SURVEY AND SUMMARY

The assembly of a spliceosomal small nuclear ribonucleoprotein particle

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ABSTRACT

The U1, U2, U4, U5 and U6 small nuclear ribonucleoprotein particles (snRNPs) are essential elements of the spliceosome, the enzyme that catalyzes the excision of introns and the ligation of exons to form a mature mRNA. Since their discovery over a quarter century ago, the structure, assembly and function of spliceosomal snRNPs have been extensively studied. Accordingly, the functions of splicing snRNPs and the role of various nuclear organelles, such as Cajal bodies (CBs), in their nuclear maturation phase have already been excellently reviewed elsewhere. The aim of this review is, then, to briefly outline the structure of snRNPs and to synthesize new and exciting developments in the snRNP biogenesis pathways.

THE STRUCTURE OF SPLICEOSOMAL snRNPs

The removal of the most abundant class of introns requires the five major spliceosomal small nuclear ribonucleoprotein particles (snRNPs; U1, U2, U4, U5 and U6). Each snRNP consists of a uridylic acid-rich small nuclear RNA (U1, U2, U4, U5 and U6 snRNAs) that is post-transcriptionally modified and a cortege of associated proteins (Table 1) (1–4). The 2,2,7-trimethyl guanosine (m_3G) capped U1, U2, U4 and U5 snRNAs (Sm snRNAs) contain an Sm site ($RAU_{3-6}GR$, where R is a purine) flanked by stem-loops, which collectively constitute domain A (5). Sm proteins (B/B′, D1, D2, D3, E, F and G) assemble into a heteroheptameric ring around the Sm site to form the core of the snRNP particle. Similarly, the γ-methyl triphosphate (γ-m-P_3) capped U6 snRNA acquires a heteroheptameric ring of LSm proteins (Like Sm). The LSm proteins (LSm2-8) assemble around the U6 snRNA 3′-terminus, which consists of a uridine tract ending in a 2′, 3′-cyclic phosphate (U_4-2′, 3′cP_i) (6–10). Proteins of the L/Sm lineage share an ancient signature motif, the Sm fold. Indeed, orthologs are ubiquitous in all three domains of life and participate in a multiplicity of RNA processing events (11–14). In addition to the core proteins, each snRNP is decorated with an ensemble of proteins unique to a given snRNP, the snRNP-specific proteins (4).

There are three variations in the Sm core structure worthy of mention here. First, SmB and SmB′ proteins are produced from an alternatively spliced transcript in mammals (15–17). In yeast, however, the single ortholog (Smb1) does not have splice variants (8). Second, mammalian SmN is a paternally expressed paralog (deleted in Prader–Willi Syndrome) of SmB/B′ for which it substitutes in brain and heart tissue (18–20). Finally, in the unicellular protist, *Trypanosoma brueci*, U2 and U4 snRNAs associate with specific spliceosomal Sm proteins (SSm) as well as a subset of canonical Sm proteins (20).

The mono-snRNPs just described do not represent their *in vivo* functional forms; rather, they are organized into higher order particles. The U4, U5 and U6 snRNPs exist largely in their functional form as a U4/U6. U5 tri-snRNP (21,22). The same holds true of the U4atac, U5 and U6atac snRNPs, which form the minor spliceosomal U4atac/U6atac.U5 tri-snRNP (23). Interestingly, minor spliceosomal counterparts of the U1 and U2 snRNPs, the U11 and U12 snRNPs, respectively, are known to assemble into the minor spliceosomal U11/U12 di-snRNP (23–25). Furthermore, penta-snRNP complexes, which consist of all five major splicing snRNPs and may represent a 'splicing holoenzyme', have been shown to exist in both yeast and humans (26–28). Although the physiological relevance of the penta-snRNP remains controversial (29), the fact that there is some degree of pre-assembly of the splicing machinery is well accepted.

The snRNPs, along with over 300 other splicing factors, assemble onto pre-mRNA to form the spliceosome, and it is this dynamic macromolecular machine that orchestrates the excision of introns and the ligation of exons through two successive transesterification reactions (30,31). Prior to participating in splicing, however, snRNPs must be assembled through a series of intricate steps that, in all organisms, begins in the nuclear compartment.

