AF (data not shown), were seen in nuclear speckles as soon as the GFP fluorescence was intense enough to be detected in the nucleus (2.5 hours after microinjection for ASF/SF2, 2 hours for U2AF35 and U2AF65). These protein splicing factors enter the nucleus faster than Sm proteins, probably because they do not have a requirement for pre-assembly onto snRNA for nuclear entry. We conclude that the initial concentration of Sm proteins in coiled bodies is not a feature of either nuclear proteins in general, or of all spliceosome factors.

The temporal patterns of Sm protein localisation do not result from a general disruption of nuclear structure

The dynamic temporal change in snRNP localisation was not caused by microinjection of the plasmid DNA as similar patterns of localisation of the GFP/YFP-tagged Sm proteins could be seen when cells were fixed at early time points following transient transfection. The expression of EYFP-SmB, EGFP-SmD1 and hGFP-SmE was less synchronous after transfection, as compared with microinjection, presumably because the cells are in contact with the DNA mixture for an extended period of time, take up the DNA with varying efficiency and therefore begin to express the exogenous genes at different times. Nevertheless, the use of transfection protocols allowed a much larger number of cells to be studied. Counterstaining of transfected cells transfected with the Y12 antibody, which recognises primarily SmB and SmB', and with anti-p80 coilin antibodies, confirmed that, at early time points, endogenous Sm proteins showed a pattern of staining in coiled bodies and speckles, and diffuse nucleoplasmic staining, identical to that seen in untransfected cells (Figure 8a-d and data not shown). In these cells, the fluorescent-labelled snRNP was diffusely distributed with accumulation in coiled bodies and nucleoli, but not in speckles. This shows that the early temporal localisation patterns of GFP/YFP-tagged Sm proteins do not result from a disruption of nuclear speckled structures caused by expression of the tagged proteins.

At late time points (64 hours after transfection), speckles containing both labelled and endogenous snRNPs were detected. Many cells expressing the tagged Sm proteins did not contain prominent coiled bodies when stained with Y12 or anti-coilin antibodies (Figure 8e-h and data not shown). Untransfected cells at late time points and both transfected and untransfected cells at all other time points contained an average of 2.7 coiled bodies per cell.

Figure 6



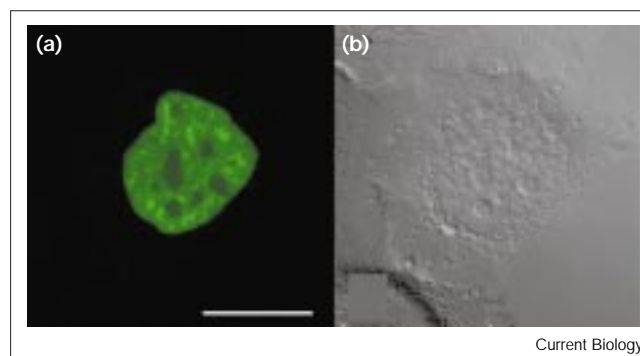
Time-lapse fluorescence microscopy demonstrates the sequential accumulation of newly made snRNPs. (a,b) Images of two different cells from a time-lapse sequence of HeLa cells transfected with pEGFP-SmD1. The images were taken every 10 min over a 240 min period. The cells initially showed a diffuse distribution of EGFP-SmD1 with additional accumulation in coiled bodies (arrows). Nucleolar accumulation was first apparent after about 60 min (arrowheads). The intensity of nucleolar signal increased throughout the time course. (c) Images from a time-lapse sequence of MCF-7 cells transfected with phGFP-SmE. The images were taken every 20 min over a 100 min period. Initially, a pattern of diffuse nucleoplasmic staining with some nucleolar accumulation and indistinct speckles was seen. During the time course, the speckles became more distinct, while the nucleolar accumulations remained unchanged. The bars represent 10 μ m.

In transfected cells at the 64 hour time point, the average was 0.6 coiled bodies per cell. It is possible that the formation of coiled bodies depends on the total level of snRNP expression, which may, in turn, be influenced by expression of tagged Sm proteins.

