

Nuclear organization of pre-mRNA splicing factors

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The splicing of mRNA precursors (pre-mRNA) in the nucleus is catalyzed by a complex machinery termed the spliceosome. In order to understand how it functions *in vivo*, it is essential to complement biochemical analyses with a detailed study of how spliceosome components are organized within the nucleus.

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Abbreviations

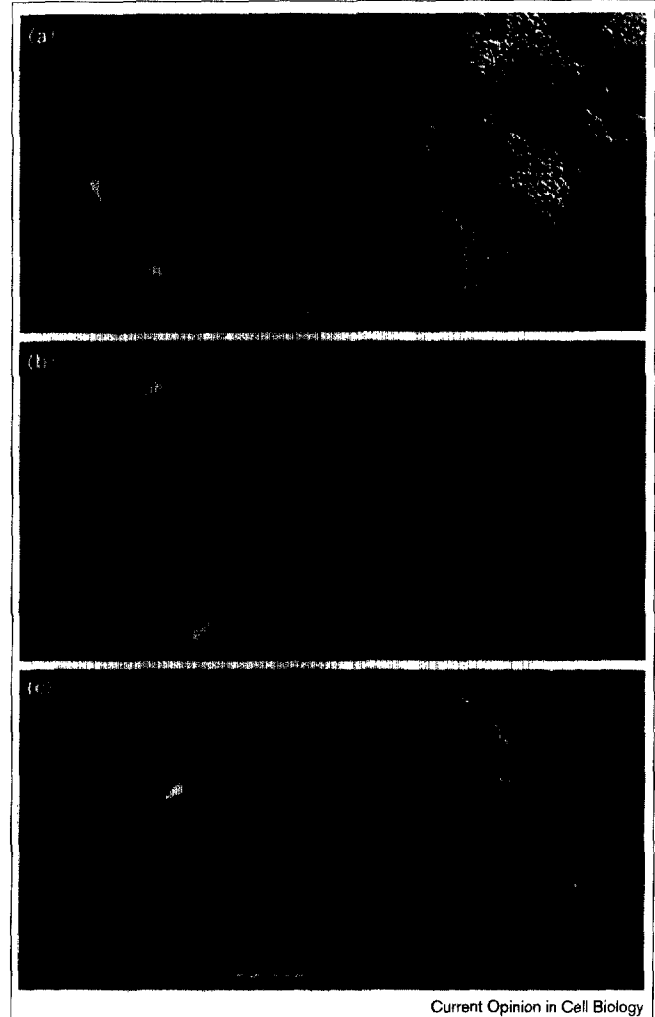
BR	Balbani ring
GFP	green fluorescent protein
PPI	protein phosphatase 1
SF3b	Splicing factor 3b
SMA	spinal muscular atrophy
SMN	survival of motor neuron
snoRNP	small nucleolar ribonucleoprotein
snRNA	small nuclear RNA
snRNP	small nuclear ribonucleoprotein
SR	Ser-Arg

Introduction – overview of splicing factor localization

Splicing of pre-mRNA takes place in the nucleus by a two step transesterification mechanism catalyzed by the spliceosome. The major subunits of the spliceosome are the U1, U2, U5 and U4/U6 small nuclear ribonucleoprotein particles (snRNPs, pronounced 'snurps') which assemble with additional protein splicing factors to form the active complex. Each snRNP comprises one or two small nuclear RNAs (snRNAs), a group of seven common snRNP proteins called Sm proteins and additional proteins specific to each snRNP (for a review see [1]). The spliceosome is a dynamic structure that goes through cycles of assembly and disassembly during the splicing of each intron. Most pre-mRNAs are probably spliced co-transcriptionally while the nascent transcripts remain associated with the site of transcription. In some cases the pre-mRNA either diffuses, or is transported, away from its cognate gene first and splicing occurs elsewhere in the nucleoplasm.