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Table 1. The composition of major mature spliceosomal snRNPs in *H. sapiens*

snRNP	12S U1	17S U2	25S U4/U6.U5		
	Ul	U2	U4	U5	U6
snRNA					
length (nt)	164	186	142	116	106
RNAP	II	II	II	11	111 |
Type	Sm	Sm	Sm	Sm	LSm
5′Cap	m_5G	m_3G	m_3G	m_3G	γ-m-P_3
3′end	3′OH	3′OH	3′OH	3′OH	U_4-cP_i
m^6A	0	1	0	0	1
m^2G	0	0	0	0	1
2′-O-m	3	10	4	5	8
Ψ	2	13	3	3	3
Heptameric Core/ Ring Proteins	B/B′ Dl D2 D3 E F G	B/B′ Dl D2 D3 E F G	B/B′ Dl D2 D3 E F G	B/B′ Dl D2 D3 E F G	Lsm2 Lsm3 Lsm4 Lsm5 Lsm6 Lsm7 Lsm8
Specific Proteins	Ul-A Ul-C U1-70K	U2-A′ U2-B″ SF3a SF3b P14 SF2/ASF hPrp5p hPrp43 SR140 CHERP U2AF Hsp60 Hsp75 PUF60 SPF45 SPF31 BRAF35	15.5K 61K	15K 40K 100K 102K 116K 200K 220K 27K[t] 65K[t] 110K[t]	20K[d] 90K[d] 60K[d]

Details regarding the snRNA are indicated, as well as the RNA polymerase responsible for its synthesis. m_3G = 5′- to 5′-linked 2,2,7-trimethyl guanosine triphosphate cap. γ-m-P_3 = γ-methyl triphosphate cap. U_4-cP_i = U_4-2′, 3′-cyclic phosphate tail. m^6A and m^2G = methlylation of exocyclic amine on A or G bases, 2′-O-m = 2′-O-methylated ribose, Ψ = pseudouridine, d = U4/U6 di-snRNP specific, t = tri-snRNP specific.

In animalia, protista and plantae, a brief transit to the cytoplasm is essential for the assembly of Sm snRNPs, but the assembly of the U6 snRNP is uninterrupted by a cytoplasmic phase (reviewed in 32,33). In contrast, it is thought that assembly of snRNPs in fungi proceeds entirely within the nucleus (33). This may not hold true for the U5 snRNP; however, as a recent study suggests that the U5 snRNP has a cytoplasmic maturation phase (34). In the discussion to follow, we describe the cellular trafficking and maturation of spliceosomal snRNPs in vertebrates, where this process is best understood, in the context of several discrete organelles, Cajal bodies (CBs; 4,35–39), splicing factor compartments (SFCs, 40–42), nucleoli (40,42) and the newly described U bodies (45).

THE BIOGENESIS OF SPLICEOSOMAL snRNPs

The assembly of all spliceosomal snRNPs begins with the transcription of a U snRNA. The genes for the U snRNAs reside in the nuclear genome and are transcribed by either RNA polymerase (RNAP) II or III (46–48). Interestingly, while there has been significant divergence in the length and sequence of the coding portion of Sm snRNAs from primitive eukaryotes to eumetazoans, both have been well conserved for the U6 snRNA (49). During evolution, multiple copies (20–100) of U1, U2, U4 and U5 genes have arisen by gene duplication; however, the U6 gene is present only in approximately five functional copies in the haploid human genome (50–54). Major clusters of the human U1 and U2 genes are present on chromosomes 1 and 17, respectively (51,52); whereas, the U6 genes are scattered throughout the genome (54). The relative sparseness of U6 genes, the essential role of the U6 snRNA in splicing and the numerous interactions occurring along most of its length explain the high degree of conservation of its primary sequence (49).

Notably, the studies that resulted in the above estimates of the number of the U snRNA genes were conducted either by Southern blot analysis or with BLAST searches on incompletely sequenced genomes. Furthermore, BLAST hits that did not conform exactly to the most abundant form (the only one known at the time) of the U snRNA were discarded. Thus, genes that encode developmentally expressed or rare forms of the U snRNAs were not taken into account. Interestingly, preliminary BLAST results on the completed human genome suggest that we may have underestimated the number (by a factor of 3) and chromosomal dispersion of U snRNA genes (our unpublished data). Empirical approaches coupled with bioinformatics studies that use completed genomic databases and take into consideration transcription regulatory elements and polymorphic variations should provide new insights into the chromosomal organization and phylogeny of U snRNA genes and pseudogenes.

As mentioned previously, the assembly of Sm snRNPs and the U6 snRNP follow two distinct pathways, which are presented in Figures 1 and 2, respectively. Individual reactions of these consensus pathways are known to occur in either the nucleus or the cytoplasm and are compartmentalized as such in both figures. Regulatory steps of the nucleo-cytoplasmic exchanges of snRNP complexes are, thus, also indicated as they directly impact snRNP biogenesis. This complex intra-cellular trafficking associated with the regulation of snRNP biogenesis is further highlighted by the interactions of snRNP complexes with several functional domains of the nucleus, CBs, nucleoli and SFCs, which are also represented in Figures 1 and 2. We will now discuss successively both cellular pathways, which despite their many differences have the same functional outcome, namely the formation of mature and nuclear spliceosomal snRNPs.