Mature GFP-tagged snRNPs return directly to 'speckles' following mitosis

We next determined whether the temporal pattern of Sm protein localisation was unique to newly assembled snRNPs, or rather was a general feature of snRNP import into the nucleus. To test this, we analysed the localisation of GFP-tagged Sm proteins in cells that had undergone mitosis and then reimported the previously assembled snRNPs into daughter nuclei. HeLa cells were fixed 48 hours after transient transfection with either pEGFP-SmD1 or phGFP-SmE. At this time point, the GFP-Sm signal was seen in speckles and coiled bodies. Mitotic cells were identified and analysed by confocal microscopy (Figure 9 and data not shown). In late telophase, the localisation pattern of re-imported EGFP-SmD1 and hGFP-SmE mirrored that of endogenous Sm proteins as

Figure 7



GFP-ASF/SF2 localises to nuclear speckles as soon as it is expressed. (a) Confocal micrograph and (b) corresponding Nomarski image of a living MCF-7 cell 2.5 h after microinjection with pGFP-ASF/SF2, encoding a fusion of ASF/SF2 to hGFP-S65T. Note the strong signal in nuclear speckles and absence of localisation to other nuclear structures. The bar represents 10 μ m.

confirmed by counterstaining with antibodies to endogenous Sm proteins (Y12) and to p80 coilin (data not shown). The speckled pattern reformed rapidly in late telophase and no preferential targeting of GFP-Sm proteins to coiled bodies was seen for snRNPs reimported after mitosis. This indicates that initial accumulation of snRNPs in coiled bodies following nuclear import is a feature of newly made snRNPs, rather than of GFP-tagged snRNPs *per se*.

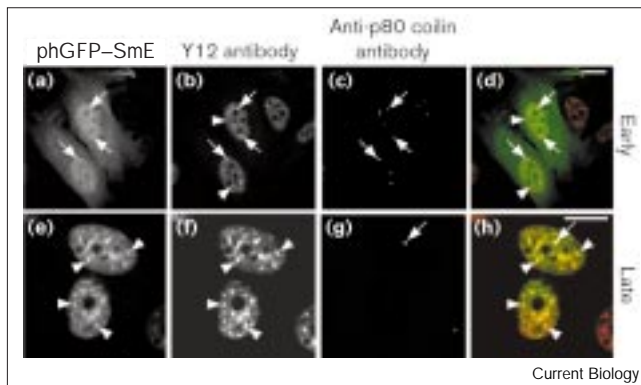
Discussion

A temporal dimension to snRNP localisation

In this study, we used GFP/YFP-tagged Sm proteins to study the kinetics of snRNP localisation when newly assembled snRNP particles first enter the nucleus. A relatively synchronous expression of the tagged protein was obtained by microinjecting the expression plasmids into HeLa or MCF-7 cells. Expression from the injected DNA template was temporary, resulting in a pulse of tagged-protein production. In both HeLa and MCF-7 cells, EYFP-SmB, EGFP-SmD1 and hGFP-SmE each showed similar changes in nuclear localisation that depended on the time of expression. Initially, the cells showed diffuse cytoplasmic and nucleoplasmic staining, then fluorescent-labelled snRNPs accumulated in coiled bodies and nucleoli (1–6 hours). At later times (6–15 hours), the GFP/YFP-tagged snRNPs appeared in the characteristic speckled pattern of endogenous snRNPs. Later still (beyond 24 hours), the labelled snRNPs showed only diffuse and speckled nuclear localisation and no longer concentrated in coiled bodies. By these late time points, the fluorescent Sm signal was entirely nuclear.

In contrast, when GFP-tagged non-snRNP protein splicing factors, such as ASF/SF2 and U2AF, were transiently expressed, they rapidly colocalised with their cognate

