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Spliceosomal snRNA modifications and their function

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Abstract

Spliceosomal snRNAs are extensively 2'-*O*-methylated and pseudouridylated. The modified nucleotides are relatively highly conserved across species, and are often clustered in regions of functional importance in pre-mRNA splicing. Over the past decade, the study of the mechanisms and functions of spliceosomal snRNA modifications has intensified. Two independent mechanisms behind these modifications, RNA-independent (protein-only) and RNA-dependent (RNA-guided), have been discovered. The role of spliceosomal snRNA modifications in snRNP biogenesis and spliceosome assembly has also been verified.

Introduction

The removal of intervening sequences, introns, from pre-messenger RNA (pre-mRNA) is of fundamental importance to gene expression. In eukaryotic organisms, the majority of introns are removed by the spliceosome, a massively large and equally dynamic complex consisting of five small nuclear (sn) RNAs (U1, U2, U4, U5 and U6) and numerous protein components.[1-4] snRNAs participate in the pre-mRNA splicing reaction as a small nuclear ribonucleoprotein (snRNP) complex, which includes a single spliceosomal snRNA in complex with a number of proteins.

Spliceosomal snRNPs are key components of the spliceosome and are absolutely required for pre-mRNA splicing. In the classical view of pre-mRNA splicing there is a step-wise assembly of the spliceosome initiated by recognition of the 5' splice-site (5' SS) by complementary base-pairing interactions with the 5'-end of U1 snRNA (**Fig. 1**).[5-14] Subsequently, the branch-site sequence (BSS) is engaged by the U2 snRNP, resulting in the formation of a pre-splicing complex, namely complex A.[11,14-24] The U2 snRNA-BSS interaction, which is mediated through base-pairing interactions, bulges out the branch point nucleotide (typically an adenosine). Addition of the U4/U6.U5 tri-snRNP, in which U4 and U6 are extensively base-paired, to complex A results in the formation of complex B1, and initiates a series of RNA-RNA rearrangements, resulting in the destabilization and release of the U1 and U4 snRNPs.[19,25-28] The result of these rearrangements is the formation of complex B2 and concomitant activation of the spliceosome, leading to the first step of splicing, in which the 2'-OH group of the bulged out branch point adenosine nucleophilically

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attacks the 5' SS. Upon the completion of the first step of splicing, Complex B2 is converted into complex C. After additional conformational changes, the second step of splicing occurs, resulting in the production of mature mRNA and the release of the excised intron and the U2, U5 and U6 snRNPs, which are recycled for further rounds of pre-mRNA splicing.

Interestingly, all five spliceosomal snRNAs are extensively posttranscriptionally modified (**Fig. 2**).[29,30] Aside from the 5' cap modification, there are essentially two types of internal modifications, namely, 2'-*O*-methylation and pseudouridylation. Pseudouridylation is a uridine-specific modification that results in the formation of the 5-ribosyl isomer of uridine, pseudouridine (Ψ), while 2'-*O*-methylation (2'-*O*me) is an RNA backbone modification that introduces a methyl group at the 2'-*O* position of the sugar ring (**Fig. 3**). Analysis of the distribution of modified nucleotides in spliceosomal snRNAs from various organisms has demonstrated conservation in the location of modifications throughout evolution. Strikingly, the majority of modified nucleotides are present in regions known to be functionally important for pre-mRNA splicing, including the regions of RNA-RNA interactions described above.[29,31]

Over the years, great efforts have been made toward understanding the mechanisms and functions of spliceosomal snRNA modifications. It is now clear that two distinct molecular mechanisms exist that are capable of site-specifically introducing modified residues within spliceosomal snRNAs. It has also become increasingly clear that modified residues are not just bystanders in the process of pre-mRNA splicing, but actually participate in and influence snRNP and spliceosome assembly. In this review we discuss the mechanisms and functions of spliceosomal snRNA modifications.