SM snRNP Biogenesis

Synthesis of pre-snRNAs by RNAPII

The Sm snRNAs are transcribed as 3′-extended (2- to 10-nt longer) precursors by RNAPII, and like all other RNAPII transcripts, they co-transcriptionally acquire a 5′- to 5′-linked N7-methyl guanosine (m_1G) cap (55,56).
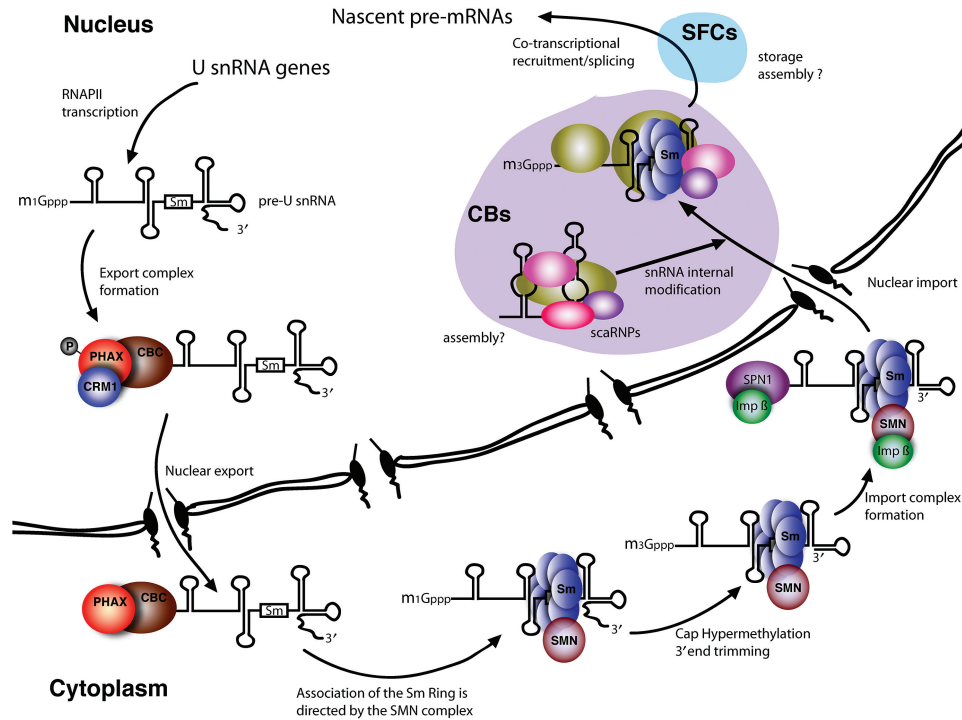
Figure 1. The Sm snRNP assembly and maturation pathway. The U2 snRNA was used here as a representative member of the Sm snRNAs. While discussed in the text, the U bodies are not shown here because of a lack of understanding of their role(s) in snRNP maturation, if any. scaRNP = small Cajal body specific ribonucleoprotein particle.
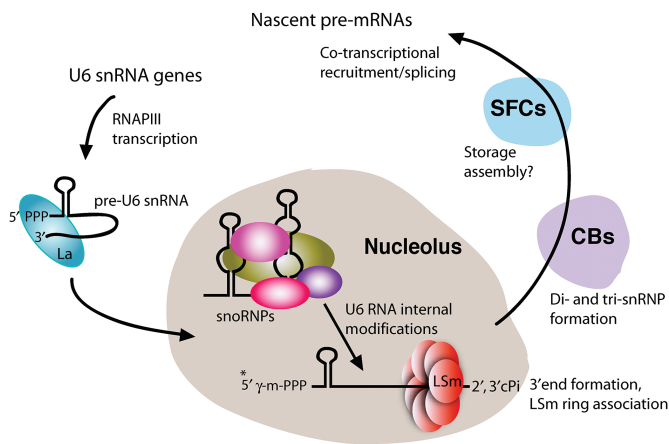

Figure 2. The U6 snRNP assembly and maturation pathway. (*) Note that the U6 snRNA in the nucleolus displays a $5'$-γ-m-P_3 cap. It is not clear, however, where and when the $5'$-capping reaction is occurring. snoRNP = small nucleolar ribonucleoprotein particle.

Although it has not been formally demonstrated, the nuclear cap binding complex (CBC) likely associates with m_1G capped U snRNAs co-transcriptionally, as it does with m_1G capped pre-mRNAs (57,58). Sm snRNA genes contain an snRNA-specific TATA-less core promoter, the proximal sequence element (PSE, -55 nt), which drives basal levels of transcription (59). The PSE helps define the $+1$ transcription start site (60) and recruits the snRNA-specific general transcription factor, PSE-binding transcription factor/snRNA gene activating protein

complex (PTF/SNAPc) (61). High levels of transcription require an upstream enhancer region, the distal sequence element (DSE, -220 nt), which consists of an OCT site and one or more closely positioned SPH sites, which recruit the POU domain-containing Oct-1 (59,62) and Zn finger-containing Sp1/Staf (63,64) transcriptional activators, respectively. Since different U snRNA variants are present in different tissues and different developmental stages, clearly other regulatory elements, yet to be identified, are responsible for their expression (65–67).