Figure 8



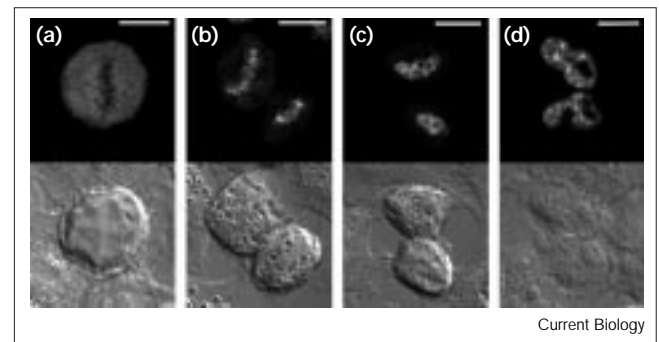
The observed localisation patterns of newly made GFP/YFP-tagged snRNPs do not result from disruption of nuclear structure. MCF-7 cells, transiently transfected with hGFP-SmE, were fixed (a–d) 16 h and (e–h) 48 h after transfection. The confocal fluorescence micrographs show (a,e) the fluorescence due to the GFP tag, and staining with antibodies to (b,f) Sm proteins (Y12 antibody) and (c,g) p80 coilin. (d,h) Overlay of (a–c) and (e–g) respectively. At 16 h, (a) the hGFP-SmE signal was diffuse throughout the cell, with clear accumulation in coiled bodies (arrows); (b) endogenous Sm proteins were, as expected, localised to speckles (arrowheads) and coiled bodies (arrows). (c) The anti-p80 coilin antibody confirmed the presence of coiled bodies (arrows). (d) The merged image shows hGFP-SmE colocalisation with endogenous Sm proteins in coiled bodies (arrows) but not speckles (arrowheads) at this early time point. (e) Later (48 h post-transfection), the hGFP-SmE signal was localised to nuclear speckles (arrowheads) and less frequently to coiled bodies. (f) Endogenous Sm proteins were also localised to nuclear speckles (arrowheads) but not to coiled bodies. (g) The anti-p80 coilin antibody showed that coiled bodies were present in only some of the cells (arrow). (h) The merged image shows colocalisation of hGFP-SmE with endogenous Sm proteins in speckles (arrowheads) at this time point. Coiled bodies were present in 60% of cells (arrow), but were not prominent. The bars represent 10 μ m.

endogenous proteins to form a speckled pattern and did not show time-dependent changes in localisation after entering the nucleus. Furthermore, the time-dependent change in snRNP localisation was not observed when mature snRNPs re-entered the nucleus after mitosis. We conclude that the sequential accumulation of snRNPs in coiled bodies and then in speckles is not a general consequence of nuclear import, but rather is a pathway for newly assembled snRNPs. We suggest that this pathway may have a role in snRNP maturation.

Accumulation of newly made snRNPs in coiled bodies

In the nucleus, accumulation of GFP/YFP-tagged snRNPs was first detected in coiled bodies and only later in speckles. It is possible that the punctate localisation pattern of mature labelled snRNP results from several independent pathways of import into the nucleus (that is, separate snRNP import pathways to coiled bodies, speckles and so on), each with different kinetics. The subsequent loss of labelled snRNP from the coiled body at later time points, when cytoplasmic signal is also absent, suggests, however,

Figure 9



Mature GFP-tagged snRNPs return directly to speckles after mitosis. Confocal fluorescence micrographs (upper panels) and corresponding phase-contrast images of (a) a metaphase cell expressing hGFP-SmE, 48 h after transient transfection, and (b–d) cells expressing hGFP-SmE at various stages of telophase. In (a), the hGFP-SmE signal is diffuse throughout the cell. In (b–d), hGFP-SmE returned directly to nuclear speckles following mitosis with no obvious accumulation in coiled bodies. The bars represent 10 μ m.

that there may be a flux of newly synthesised snRNPs through the coiled body. If the targeting of snRNPs to coiled bodies was simply faster, or of higher affinity, than targeting to the nucleolus or speckles, it might be expected that the interaction of labelled snRNPs with the coiled body would also outlast their interactions with other nuclear components and this is not the case. Furthermore, following mitosis, tagged Sm proteins were re-imported directly back into speckles, without showing any preferential localisation in coiled bodies at early time points. Again, this suggests that the observed accumulation of labelled snRNPs in coiled bodies at early time points does not result from more rapid kinetics of entry into, or a higher affinity for retention in, coiled bodies. The speckled structures are present in the nucleus and contain endogenous snRNPs at the early time points of GFP/YFP-tagged Sm protein expression. Despite speckles being more numerous and occupying a larger nuclear volume than coiled bodies, we show here that newly imported snRNPs do not initially concentrate in speckles. The data thus demonstrate that the distribution of snRNPs between coiled bodies and speckles is directed and not random.