Mechanisms of Spliceosomal snRNA Modifications

RNA-dependent mechanism

Posttranscriptional modification of spliceosomal snRNAs can occur via two distinct pathways, known as RNA-dependent and RNA-independent mechanisms (see below). In the RNA-dependent mechanism, small noncoding RNAs, namely Box H/ACA or Box C/D RNAs, are responsible for the site-specific posttranscriptional pseudouridylation and 2'-*O*-methylation of substrate RNAs, respectively (**Fig. 4A and B**). Both RNAs assemble with an evolutionarily conserved, yet distinct set of four core proteins [C/D RNAs: Nop1p, Nop56p, Nop58p and Snu13p; H/ACA RNAs: Cbf5p (Dyskerin in humans), Nhp2p, Nop10p and Gar1p].[31-47] While the RNA component is responsible for dictating site-specificity through complementary base pairing interactions with the substrate RNA, the catalytic activity, i.e., modification activity, is provided by one of the core protein components (Nop1p for 2'-*O*-methylation, and Cbf5p for pseudouridylation).[48-51]

Analyses of the subnuclear localization of guide-RNAs directing snRNA modification have revealed that they primarily reside within Cajal bodies, a subnuclear compartment present in eukaryotic cells.[52-54] Thus, these RNAs have been referred to as small Cajal body-specific RNAs (scaRNA).[54] Recently, the mechanism behind Cajal body localization has received significant attention, and it has been demonstrated that Cajal body retention of H/ACA RNAs in mammalian cells is mediated by a 4-nucleotide (nt) sequence (5'-ugAG-3', refereed

to as the CAB box: lower case letters are less conserved) located within the apical loop of either hairpin (see **Fig. 4B**).[55] Interestingly, the Sm proteins, SmB and SmD3, have been shown to specifically interact with the CAB box of both H/ACA and telomerase RNAs by immunoprecipitation and northern blot analysis.[56] However, whether these interactions are necessary for Cajal body localization has not been addressed. More recently, the Steitz group has shed light on the mechanism of Cajal body retention and identified a CAB box for Drosophila C/D RNAs (5'-cgaGUUAnUg-3': lower case letters are less conserved).[57] Using a UV crosslinking approach, a Drosophila WD40 repeat protein, p70, was identified which recognizes the Drosophila C/D RNA CAB box. In addition, both p70 and its human homologue, WDR79, were shown to interact with both human and Drosophila C/D, and H/ACA RNA CAB boxes. Importantly, this interaction was shown to be required for Cajal body retention.[57]

While Cajal bodies are considered to be the site of spliceosomal snRNA modification, there is growing evidence suggesting that RNA-guided modification is not restricted to Cajal bodies. For example, nuclear fractionation and northern blot analysis indicate that pugU2-34/44, a Xenopus H/ACA RNA that directs U2 snRNA pseudouridylation at two different positions, resides within the nucleoplasm.[58] Furthermore, it has recently been shown that while flies null for coilin lack detectable Cajal bodies, their spliceosomal snRNAs are efficiently posttranscriptionally modified.[59,60] Analysis of scaRNA localization by fluorescent in situ hybridization failed to detect any sites of scaRNA accumulation.[59] Taken together, these results strongly suggest that the modification machinery is dispersed throughout the nucleoplasm (rather than being present exclusively in the Cajal bodies). In addition, our lab has recently shown that in *Saccharomyces. cerevisiae* artificial C/D RNAs are capable of site-specifically modifying pre-mRNA.[61] These data strongly suggest that the guide-RNA mechanism of modification is functional in the nucleoplasm and raises the possibility that other nuclear RNAs, i.e., mRNA, may be natural targets of the RNA-dependent modification scheme. The apparent lack of detectable H/ACA and C/D RNAs in the nucleoplasm maybe a result of being too dilute within this compartment.