The $3'$-ends of the pre-snRNAs are generated by an RNA processing event that is coupled to PSE-directed transcription, rather than to a transcriptional termination event (68–70). A conserved $3'$-box (GTTTN$_{0-3}$AA APuNNAGA, where Pu = purine and N = any nucleotide) marks the cleavage site which resides $\sim$10-nt upstream (71–73), and a heterododecameric metallo β-lactamase complex (Integrator) contains the enzymatic activity for $3'$-end formation (74). The Integrator was identified as a component of RNAPII holoenzymes and was found to associate with the C-terminal domain (CTD) of its largest subunit, RPB1 (74). Accordingly, $3'$-end cleavage was inhibited when the phosphorylation status of the CTD was altered (75–77). Interestingly, two subunits of the Integrator are paralogous to components of the cleavage and polyadenylation specificity factor (CPSF) complex (74). Indeed, many similarities exist between $3'$-end formation in pre-snRNAs and pre-mRNAs. More recently, phosphorylation of serine 7 of the CTD was shown to be specifically required for the expression of Sm snRNAs and the recruitment of the integrator complex (78).

Are CBs involved in regulating pre-snRNA synthesis?

CBs have been observed to associate with a specific set of gene loci in diptera, amphibians and mammals with high frequency (reviewed in 38). Included in this group are the U snRNA genes (79–81). In HeLa cells, roughly 45% of U2 loci and 25% of U1 loci examined associated with CBs (81). U4 and the minor splicing U11 and U12 snRNA genes also associated with CBs although with reduced frequency, but the U6 locus showed no preference for CB association (82). It remains to be examined as to whether the U5, U4atac and U6atac loci associate with CBs. Although it is unlikely that the association of CBs with gene loci is merely fortuitous given the specificity and frequency of interactions, the physiological relevance of this association is not immediately clear.

Investigations into the requirement for transcription and nascent snRNA at the U2 locus have resulted in conflicting conclusions. A study conducted in HeLa cells suggested that neither transcription of endogenous U2 genes nor nascent U2 snRNA is required for the association of CBs (81). In contrast, it was found that the frequency of association of CBs with artificial arrays of U1 and U2 genes was proportional to the level of transcription, and in the case of U2 genes, the association was dependent on the coding sequence of the U2 snRNA (83,84). In the latter studies, CBs were further speculated to play a role in regulating set point snRNA levels, possibly by negative feedback inhibition (84). A more recent study further supports this idea by showing that pre-existing CBs associate with an artificial array of U2 genes only after the induction of transcription (85). In another work, the finding that RNAPII, PTFγ and TATA box binding protein (TBP) were found in domains associated/overlapping with both CBs and U2 gene loci was used to advance the argument that CBs may be positive regulators of snRNA transcription (86).

The nuclear export of pre-snRNAs

The newly transcribed pre-snRNAs must be transported to the cytoplasm to continue their maturation, necessitating the assembly of an export competent complex (32,87). To this effect, the nuclear CBC, consisting of CBP20 and CBP80, first associates with the m_1G cap of the RNA (88,89). Next, the phosphorylated adaptor for RNA export (PHAX) binds the CBC–RNA complex (90,91). The export receptor, Exportin 1/Chromosome Region Maintenance 1 (Xpo1/CRM1), recognizes the export adaptor, PHAX, in its phosphorylated form bound to its CBC/pre-snRNA cargo and binds to this complex together with RanGTP (90,91). While all of the above interactions are individually quite weak, cooperative binding ensures the formation of a stable export complex. The possibility that CBs may participate in the formation of an export competent pre-snRNA has been suggested by the detection in CBs of pre-U2 snRNAs (81), PHAX (83,92,93) and Xpo1 (92–94). In addition, fluorescently labeled U2 snRNAs injected into the nucleus of *Xenopus laevis* oocytes accumulate in CBs prior to export (95). Whether other pre-U snRNAs are found in CBs has not been investigated yet.

It was shown recently that the assembly of an export competent mRNA begins at the transcriptional unit (96,97). It will be interesting to see if symmetry exists between RNAPII transcribed coding and non-coding RNAs. In particular, the co-transcriptional recruitment of PHAX to snRNA gene loci would suggest that the assembly of an export competent pre-snRNA also begins co-transcriptionally. Differences in the transcriptional machinery could then account for the differences in the export pathways for U snRNAs and mRNAs. In essence, the fate of the RNA would be governed by the promoter structure of its corresponding gene.

After assembly, the entire complex translocates through the nuclear pore complex (NPC). Radiolabeling experiments have demonstrated the presence of labeled pre-snRNAs in the cytoplasm within 4 min after a pulse of P-32 UTP (55,98,99). Therefore, their transcription, 3′-end formation, assembly into an export complex, putative transit through CBs and translocation through the NPC are rapid processes.