Not all splicing factors *in vivo* are always bound to pre-mRNA and assembled into active spliceosomes. Because the proportion of 'active' splicing factors can vary, staining with an antibody that recognises a splicing factor will not only detect spliceosomes and sites of splicing, it can also label sites of assembly, transport or storage of spliceosome components. Splicing factors show both

Figure 1



'Speckled patterns' – the nuclear organization of splicing factors. Detection of splicing factors in paraformaldehyde-fixed human cells. **(a)** Confocal fluorescence micrograph of MCF-7 cells stained with an antibody to the SR protein, SC-35 [59], together with the corresponding DIC (differential interference contrast) image. α -SC-35 gives a pattern of very pronounced speckles (arrows), with a small amount of diffuse nucleoplasmic staining. **(b)** Confocal fluorescence micrograph of MCF-7 cells stained with an antibody (rabbit polyclonal serum 856 [60]) to the U1 snRNP-specific protein, U1A, together with the corresponding DIC image. U1A staining shows speckles (arrows) together with a significant amount of diffuse nucleoplasmic signal. **(c)** Confocal fluorescence micrograph of MCF-7 cells stained with an antibody (mouse monoclonal Y12 [61]) to the core snRNP Sm proteins together with the corresponding DIC image. In addition to the 'speckles' and diffuse nucleoplasmic compartment, Sm proteins localize to coiled bodies (arrow). Bar = 10 μ m.

diffuse and punctate (speckled) staining because they associate with several different subnuclear structures (Figure 1).

A comparison of immunofluorescence and immunoelectron microscopy data shows that splicing factors are located in clusters of interchromatin granules, perichromatin fibrils, interchromatin granule-associated zones, coiled bodies and gems. Splicing factors can also move between these structures. The pattern of staining can vary for different splicing factors or for the same factor in different cell types or under different growth conditions. For example, when cells are treated with transcription inhibitors that block the production of splicing substrates, the diffuse staining disappears and virtually all the splicing factors move into 10–15 large clusters. Most of the splicing factors assembled into active spliceosomes probably form the diffuse staining component (largely perichromatin fibrils) that chases away when transcription is inhibited. Most active genes are either excluded from, or at the external periphery of, 'speckles' (largely clusters of interchromatin granules). These punctate structures may act as storage sites for inactive splicing factors although it is possible that they also play other roles in the splicing pathway. A major goal of current research is to characterize the assembly and function of the different splicing factor compartments and to determine mechanisms involved in the movement of splicing factors between them. For relevant reviews see [2–8]. Here we review recent progress in analyzing the nuclear organization of splicing factors. We have given priority to studies that emphasize the relationship between nuclear structure and biological function.

Analysis of splicing factors

The distribution of snRNPs and protein splicing factors has been analysed using either antibodies for immunofluorescence studies, or *in situ* hybridization with DNA or RNA probes to detect snRNAs (reviewed by [9,10]). The increasing availability of cDNA clones encoding splicing factors also allows their detection as epitope-tagged proteins following transfection into cells. A particularly exciting development in this area has been the recent use of green fluorescent protein (GFP) fusions to detect splicing factors in living cells [11,12,13*]. Although most splicing factors show a punctate or speckled pattern (Figure 1), snRNPs are usually also detected in coiled bodies, while most non-snRNP protein splicing factors are not.

Recent studies have reported the identification and cloning of new components of the splicing apparatus and investigated their nuclear organization. A large scale analysis of purified mammalian spliceosomes, using combined mass spectrometry and database searching, identified 19 new human genes encoding spliceosome associated proteins [14*]. All the new splicing factors tested showed the characteristic speckled pattern of localization. A separate analysis of nuclear structural proteins in *Xenopus* identified a novel conserved 146 kDa nuclear protein that was shown to associate with the splicing machinery *in vitro* and is likely to be a component of the splicing factor complex SF3b (splicing factor 3b) [15,16]. It was not found in coiled bodies, but otherwise colocalized with Sm proteins *in vivo*.