RNA-independent mechanism

To date, the majority of spliceosomal snRNA modifications have either been predicted or proven to be catalyzed by the RNA-dependent mechanism (**Tables 1 and 2**). However, while investigating whether *S. cerevisiae* spliceosomal snRNAs are posttranscriptionally modified, Massenet et al. demonstrated that Ψ44 of U2 snRNA is catalyzed by a single polypeptide enzyme known as Pus1p, demonstrating the existence of a second mechanism for spliceosomal snRNA modification, the RNA-independent or protein-only mechanism.[62] In this mechanism, an enzyme is responsible for both substrate recognition and catalysis. While this was an interesting finding, the protein-only mechanism had been known for decades to catalyze the modifications of tRNA (both in prokaryotes and eukaryotes) and rRNA (in prokaryotes and in 5S rRNA of *S. cerevisiae*).[63,64] In fact, Pus1p had already been shown to be responsible for eight different uridine-to-pseudouridine conversions in tRNA.[62] Additionally, using a yeast GST-ORF genomic library, Ma et al. identified the previously uncharacterized ORF YOR243c as being responsible for Ψ35 formation in U2 snRNA.[65] ORF YOR243c was subsequently renamed as PseudoUridine Synthase 7, PUS7.

Surprisingly, when the amino acid sequence of Pus7p was compared with those of other known pseudouridine synthases, namely those of the TruA, TruB, RluA and RsuA families, no significant homology was identified. Thus, Pus7p represented a novel family of pseudouridine synthases. Furthermore, a BLAST search of available databases indicated the presence of Pus7p homologues in many organisms, including *Schizosaccharomyces pombe*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Xenopus laevis* and humans. The Xenopus and human Pus7p homologues have been cloned, and both of them are capable of catalyzing U2 pseudouridylation at position 34, which is equivalent to position 35 of yeast U2 (Ma and Yu YT, unpublished data). Shortly following the identification of Pus7p, an *E. coli* pseudouridine synthase, TruD, was identified which contained homology to Pus7p.[66] Pus7p has since been classified as a member of the TruD family of pseudouridine synthases. Analysis of Pus7p substrates has revealed the importance of a 7 nucleotide-sequence flanking the target uridine in substrate recognition and catalysis.[67]

RNA-dependent mechanism versus RNA-independent mechanism: which came first?

It appears that both RNA-dependent and RNA-independent mechanisms co-exist in various organisms. In higher eukaryotes, while spliceosomal snRNA modifications appear to be predominantly catalyzed by the RNA-dependent mechanism, at least one such modification (the pseudouridylation of human and Xenopus U2 at position 34) appears to be catalyzed by the RNA-independent mechanism as well. Thus, two mechanisms, RNA-dependent and RNA-independent, act at the same site. However, in *S. cerevisiae*, spliceosomal snRNA pseudouridylation at a given site can only be catalyzed by one of the two mechanisms, either RNA-dependent or RNA-independent mechanism, but not by both. For instance, snR81, a H/ACA RNA, directs pseudouridylation of position 42, and Pus1p and Pus7p, protein-only enzymes, pseudouridylate position 44 and 35, respectively.[62,65,68]

The coexistence of the two mechanisms and their distinct usage in *S. cerevisiae* and other organisms is rather interesting from an evolutionary point of view. It is possible that the RNA-dependent mechanism evolved from the protein-only mechanism in both higher eukaryotes and *S. cerevisiae*. However, in yeast, while some modifying enzymes evolved (e.g., snR81), others (e.g., Pus1p and Pus7p) may have remained unchanged (or evolved but were subsequently lost from the genome; e.g., via chromosomal deletion). Conversely, it is equally possible that the RNA-dependent mechanism is the most ancient mechanism, given that archaeal H/ACA RNPs, C/D RNPs, and ribosomes share a common core protein, L7 (homologous with Nhp2p (H/ACA RNP) and Snu13p (C/D RNP) in Eukarya).[69] Thus, it is possible that the RNA-dependent mechanism evolved from the ancient translation apparatus, rather than from the RNA-independent mechanism. While the issue of which came first remains controversial, the preservation of the putatively ancient RNA-dependent (or RNA-independent) modifying mechanism throughout evolution implies that such modifications are functionally important.