The cytoplasmic phase: assembly of core Sm snRNPs

Upon entry in the cytoplasm, PHAX is dephosphorylated by protein phosphatase 2A (90,91,100), but remains associated with the CBC/pre-snRNA complex, presumably until the m_1G cap is hypermethylated (91,100). This association may potentially prevent an illicit interaction with the translation initiation factor eIF4E, which would target the RNA for translation. CBC and PHAX are subsequently recycled to the nucleus, where the latter is phosphorylated by casein kinase 2 (CK2) to initiate another round of pre-snRNA export (100).

The cytoplasmic phase of snRNP maturation is orchestrated by a large 20S assemblysome called the survival of motor neuron protein (SMN) complex, which consists of the SMN, seven distinct Gemin proteins (Gemin 2-8), and several other protein factors (101–103). Mutations in the human telomeric SMN gene (*SMN1*) results in spinal muscular atrophy (SMA), an autosomal recessive condition characterized by the degeneration of the motor unit. A discussion of SMA, its etiology, and its selective influence on the motor unit can be found elsewhere (104–106). Interestingly, two members of the galectin family (galactose-binding lectins), galectin-1 and galectin-3, associate with the SMN complex through Gemin 4. While there is evidence that these galectins associate with spliceosomes and are critical in pre-mRNA splicing *in vitro*, their role in the biogenesis of snRNPs remains an exciting but unexplored possibility (reviewed in 107).

The SMN complex participates in all three snRNP maturation events in the cytoplasm: (i) the assembly of an Sm ring onto the Sm site; (ii) the hypermethylation of the m_1G cap; and (iii) the trimming of the pre-snRNA's 3′-end. Indeed, the SMN complex associates with a distinct set of snRNP populations, each representing different stages in their cytoplasmic maturation: a disassembled export complex, the core Sm snRNP and an import complex (108).

(i) The assembly of the core snRNP begins with the formation of the Sm ring around the Sm site. Although

the Sm proteins do not form rings in the absence of the snRNA, they exist as dimers (B/B′-D3, D1-D2) or trimers (E-F-G) (109). First, the SMN complex facilitates the formation of a semi-stable open ring complex consisting of D1-D2-E-F-G proteins around the Sm site of pre-U snRNAs (109,110). Then, the SMN complex completes the formation of a 7-membered ring (-D3-B/B′-D1-D2-E-F-G-) upon integration of the B/B′-D3 heterodimer (109,110). While the Sm core can be assembled *in vitro* on essentially any RNA with a short stretch of uridines, the SMN complex likely serves as a specificity factor—in addition to an assembly factor—that ensures the assembly of the Sm ring only on RNAs with the appropriate snRNP code (111). The WD repeat containing subunit of the SMN complex, Gemin 5, recognizes this code on the snRNA, which consists of the Sm site and parts of the adjacent stem-loop structure(s) (112). The U1 snRNA is distinct in that its code consists of stem-loop I (SL1) (102,113). SL1, however, is not a strict requirement as an SL1-deleted U1 snRNA still acquires its Sm complement and is recruited to the nucleus (96).

SMN binds with high affinity to symmetric dimethyl arginines (sDMAs) in the arginine–glycine rich (RG) motif of B/B′, D1 and D3 through its tudor domain (114–118). SMN may, thus, directly transfer Sm subcomplexes onto the Sm site. Gemin 6 and 7 contain a non-canonical Sm fold and form a heterodimer (119). An interesting hypothesis is that this Gemin 6/7 heterodimer serves as a surrogate to B/B′-D3 in order to stabilize the open ring intermediate (119). Given that all components of the SMN complex, with the exception of Gemin2, associate with Sm proteins (102), how the SMN complex contributes to the assembly of the Sm ring onto an Sm site is difficult to evaluate mechanistically. Recent crystal structures of SMN subcomplexes (116,117,119) and interaction maps of the SMN complex (120) should facilitate future studies.

It is clear that the methylation status of the Sm proteins influences their interaction with the SMN assemblysome, and there have been important new developments in our understanding of Sm protein methylation. Asymmetric and symmetric dimethylations of arginine residues are catalyzed by type I and type II protein arginine methyltransferase (PRMTs), respectively. Each of these modifications are encountered on the RG motifs of Sm proteins. Symmetric dimethylation was long thought to be exclusively catalyzed by the type II PRMT, PRMT5 (121–123). More recently, two other type II PRMTs, PRMT7 and PRMT9, have been shown to also symmetrically dimethylate Sm proteins in mammals (124,125). The same was also demonstrated in flies for PRMT7 (126). In contrast, the enzyme(s) involved in synthesizing asymmetric DMAs on Sm proteins *in vivo* remains to be determined. However, PRMT4, a type I PRMT, is a good candidate since it was shown to asymmetrically dimethylate SmB *in vitro* (127).