A speckled staining pattern that colocalizes with splicing factors has been reported for a nuclear form of Protein 4.1 [17]. Most of Protein 4.1 is found in the cytoplasm where it binds to the cytoplasmic side of the cell membrane and links transmembrane proteins to the spectrin/actin cytoskeleton. The nuclear form is the 4.1E isoform and biochemical data indicate that it can interact with the splicing factor U2AF35 [18*]. The authors suggest that this nuclear isoform might be a structural component of splicing factor domains rather than an active splicing factor.

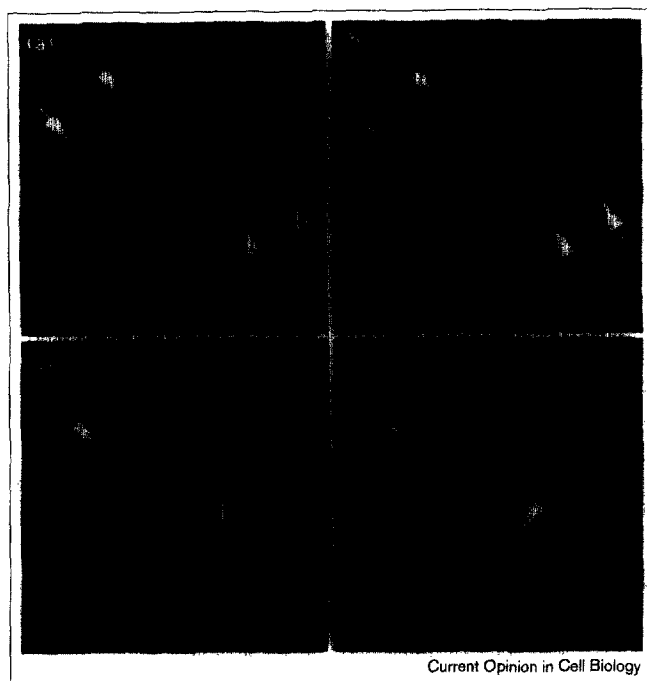
Splicing factors in *Chironomus* salivary gland nuclei show mostly diffuse staining with little or no accumulation in speckles. This is clearly different to plant or mammalian cells but is consistent with the view that active splicing factors are largely in the diffuse compartment. This may reflect the highly specialized nature of gene expression in these cells. *Chironomus* hrp23 is a component of Balbiani ring (BR) RNPs, related in structure to SR (Ser-Arg) protein splicing factors and the RBD-Gly RNA binding proteins involved in many types of RNA processing reaction [19*]. Immunoelectron microscopy showed that hrp23 joins nascent BR transcripts and is released immediately prior to their translocation through the nuclear pore complex. Interestingly, hrp23 — a novel protein, which is structurally related to proteins involved in mRNA splicing and other RNA processing reactions — was also detected in nucleoli, apparently dependent upon interaction with RNA. There is increasing evidence that the nucleolus may play roles in aspects of nuclear function other than ribosome production (reviewed in [7]).

Coiled bodies

Coiled bodies are a class of nuclear body that contain splicing snRNPs and a human autoantigen p80-coilin, which is widely used as a specific marker of these structures. Coiled bodies vary in size (~0.2–1.5 µm) and in number (~1–10) in different cell types and under different growth conditions. Changes in coiled body organization can be used as a marker of cell differentiation, as shown for rat hippocampal neurons [20], bovine lens cells [21] and *Arabidopsis* root tip epidermis [22]. They contain nucleolar antigens, including fibrillarin, Nopp140 and small nucleolar ribonucleoproteins (snoRNPs) but not rRNA, pre-mRNA, DNA or non-snRNP protein splicing factors such as SR proteins. They are, therefore, unlikely to be sites of either splicing or ribosome production. Several recent studies present evidence that coiled bodies have a role in controlling snRNP production and/or transport.