Localization of snRNA-specific modifying enzymes in *S. cerevisiae*

With regard to subnuclear localization, it is worth noting that a coilin homologue has thus far escaped detection in *S. cerevisiae*. While it is possible that *S. cerevisiae* lacks a coilin homologue, it does however possess a structure analogous to Cajal bodies, the nucleolar

body.[70] The notion that the nucleolar body is the functional homologue of the Cajal body comes from several lines of evidence. For instance, when the human-Cajal body specific protein, survival of motor neuron (SMN), is ectopically expressed in *S. cerevisiae*, it concentrates to the nucleolar body.[70] Furthermore, the 5'-cap hypermethylase, Tgs1p, responsible for spliceosomal snRNA 5'-cap hypermethylation, specifically localizes to the nucleolar body.[71,72] However, analysis of spliceosomal snRNA localization through indirect immunofluorescence using an anti-m_3G antibody has proposed that the nucleolus is the site of snRNA accumulation.[73] While this would stand in direct opposition to the localization of spliceosomal snRNAs in mammalian cells, this interpretation of the data relies on the assumption that there are no other m_3G-capped small nuclear RNAs. In fact, numerous yeast snoRNAs, including members of the H/ACA and C/D RNA families, are m_3G-capped.[74] Thus, a detailed and systematic analysis of spliceosomal snRNA localization in *S. cerevisiae* has yet to be carried out. Likewise, the specific subnuclear sites, to which the spliceosomal snRNA-specific modification enzymes (RNA-dependent and RNA-independent) localize, are yet to be determined. Thus, whether nucleolar bodies are the premier sites of spliceosomal snRNA modification remains uncertain.

Functions of Spliceosomal snRNA Modifications

Posttranscriptional modification provides a means to expand the vocabulary of nucleotides in the genetic code. Importantly, it is clear that modified nucleotides have distinct chemical properties from their unmodified counterparts. Thus, they have the potential to impact numerous aspects of the modified RNA, including structure, thermal stability and biochemical interactions.[75] In each case, the structural, thermodynamic and biochemical contributions imparted by the modified nucleotide depend on the structural context and can extend beyond the site of modification. Indeed, ^{1}H NMR, UV, and CD (circular dichroism) spectroscopy have demonstrated that short RNA fragments containing pseudouridine are more stable than if the same RNA contained uridine.[76] Conformational stabilization appears to be an intrinsic property of pseudouridine at the nucleotide level, and is mediated by both an increase in base stacking and the ability to coordinate a water molecule through the extra hydrogen bond present.[76-78] Similarly, 2'-*O*-methylation promotes increased stability in RNA conformations. For instance, 2'-*O*-methylation alters the hydration sphere around the oxygen resulting in the blockage of sugar edge interactions.[79-81] In addition, methylation of the 2'-OH alters the ability of the ribose to participate in hydrogen bonding interactions.

In the context of the spliceosome, posttranscriptional modifications have the potential to influence numerous aspects of pre-mRNA splicing, including (1) RNA-RNA interactions, (2) interactions of spliceosomal snRNAs with spliceosomal proteins, and (3) directly participating in the catalytic reactions. To date, not much data has been generated regarding the latter two. Thus, we will primarily focus on the role of the modified nucleotides engaged in RNA-RNA interactions.