Interestingly, while sDMAs were detected on both nuclear and cytoplasmic Sm proteins, aDMA were only detected in nuclear Sm proteins (128), which may bear important functional significance. For instance, sDMA appear to be important for core snRNP assembly (124,129), possibly through interactions with the tudor domain of SMN, as noted earlier. These modifications might not be essential, however, since methylation mutants of SmD3 are still integrated into core snRNPs (130). In contrast, aDMA may serve a role in the complex trafficking of snRNPs through various nuclear compartments and/or in the regulation of alternative splicing (127).

(ii) The hypermethylation of the m_1G cap occurs after the assembly of the Sm ring. Trimethyl guanosine synthase 1 (Tgs1), an SMN complex-associated methyltransferase, recognizes SmB/B′ in the context of an Sm core as well as the m_1G cap on the snRNA and subsequently transfers two methyl groups to position 2 of the m_1G cap forming the m_3G cap (131,132). Since the addition of B/B′-D3 heterodimer completes the assembly of the Sm ring, the association of Tgs1 to B/B′ ensures that only snRNAs with fully assembled Sm rings are hypermethylated.

(iii) Nucleolytic trimming of the 3′-end of the pre-snRNA generates the mature length snRNA. Sm core assembly is required for 3′-end trimming (133,134). However, whether 3′-end trimming is required for nuclear import remains debatable (56,135–137), and the factor(s) responsible for this 3′-end maturation event has yet to be identified in metazoans. However, the presence of a ladder of m_3G capped RNAs spaced 1-nt apart from the pre-snRNA to the mature snRNA in the cytoplasm suggests that the enzyme is primarily a cytoplasmic 3′- to 5′-exonuclease (56,138,139). In addition, several endonucleases are required to generate the mature 3′-end of U snRNAs in yeast (140,141) and possibly in mammals (142). Interestingly, smaller ladders have also been observed in the nucleus, suggesting that 3′-end maturation is completed in the nucleus (56,139,143). Supporting this idea is the finding that the interferon-stimulated gene product of 20 kDa (ISG20), a 3′- to 5′-exonuclease component of the exosome, co-immunoprecipitates with the SMN complex and several U snRNAs in nuclear fractions (144). In addition, the nuclear exosome is known to be required for the maturation of U snRNAs in yeast (140).

While the molecular mechanisms regulating the cytoplasmic maturation events of snRNAs have been extensively studied, the spatial arrangement of these events within the cytoplasm remains poorly understood. A recent study suggests that snRNP maturation might partly occur in discrete cytoplasmic bodies (45). These organelles were named the 'U bodies' because they contain the major U snRNPs. While they were described for the first time in *Drosophila* oocytes, similar structures were also found in many other cell types, including cultured human and amphibian cells (45) and, thus, may be universal organelles. Importantly, the enrichment of SMN within the U bodies and their association with P bodies directly implicates them in snRNP metabolism.

The nuclear entry of assembled snRNPs

The core snRNP must be brought into the nucleus to continue its maturation and, afterwards, participate in pre-mRNA splicing. The requirements for nuclear

import vary depending on the particular snRNP and on the cell system. In general, the m$_3$G cap and the Sm core are considered to be nuclear localization signals (NLS) that utilize the same import receptor importin β (Imp β) but distinct import adaptors, snurportin-1 (SPN1) and possibly SMN, respectively. While the m$_3$G cap-dependent pathway is well understood (reviewed in 145), much less is known about the Sm-dependent pathway. Remarkably, the SMN complex was shown to be sufficient, in presence of Imp β, for the nuclear entry of snRNPs in digitonin permeabilized HeLa cells (146,147). These data strongly suggest two essential properties of the SMN complex: (i) the SMN complex can serve as an adapter between the Sm core snRNP and the nuclear import machinery; (ii) the SMN complex can participate in the nuclear import of snRNPs independently from the SPN1 pathway. The latter property is further supported by the fact that SPN1 and Imp β, in an identical experimental system, was shown to be sufficient for the nuclear import of snRNPs (148). It was also proposed that the release of the Sm cargo upon nuclear entry is mediated by the CB signature protein coilin. Indeed, coilin was shown to associate with SmB and to compete with SMN for binding SmB *in vitro* (149). In addition, coilin was previously proposed to target snRNPs to CBs (150–152).