Evidence for the association of coiled bodies with snRNA gene loci ([37–39]; reviewed in [40]) has been used to propose an autologous feedback mechanism, whereby a proportion of mature or partially mature snRNPs return to coiled bodies adjacent to snRNA genes and regulate their transcription [41]. A flux of newly made snRNPs through coiled bodies would provide a mechanism to link transcription of snRNA with maturation of snRNPs in such a feedback mechanism. The present data are consistent with this model. It is not clear whether all newly made snRNPs, or just some of them, pass through the coiled

body. Preliminary data using FRAP suggested, however, that labelled snRNPs flux quickly through the coiled body (our unpublished data). EYFP-SmB re-accumulated in coiled bodies within 1 minute following its bleaching with a 488 nm laser. Our initial evidence from time-lapse fluorescence microscopy indicated that coiled bodies containing newly made snRNPs do not show large movements, either relative to each other or to nucleoli. If coiled bodies are involved in a snRNP maturation pathway, this suggests that their role may be as sorting points, rather than transport vehicles. Nevertheless, we do not exclude the possibility that, under some circumstances, coiled bodies containing snRNPs also move through the nucleoplasm. We have recently recorded examples of coiled body movements in HeLa cells (M. Platani, J. Swedlow and A.I.L., unpublished data). Movement of nuclear coiled bodies has also been described in plants expressing GFP fused to the U2 snRNP B'' protein [42].

A role for coiled-body interaction with the nucleolus in snRNP maturation

GFP/YFP-tagged Sm proteins were first detected as a diffuse signal followed by accumulation in coiled bodies. In many cells, nucleolar accumulation was also seen. It is not clear whether snRNPs interact first with the coiled body and then with the nucleolus, or whether these are independent events. Both were early events, however, and preceded the appearance of tagged Sm proteins in speckles. Previous evidence points to a functional interaction between coiled bodies and nucleoli. For example, expression of mutants of the coiled-body protein, p80 coilin, can disrupt nucleolar structure [43]; conversely, mutant forms of the nucleolar protein, Nopp140 [44], affect coiled-body structure. In all tissue culture lines that we have tested, a proportion of cells show low levels of nucleolar localisation of snRNPs and p80 coilin. The present data suggest that these accumulations may specifically represent newly synthesised snRNPs. The level of snRNP in nucleoli can be increased significantly either by inhibition of protein phosphorylation with okadaic acid or by transient expression of a p80 coilin point mutant with a single serine to aspartate substitution (S202D) [35,45]. Thus, protein phosphorylation may have a role in regulating snRNP localisation in nucleoli. These observations will be used in future studies to investigate whether a pathway exists for the directed movement of snRNPs between coiled bodies and nucleoli.

It has been reported recently that fluorescently labelled U3 snoRNA, following its injection into the nuclei of *Xenopus* oocytes, accumulates in sphere organelles (also known as *Xenopus* coiled bodies) before nucleoli [46]. The authors suggested a role for the *Xenopus* coiled body in the biogenesis and/or intranuclear transport of nucleolar snoRNAs, and the work provides further evidence for functional interactions between coiled bodies and nucleoli. The *Xenopus* data are consistent with our present studies on

splicing snRNPs and the proposed role for the mammalian coiled body in the maturation of nuclear RNP particles.

Nuclear speckles

In interphase nuclei, some nucleoplasmic splicing snRNPs localised to speckles, which correspond largely to clusters of interchromatin granules [14,15]. These nuclear speckles were the last sites in which newly synthesised snRNPs, detected by tagging with fluorescent protein, accumulated following entry into the nucleus. Analysis of a GFP-tagged version of the non-snRNP protein splicing factor ASF/SF2 showed that it localised rapidly to speckles without prior accumulation in coiled bodies. The endogenous ASF/SF2 also showed speckled staining along with a diffuse nucleoplasmic pool. Recruitment of ASF/SF2 from speckles to sites of active transcription has been demonstrated [47,48]. Additionally, overexpression of Clk/Sty or SRPK1/2 protein kinases, which have been shown to phosphorylate ASF/SF2, results in a decrease in the localisation of ASF/SF2 in speckles [49–52]. Our present data implicate speckles as the probable end point of a putative snRNP maturation pathway. The snRNPs accumulating in speckles are therefore likely to be mature. This is consistent with the recent evidence showing that active splicing factors are able to cycle between speckles and sites of RNA polymerase II transcription.