U1 snRNA

Within the initial step of pre-mRNA splicing, recognition of the 5'SS by the 5'-end of U1 snRNA, there are four modified nucleotides that can influence the U1 snRNA-pre-mRNA base pairing interaction (**Fig. 2**). Interestingly, however, U1 snRNA-depleted mammalian

splicing extracts can be successfully reconstituted using in vitro transcribed U1 snRNA (presumably lacking modifications).[82] Taken at face value these results suggest that modified nucleotides within the 5'-end of U1 snRNA are not necessary for pre-mRNA splicing; however, this relies on the assumption that the spliceosomal snRNAs are not capable of being modified in the splicing extracts. In fact, based on our own experience and that of others, in vitro transcribed U2 snRNA is readily modified when added to yeast splicing extracts (perhaps mammalian extracts as well). Furthermore, as only a strong splicing substrate was analyzed, whether modified nucleotides are required for the splicing of a suboptimal 5'SS was not addressed. Indeed, in vitro splicing assays in which two 5'SS are in competition with each other suggest that the presence of pseudouridine within the 5' end of U1 snRNA provides an advantage in 5'SS discrimination.[83] Furthermore, a Ψ-G base pair was shown to contribute to the stability of the U1 snRNA interaction with the 5'SS of HIV-1 SD4 RNA.[84] However, a complete functional dissection of the role of U1 snRNA posttranscriptional modification will have to wait for the identification of the enzymes responsible for their formation.

U2 snRNA

Of the spliceosomal snRNAs, U2 snRNA has the most posttranscriptional modifications. Human U2 snRNA contains ten 2'-*O*-methylated residues and 13 pseudouridines within the 5' half of the molecule (**Fig. 2**). It is perhaps for this reason that the functions of U2 snRNA posttranscriptional modifications have been the most extensively studied.

U2 snRNA modifications, snRNP biogenesis and spliceosome assembly—The initial experiments of Patton in the early 1990s provided the first functional analysis of U2 snRNA modification.[85,86] Using HeLa cell S100 and nuclear extracts he demonstrated that the incorporation of 5-fluororidine (5-FU) in to U2 snRNA blocked U2 snRNA pseudouridylation. In addition, while it was observed that 5-FU-substituted U2 snRNA was able to form an U2 snRNP, the snRNP was overwhelmingly susceptible to salt dissociation.[85] A more detailed and systematic analysis of the effects of U2 snRNA pseudouridylation on pre-mRNA splicing eventually established a nice correlation between modification status, pre-mRNA splicing competency, and U2 snRNP biogenesis.[87] Using Xenopus oocytes, Yu et al. demonstrated that while in vitro transcribed U2 snRNA was unable to rescue splicing in U2 snRNA-depleted oocytes, upon longer reconstitution periods in vitro transcribed U2 snRNA gained the ability to reconstitute splicing activity. Strikingly, the pseudouridylation status of U2 snRNA mirrored the ability of U2 snRNA to reconstitute pre-mRNA splicing, that is, in vitro transcribed U2 snRNA became pseudouridylated following the longer reconstitution periods. Further analyses using anti-snRNP immunoprecipitation in conjunction with glycerol gradient sedimentation demonstrated that while U2 snRNA lacking pseudouridine is able to form nonfunctional 12S U2 snRNP particles, it is unable to detectably form functional 17S particles.[87] Consequently, U2 snRNA lacking pseudouridine is unable to participate in spliceosome assembly. Furthermore, Zhao and Yu (2004) were able to show that pseudouridine residues within the branch site recognition region of Xenopus U2 snRNA are essential for U2 snRNP assembly and spliceosome assembly. Interestingly, the rate at which in vitro transcribed U2 snRNA is

modified within the branch site recognition region is significantly faster than within the 5' region of U2 snRNA, when injected into Xenopus oocytes.[88]

In 2004 the Lührmann group carried out an extensive analysis on the role of modified nucleotides in the first 24-nt of human U2 snRNA.[89] Interestingly, 2'-*O*-methylations at positions 1, 2, 12 and 19, were individually shown to be required for pre-mRNA splicing, while pseudouridines located within this region were shown to have a cumulative effect on splicing, as none were absolutely required for pre-mRNA splicing.[89] Interestingly, their study demonstrated that the internal modifications are required for E complex formation. Taken together, the data accumulated thus far have clearly demonstrated that most modified nucleotides in U2 snRNA, including those residing within the 5'-end region and the branch site recognition region, are functionally important.