The nuclear phase: formation of mature snRNPs

The nuclear phase is the least understood part of the entire snRNP biogenesis pathway, and it involves a multiplicity of processes and factors, as well as trafficking to several subnuclear domains. In particular, extensive internal modifications of the U snRNAs by 2′-*O*-methylation and pseudouridylation represent a critical step in the making of a fully functional snRNP. The requirement of such modifications was especially well demonstrated for the U2 snRNP both *in vitro* (153), and *in vivo* (154). Using HeLa cell extracts, Donmez *et al.* demonstrated that the modifications within the first 20 residues of U2 snRNA do not prevent the assembly of a 17S U2 snRNP, but are critical for the formation of the early spliceosomal E complex. In the *Xenopus* oocyte, Yu *et al.* (95) showed that internal modifications of U2 snRNA are important for the assembly of the 17S U2 snRNP and splicing. Several domains such as CBs (32), the nucleolus (95) and the nucleoplasm (155), were directly implicated in the regulation of these internal modifications. CBs appear to play a predominant role as they were shown to contain a novel class of guide RNAs called small CB-specific RNAs (scaRNAs), which direct the 2′-*O*-methlylation and pseudouridylation of the Sm snRNAs (133). In addition, CBs may also be the site where snRNP-specific proteins are acquired (156). Finally, the assembly of the U4/U6 di-snRNP and U4/U6.U5 tri-snRNP may occur in or be facilitated by CBs (157,158). Eventually, snRNPs are recruited to SFCs where further packaging and/or storage together with other splicing factors may occur and are subsequently released when required for spliceosomal assembly (reviewed in 40).

U6 snRNP Biogenesis

Synthesis of pre-U6 snRNA by RNAPIII

Like the biogenesis of Sm snRNPs, the biogenesis of the U6 snRNP begins with the synthesis of its RNA component. The transcription of the pre-U6 snRNA is directed by many of the same *cis*-acting sequences as the Sm type RNA genes. In particular, U6 snRNA genes contain a PSE and DSE that are structurally similar and functionally interchangeable with that of the Sm snRNA genes (159–161). A major difference, however, resides in the presence of a TATA box within the U6 gene promoter, and while the molecular mechanisms are not yet understood, the PSE, DSE and TATA box of the U6 gene presumably act in concert to specify the recruitment of RNAPIII. Indeed, when the TATA box is deleted from the U6 snRNA gene, transcription is directed by RNAPII. Reciprocally, when the U6 TATA box is placed within the promoter of the Sm snRNA genes, they switch polymerase specificity to RNAPIII. Surprisingly, it was recently found in HeLa cells that RNAPII accumulates ~300-bp upstream of the U6 genes that are actively transcribed by RNAPIII (162). However, RNAPII was not enriched on inactive U6 genes. These results collectively suggest an involvement of RNAPII in U6 pre-snRNA synthesis. A requirement of RNAPII activity was further evidenced by the fact that the transcription of the U6 genes was inhibited by the RNAPII inhibitor α-amanitin. This result contrasts sharply from an earlier work, which established that a specific inhibition of RNAPII activity with α-amanitin (1 μg/ml) has little or no effect on U6 transcription (163). This work was carried out in S100 HeLa cell extracts, however, which could be a source of experimental differences. It is currently speculated that active RNAPII recruits chromatin-modifying enzymes to make the U6 promoter accessible to RNAPIII (162). While both Sm and U6 snRNA genes require TBP for their transcription, each requires a TBP-associated factor (TAF) that is distinct from each other and from other RNAPII and III genes, respectively (164). The TBP–TAFs complex for U6 genes has been identified as TFIIIB-α (165). The promoter for the U6 snRNA genes is referred to as a Type III RNAPIII promoter and is shared by other small metabolically stable RNA genes, such as U6atac and 7SK RNAs (reviewed in 46,166). Type III promoters represent an unusual class of RNAPIII promoters in that they are extragenic sequences; whereas, Type I (box A and box B, 5S rRNA genes) and Type II (box C, tRNA genes) promoters are both intragenic control elements.

Unlike that for Sm pre-snRNAs, the formation of the 3′-end of the pre-U6 snRNA is a transcription termination event, rather than an RNA processing event. The terminal poly(U) sequence serves as a transcription termination signal, which is the general mechanism encountered for all RNAPIII genes (46). After pre-U6 snRNA is transcribed, it undergoes many of the same maturation events that pre-Sm snRNAs undergo. In the case of the pre-U6 snRNA, however, it is thought that maturation is confined exclusively to the nucleus. Interestingly, U6 snRNP was demonstrated in the cytoplasm of yeast

heterokaryons (167) and mouse fibroblast cell lines (168), and more recently, within the cytoplasmic U bodies of *Drosophila* oocytes (45). Whether the cytoplasmic presence of the U6 snRNP is required for its maturation or bears a new functional significance is not known.