In some cell types a dramatic association between coiled bodies and snRNA gene loci has been detected, prompting the idea that coiled bodies may provide a feedback mechanism for modulating the expression of snRNA genes [4]. This idea is supported by a recent study showing that artificial tandem arrays of human U1 and U2 snRNA genes can colocalize with coiled bodies. Colocalization was dependent on transcription of the exogenous snRNA gene

Figure 2



Coiled bodies and gems – two sides of the same coin? Detection of coiled bodies and gems in different strains of HeLa cells. (a) Confocal fluorescence micrograph of HeLa cells (from American Tissue Culture Collection) stained with anti-SMN (MANSMA1, mouse monoclonal) to detect gems. (b) The same cells stained with anti-p80-coilin (rabbit polyclonal serum 204,10 [27]) to detect coiled bodies. p80-coilin shows diffuse nucleoplasmic staining in addition to coiled bodies. SMN shows cytoplasmic staining in addition to gems. In these cells the coiled bodies and gems colocalize, (arrows in [a] and [b]). (c) Confocal fluorescence micrograph of HeLa cells (strain D) stained with anti-SMN. (d) The same cells stained with anti-p80-coilin. In these cells, coiled bodies and gems associate less closely and are sometimes seen as completely separate structures (arrows in [c] and [d] point to a coiled body with no anti-SMN staining, arrowheads point to a gem with no anti-p80-coilin staining). Bar = 10 μ m.

array and upon it containing snRNA gene sequences [23**]. The association of coiled bodies with snRNA genes may thus be mediated by snRNAs rather than by DNA sequences or DNA-bound proteins. This may explain why several transcription factors required for snRNA expression are detected in coiled bodies [24,25*].

An alternative idea is that coiled bodies have a role in transporting snRNPs between the nucleolus and nucleoplasm [26,27]. This could explain the frequent association between coiled bodies and the nucleolar periphery. Selective disruption of nucleoli and coiled bodies caused by expression of certain p80-coilin mutants indicates a functional relationship between the two structures. The nucleolar protein Nopp140 might play a role in the mechanism linking coiled bodies with nucleoli, possibly acting as a shuttling factor between the two structures [28**]. Transiently expressed Nopp140 accumulates first in nucleoli and only later in coiled bodies. Nopp140 can also

interact with p80-coilin in a yeast two-hybrid assay [28**]. Transient expression of a gene encoding a Nopp140 mutant containing only the carboxyl terminus caused the dispersal of nucleoplasmic coiled bodies.

In considering a link between nucleoli and coiled bodies, it is interesting that a small nucleolar RNA, mgU6-77, can act as a guide RNA to direct 2'-O-methylation of both 28S rRNA and U6 snRNA [29**]. If nucleolar modifying enzymes act on snRNAs as part of the snRNP maturation pathway, coiled bodies might play a role as transport or sorting organelles that ferry new snRNPs to and/or from the nucleolus [26,27]. Evidence for movement of coiled bodies both into and out of nucleoli has been reported. A reduction in the association of p80-coilin with nucleoli, caused by acute osmotic stress in rat neurosecretory neurons, might represent exit of coiled bodies from the nucleolus [30]. More recently, a U2 snRNP B"-GFP fusion protein was used to visualize coiled bodies in plant nuclei moving through the nucleoplasm towards nucleoli and entering the nucleolar periphery (K Boudonck, P Shaw, personal communication).

A family of coiled-body related structures?

Nuclear structures in *Xenopus* oocytes that were originally called sphere organelles have been renamed 'coiled bodies' because they contain splicing snRNPs and the sph-1 (sphere-1) protein, which is related in sequence to p80-coilin [31,32,33*]. A subset of both mammalian coiled bodies and *Xenopus* sphere organelles are found associated with histone gene loci [34,35]; however, it is not certain whether all somatic coiled bodies are identical to sphere organelles. The term 'coiled body' may include a population of related structures some of which have different protein components and perform different functions. For example, an immunofluorescence study of coiled bodies in endothelial cells reported variation in their protein composition [36*]. It is possible that one class of coiled body acts to regulate snRNA gene expression, another functions at histone gene loci and another shuttles components to the nucleolus.