U2 modification and splicing efficiency—In addition to playing a role in the assembly of catalytically competent snRNPs and splicing complexes, posttranscriptional modifications, in particular pseudouridylation within the branch site recognition region of U2 snRNA, has been demonstrated to influence, directly or indirectly, the catalytic phase of pre-mRNA splicing. For instance, deletion of the gene encoding Pus7p, responsible for Ψ35 formation in *S. cerevisiae* U2 snRNA (see above), although viable, displayed reduced fitness under conditions of high salt or when in competition with wildtype yeast.[65] Further analysis demonstrated that loss of Ψ35 in conjunction with U40G or U40Δ mutations in U2 snRNA severely reduced the organism's fitness.[90] Analysis of pre-mRNA splicing by semi-quantitative RT-PCR indicated an accumulation of pre-mRNA in the *pus7*Δ U2-U40G and *pus7*Δ U2-U40Δ strains, while any single mutation resulted in minimal if any accumulation of pre-mRNA. In line with the notion of pseudouridylation within the branch site recognition region of U2 snRNA affecting the catalytic phase of pre-mRNA splicing, the change of a single uridine (U35) to pseudouridine (Ψ35) significantly enhances the production of X-RNA, a product generated by a splicing related reaction in a cell- and protein-free system.[91,92] It should be noted that Ψ35 is the nucleotide nearly opposite the branch-point adenosine.

Nucleophile positioning via U2 snRNA pseudouridylation—In recent years, the role of U2 snRNA pseudouridylation has been extensively investigated using various biophysical techniques. In this regard, the crystal structure of a self-complementary RNA designed to mimic the *S. cerevisiae* U2 snRNA-branch point interaction was determined in the absence of pseudouridine.[93] Surprisingly, the adenosine 5' of the expected branch point adenosine was bulged out. Subsequently, the Greebaum group determined solution structures of the *S. cerevisiae* U2 snRNA-branch point interaction either in the presence or absence of pseudouridine (Ψ34, corresponding to Ψ35 in mammals).[94,95] Interestingly, NMR data coupled with 2-aminopurine fluorescence titration data indicated that the presence of the pseudouridine was required for the bulging out of the expected branch point adenosine.[95] However, in the NMR structure, the bulged adenine base was inserted in the minor groove, burying the 2'-OH, the nucleophile in the reaction, making it unlikely to participate in the splicing reaction. More recently, Lin and Kielkopf determined the crystal structure of the U2 snRNA-branch point interaction in the presence of pseudouridine and

observed an extra-helical branch point adenosine in which its 2'-OH was prominently exposed and available for attack on the 5'SS.[96] Thus, the structure proposed by Lin and Kielkopf is more likely to be functionally relevant for catalysis.

Taken together, U2 snRNA modification is required for pre-mRNA splicing. Biochemical and molecular data have clearly established links between spliceosomal snRNP biogenesis, spliceosome assembly, and splicing efficiency with the status of U2 snRNA modification. In addition, biophysical data have provided detailed structural information indicating that U2 snRNA pseudouridylation is required for proper positioning of the 2'-OH of the branch-point adenosine so that it is accessible and exposed for recognition and nucleophilic activity.