U6 snRNP maturation

After the transcription of the pre-U6 snRNA, the La auto-antigen binds both its 5′ and 3′-ends. La binds the 5′-triphosphate cap through its Walker A box (169) and the 3′-uridine tract through its winged-helix motif (170). The association of the La protein is believed to protect a subset of RNAPIII transcripts from ribonuclease degradation and to target them to nucleoli (171). A poly(U) polymerase (U6 Terminal Uridyl Transferase, U6-TuTase) (172–174), enriched in nucleoli, and an U6-specific 3′-exonuclease (175) extend and trim, respectively, the 3′-tail of the pre-U6 snRNA to form the mature length U6 snRNA. The mature 3′-end is eventually generated by the formation of a $2′, 3′cP_i$ (176). However, the factor(s) responsible for the required enzymatic activity have yet to be identified. A specific 130 kDa methyltransferase catalyzes the addition of a methyl group to the 5′-triphosphate cap to form the mature 5′-end, a γ-m-P_3 cap (177). The formation of γ-m-P_3 cap and $2′, 3′ cP_i$ tail precludes the association of the La protein (169,178). However, the LSm proteins are able to associate with this modified 3′-end to form the core domain of the U6 snRNP (7). Unlike the Sm proteins, the LSm proteins form a heteroheptameric ring complex in the absence of RNA (7). Thus, LSm core assembly onto the U6 snRNA may very well be a single step process. Importantly, the LSm proteins were recently demonstrated to serve as a nuclear retention signal for the U6 snRNA (179,180). Like the Sm snRNAs, the U6 snRNA is 2′-*O*-methylated and pseudouridylated. However, these modifications are guided by small nucleolar (sno) RNAs, rather than scaRNAs (181,182). It is well established that the U6 snRNA transiently localizes to nucleoli after its transcription and prior to accumulating in CBs (183). The snoRNA- and U6-TuTase-directed modifications of the U6 snRNA most likely occur within the nucleolus (172,182). Given that the U6 snRNP also localizes to CBs (183), it is still possible that some modifications also occur in these nuclear domains. The order and compartmentalization of U6 snRNA metabolism remains an active area of investigation.

BEYOND MONO-snRNPs

The two complex maturation pathways that we just described result in the assembly of individual snRNPs containing a complement of Sm or LSm proteins, respectively. In the cell nucleus, however, another level of assembly of the splicing machinery occurs and snRNP-containing complexes are formed prior to spliceosomal assembly. To which extent and where in the nucleus preassembly of the splicing machinery occurs is still unclear. It is a critical regulatory step to consider, however, as it directly precedes the recruitment of snRNPs to nascent transcripts.

The U4, U5 and U6 snRNPs are known to assemble into a U4/U6.U5 tri-snRNP prior to engaging their pre-mRNA substrates. The formation of the U4/U6 di-snRNP occurs through the extensive base pairing of the U4 and U6 snRNAs followed by the addition of di-snRNP-specific proteins (184). Tri-snRNP assembly follows with the incorporation of the U5 snRNP into the U4/U6 di-snRNP and the association of tri-snRNP-specific proteins (184). The only known U6 snRNP-specific protein is SART3/p110/Prp24p (185). The absence of SART3 in the U4/U6 di-snRNP and the enhanced formation of di-snRNPs in the presence of SART3 suggest that it is a di-snRNP assembly factor (185,186). Consistent with their role in snRNP maturation, CBs are enriched in SART3 (158). Furthermore, the U4/U6 di-snRNP accumulates in CBs when hPrp31 is knocked down. Since hPrp31 is implicated in bridging the interaction between the U5 snRNP and the U4/U6 di-snRNP, this result suggests that the U4/U6.U5 tri-snRNP may also be assembled in CBs (157).

In the canonical model of spliceosomal assembly, the U1snRNP and U2 snRNP are proposed to first associate with the pre-mRNA template onto the 5′-splice site and the A-branch point sequence, respectively. The tri-snRNP is then recruited to complete the assembly of the spliceosome. This textbook model was recently challenged by the demonstration of a large nuclear RNP complex containing the five spliceosomal snRNPs and at least 13 other proteins. This complex was characterized in yeast (27) and mammals (26), and was called the penta-snRNP as it contains stoichiometric amounts of the five snRNPs. Remarkably, the penta-snRNP forms in absence of pre-mRNA, thus, distinguishing it from a spliceosome. In addition, the penta-snRNP was shown to splice RNA substrates upon complementation with a snRNP-depleted extract. These findings, thus, reveal a possible new level of snRNP assembly that supports a model in which the splicing machinery would be pre-assembled, possibly within CBs (36), prior to engaging pre-mRNAs (27).

THE PAST, PRESENT AND FUTURE

The last decade was rich in providing new insights into the molecular processes involved in the biogenesis of fully functional splicing snRNPs and the regulation of their nucleo-cytoplasmic exchanges. The intra-nuclear trafficking of snRNPs was also well documented, in particular with respect to several organelles, such as CBs, nucleoli and SFCs, which are currently thought to orchestrate several aspects of their maturation, assembly and storage. Similarly, the recently discovered U bodies are potential structures organizing the cytoplasmic maturation phase of snRNPs. How the trafficking of snRNPs within these various discrete cellular structures is regulated and how it influences pre-mRNA splicing are two fundamental and related questions that remain to be answered.

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