Conclusions

The complex localisation pattern of splicing snRNPs includes speckles and coiled bodies in addition to a diffuse nucleoplasmic pool. This study suggests that the association of snRNPs with different nuclear structures may result, at least in part, from differences in the maturity of the snRNPs, with newly made snRNPs accumulating in the coiled body and nucleolus, whereas more mature snRNPs accumulate in speckles. The existence of a nuclear trafficking pathway for snRNP maturation raises many interesting questions concerning the mechanism of snRNP movement and how coiled bodies and other subnuclear structures are assembled and regulated. The Sm fusion proteins described here may be useful for future studies addressing nuclear dynamics in living cells.

Materials and methods

Plasmid constructs

The full-length human SmB cDNA was fused in frame with the EYFP coding sequence as an *EcoRI*–*SalI* fragment in pEYFP-C1 (Clontech) to make pEYFP-SmB. The full-length cDNA for human SmD1 was fused in frame with the EGFP coding sequence as a *BamHI*–*PstI* fragment in pEGFP-C1 (Clontech) to make pEGFP-SmD1.

Cell culture and transfection assays

HeLa, 293 and MCF-7 cells, obtained from ATCC, were grown in Dulbecco's modified Eagles' medium supplemented with 10% foetal calf serum and 100 U/ml penicillin and streptomycin (Life Technologies). For immunofluorescence assays, cells were grown on coverslips and transfected using Fugene 6 (Roche) according to the manufacturer's instructions. For the preparation of cell lysates, cells were grown in 10 cm diameter dishes and transfected using a modified calcium-phosphate-mediated transfection

procedure with 1 µg/ml DNA per plate [53,54]. The cells were incubated for 48 h before lysis.

Preparation of cell lysates and immunoblotting

Transfected cells were washed twice with ice-cold PBS and then lysed in 0.5 ml 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 repeatedly passed through a 21G hypodermic needle to break up the DNA and then cleared by centrifugation for 15 min at 4°C and 13000 × g. Lysates were electrophoresed on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes for immunoblotting. Primary antibodies used were anti-GFP mouse monoclonal antibody (Roche) and Y12 anti-Sm mouse monoclonal antibody [36].

Immunoprecipitation of snRNP complexes

Lysates from HeLa cells expressing EGFP–SmD1 or EGFP were incubated for 1 h on a shaking platform with 20 µl agarose beads coupled to anti-TMG antibodies (Calbiochem) or control, uncoupled beads. The beads were washed with 20 mM HEPES pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 10% glycerol. The remaining lysate (unbound fraction) was precipitated with 20% trichloroacetic acid. The beads and unbound lysate were separated on an 8% SDS–polyacrylamide gel and transferred to nitrocellulose membranes for immunoblotting with anti-GFP and anti-Sm (Y12) antibodies.

Fixation and immunofluorescence

Cells were washed in PBS and fixed for 5 min in 3.7% (w/v) paraformaldehyde in CSK buffer (10 mM PIPES pH 6.8, 10 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 2 mM EDTA) at room temperature. Permeabilisation was performed with 1% Triton X-100 in PBS for 15 min at room temperature. Immunofluorescence staining was carried out as described [55]. Antibodies used were anti-Sm monoclonal antibody (Y12) [36] (dilution 1:500) and 204,10 rabbit polyclonal anti-p80 coilin antibody [43] (dilution 1:350). TRITC-conjugated goat anti-mouse and Cy5-conjugated goat anti-rabbit secondary antibodies were used (Jackson Immunochemicals). Fluorescence microscopy of fixed cells was carried out using a Zeiss LSM 410 confocal laser-scanning microscope. Excitation wavelengths were 488 nm (EGFP/EYFP), 543 nm (TRITC) and 633 nm (Cy5).

Microinjection of constructs expressing fluorescent-protein-tagged Sm, and observation of live cells

Plasmid DNA was diluted to 20 µg/ml with 100 mM glutamic acid pH 7.2 (with citric acid), 140 mM KOH, 1 mM MgSO₄ and 1 mM DTT before injection into living MCF-7 or HeLa cells using an Eppendorf 5242 microinjector. Cells were maintained at 37°C in a POC chamber (Helmut Saur) in Dulbecco's modified Eagles' medium without phenol red. Time-lapse sequences were taken using a Zeiss axioptan epifluorescence microscope equipped with a KAF-1600, 1532 × 1025 pixel cooled CCD camera (Digital Pixel Advanced Imaging Systems) using IP lab image acquisition software. Exposure times of 200 msec were used.

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