U5 snRNA

The U5 snRNA plays a critical role in juxtaposing the 5' SS, 3' SS and the BSS during pre-mRNA splicing. To date all U5 snRNAs examined possess an 11-nt loop, called loop 1, which contains the conserved 9-nt sequence 5'-$G_1C_2C_3U_4U_5U_6U_7A_8C_9$-3'.[97] Loop 1 engages the 5' exon before the first step of splicing and this interaction is maintained throughout the second step of splicing. In contrast, the loop 1-3' exon interaction can not be detected until the second step.[98-100] Strikingly, there are 4 post-transcriptional modifications within the conserved 9-nt sequence of loop 1 (2'-*O*me at positions 1, 5 and 9; Ψ at position 7; **Fig. 2**). Furthermore, nucleotides at positions 5 and 7 have been shown to interact with the splicing substrate by photochemical crosslinking and genetic suppression analyses.[14,99,101-106] Unfortunately, the mechanism of U5 snRNA modification has not been elucidated, thus precluding a functional analysis of these modifications in pre-mRNA splicing. However, based on the known function of loop 1 in pre-mRNA splicing, it is reasonable to speculate a role for these modifications in influencing the stability of the pre-mRNA-U5 snRNA interaction. Alternatively, posttranscriptional modification of U5 snRNA loop 1 may be required for the Prp8p-mediated stabilization of exon-U5 snRNA loop 1 interactions during pre-mRNA splicing.[107-109]

U4 and U6 snRNAs

U4 and U6 snRNAs enter the pre-mRNA splicing reaction as the U4/U6.U5 tri-snRNP. Following tri-snRNP addition, a series of RNA-RNA rearrangements proceed, resulting in the exclusion of the U1 and U4 snRNAs from the spliceosome (see above). U6 snRNA is one of three snRNAs present in active spliceosomes (U2 and U5 are also present). However, evidence suggests that it is U2 and U6 that are directly involved in the catalytic steps (see above).[98,110-112]

Within the tri-snRNP, there are extensive base-pairing interactions between U4 and U6. Strikingly, this interaction is particularly strong with an experimentally determined T_m for affinity purified yeast U4/U6 di-snRNP of 55°C; on the other hand, human U4/U6 di-snRNP has two T_ms, a lower T_m of 37°C and a higher T_m of 55°C.[113] It should be noted that the human di-snRNP also presented with two alternate mobilities by native gel analysis.[113] Interestingly, between human U4 and U6 snRNAs there are six Ψs and 12 2'-*O*me residues (**Fig. 2 and Tables 1 and 2**), and half of them map to the regions involved in U4/U6 snRNA base-pairing interactions (**Fig. 2**). It has previously been proposed that the ATP-dependent

unwinding of this duplex provides a proofreading step to ensure proper positioning of the tri-snRNP on the spliceosome,[114] and timely formation of the catalytic center (U2-U6 duplexes). Within this context one could imagine that the extra thermodynamic stability afforded to the base-pairing interactions by the posttranscriptional modifications influences the rate of unwinding. Alternatively, as the binding positions of the helicases responsible for di-snRNP unwinding have not been mapped on to the snRNAs, modifications may induce a structure conducive to recognition by helicases.

Minor Spliceosomal snRNAs are Pseudouridylated

While the majority of introns are removed by the aforementioned spliceosome (or the major spliceosome), there exists a rare class of introns (~1–300) that are removed by a functionally similar, yet structurally distinct spliceosome, which is of much lower abundance (~104 copies per cell) relative to components of the major spliceosome.[115,116] Thus, this spliceosome is referred to as the minor spliceosome. The activity of the minor spliceosome requires four distinct spliceosomal snRNAs, namely U11, U12, U4atac and U6atac, while sharing the U5 snRNA with the major spliceosome (**Fig. 5**).[115] Analysis of minor spliceosomal snRNAs from HeLa cells has demonstrated that they too are posttranscriptionally modified (**Fig. 5**). To date, four pseudouridines have been identified in the minor spliceosomal snRNAs, two within U12, and one each within U4atac and U6atac.[117] A single 2'-*O*-methylation has been detected in U12.[118] While all of the modifications present in the minor class spliceosomal snRNAs have been predicted to be guided via the RNA-dependent mechanism, none have had their mechanism of formation experimentally determined (**Table 2**).