The name 'gem' (gemini of the coiled body) was given to a nuclear body that contained the survival of motor neuron (SMN) protein [37]. Mutations in the SMN gene are responsible for the inherited motor neuron degenerative disease, spinal muscular atrophy (SMA). Initial studies indicated that gems were distinct from coiled bodies, though they behaved similarly and were often found closely associated. More recent data point to a closer relationship and in many cell types coiled bodies and gems appear to be the same structure (Figure 2; for discussion see [3]). Gems may thus represent an example of coiled body heterogeneity. Biochemical studies on the SMN protein have shown that it interacts both with Sm proteins and with SIP1, which is a homologue of the yeast Brr1 gene product [38]. The SMN-SIP1 complex has an essential role in splicing snRNP biogenesis in *Xenopus* oocytes whereas mutations in Brr1 affect snRNP biogenesis in yeast [39,40]. These data support a role for gems/coiled bodies in snRNP maturation. Interestingly, transient

expression of a gene encoding an amino terminal deletion mutant of the SMN protein results in an altered pattern of snRNP localization and gem/coiled body structure [41**]. The same amino-terminal SMN deletion mutant inhibits splicing *in vitro*. SMN was not found amongst the spliceosome associated proteins reported by Neubauer *et al.* [14*] but a novel SMN-related protein, (S10), was identified in the spliceosome [14*]. Further insights into the relationship between coiled bodies/gems, SMN and pre-mRNA splicing are likely to emerge in the near future.

Pathways involving reversible protein phosphorylation

Reversible protein phosphorylation on serine/threonine residues affects the localization of splicing factors (reviewed in [42]). Several kinases have been shown to phosphorylate the SR family of protein splicing factors [43–48,49*,50]. Upon transient overexpression of either the Clk/Sty or SRPK1/SRPK2 (SR protein kinase 1/SR protein kinase 2) kinases, splicing factor localization becomes more diffuse. Transcriptional activation of genes results in the relocation of splicing factors from speckles to sites of transcription [12]. For SR proteins, this relocation depends upon the presence of serine phosphorylated Ser–Arg repeats [51**].

Nuclear protein phosphatase activities can also influence splicing factor localization. For example, experiments using permeabilized HeLa nuclei showed that inhibition of a nuclear protein phosphatase I (PP1) activity prevented the movement of splicing factors into speckles upon subsequent inhibition of transcription. Addition of the PP1 catalytic subunit to transcriptionally active nuclei increased the diffuse distribution of splicing factors [46]. Treatment of living cells with low levels of the selective serine/threonine-specific protein phosphatase inhibitor, okadaic acid, results in snRNP-containing structures resembling coiled bodies forming within nucleoli. A similar effect results from transient expression of a myc- or GFP-tagged mutant of p80-coilin Ser202→Asp, suggesting that protein phosphorylation may modulate interactions between splicing snRNPs and the nucleolus [13*,52]. Two nuclear PP1 binding proteins, NIPP1 and p99/PNUTS, have been identified in fractionated nuclear extracts and the genes encoding them cloned [53–56]. NIPP1 colocalizes in the nucleus with splicing factors [57*]. Biochemical experiments show that NIPP1 is in a complex containing Sm proteins whereas mutation of the PP1 binding site in the gene encoding NIPP1 produces a dominant-negative mutant protein that can inhibit splicing *in vitro*. It is likely that multiple phosphorylation pathways influence splicing factor localization and may act to regulate splicing during the cell division cycle and/or in response to metabolic stimuli. In this regard it is interesting that cyclin E has been found to associate with components of the pre-mRNA splicing machinery [58*].

Conclusions

Splicing factors are highly dynamic *in vivo* and interact with different subnuclear structures. As yet we do not

have a detailed understanding of the roles of each structure, or of the mechanisms whereby splicing factors move between them. Future advances in this area will be aided by the development of new assay systems, either in living or semi-permeabilized cells, which facilitate functional studies on nuclear organization. An important step in this direction has been the advent of GFP fusion proteins and their application to the analysis of splicing factors in living cells. Another important goal for the future will be the biochemical isolation of specific subnuclear bodies that contain splicing factors and the characterization of their components.

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