Although fewer pseudouridine residues are present in the minor spliceosomal snRNAs when compared to the major spliceosomal snRNAs, the positions of pseudouridylation for U12 and U4atac are homologous to those within U2 and U4, respectively, thus suggesting that these pseudouridines are important for the splicing of minor introns. In fact, Ψ19 of U12 snRNA, which is adjacent to the branch point adenosine, is present in equivalent positions in U2 snRNA in human (Ψ34), plant (Ψ34) and *S. cerevisiae* (Ψ35). Interestingly, introns removed by the minor spliceosome contain more constrained consensus sequences at the 5' end of the intron and BSS.[119-121] Thus, it is reasonable to hypothesize that the increased amount of modified nucleotides present in the major spliceosomal snRNAs, relative to the amount present in the minor spliceosomal snRNAs, is necessitated by the fact that major class (U2-type) introns contain less conserved consensus splice site sequences than the minor class (U12-type) introns. In support of this hypothesis, the introns of *S. cerevisiae* contain highly conserved consensus splice site sequences, while the spliceosomal snRNAs contain relatively few modified residues.

Spliceosomal snRNA Pseudouridylation as a Therapeutic Target

5-FU is commonly used in the treatment of a variety of solid tumors such as colorectal, breast, and liver carcinomas.[122,123] Although nearly six decades have passed since the initial uses of 5-FU as a chemotherapeutic agent, its mechanism of action is one of debate. Initially it was hypothesized that 5FU affects DNA metabolism through inhibition of the enzyme

thymidylate synthase, which is required for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP).[124,125] A reduction in the amount of dTMP, in turn, results in an inhibitory effect on the production of its downstream product deoxythymidine triphosphate (dTTP). Consequently, dUMP accumulates, resulting in elevated synthesis of its downstream product, deoxyuridine triphosphate (dUTP), and the incorporation of dUTP into DNA, thus resulting in DNA damage.[126,127] Paradoxically, however, when 5FU-exposed cells are treated with thymidine, which can be converted to dTMP through the action of thymidine kinase (a pathway independent of the thymidylate synthase pathway), 5FU-mediated cytotoxic and apoptotic effects remain, suggesting that DNA metabolism is not the primary target of 5FU.[128,129]

Given that 5FU can be readily converted into 5-fluorouridine triphosphate (5FUTP), a ribonucleotide analog that can be incorporated into RNA, it has been proposed that 5FU may directly affect RNA metabolism.[122,123] Indeed, 5FU-treated HeLa cells show a dramatic accumulation of pre-mRNA.[130] In addition, thin-layer chromatography analysis of U2 snRNA isolated from 5FU-treated HeLa cells demonstrated the presence of 5FU and a reduction in the amount of pseudouridine present. Furthermore, while U2 snRNA isolated from uracil-treated HeLa cells can efficiently reconstitute pre-mRNA splicing in U2 snRNA-depleted Xenopus oocytes, U2 snRNA purified from 5FU-treated HeLa cells failed to reconstitute pre-mRNA splicing.[130] Thus, some of the therapeutic effect of 5FU can be attributed to the inhibition of pre-mRNA splicing as a result of precluding pseudouridine formation.

Conclusions

The past decade has seen remarkable progress towards elucidating the mechanism and function of spliceosomal snRNA modification. However, relative to the progress made on DNA and protein modifications, research on RNA modifications has lagged behind. The key to addressing the in vivo role of spliceosomal snRNA modifications is to identify all the gene products responsible for their formation. While in vitro studies and studies using small molecule inhibitors of modification, i.e., 5FU, can offer insight in to the function of modifications, they are no substitute to a clean loss of that particular modification. To date, only 16 of the 24 known sites of pseudouridylation within the major spliceosomal snRNAs (U1, U2, U4, U5 and U6), and two of four for the minor spliceosomal snRNAs (U11, U12, U4atac and U6atac) of mammals have had the enzymes responsible for their formation proven or predicted. In addition, only three of six pseudouridines have had their modifying enzyme identified in *S. cerevisiae* (**Table 1**). Thus, a daunting task of identifying the enzymes responsible for the remaining modifications lies ahead.

It should be reiterated that the only spliceosomal snRNA which has had its modifications subjected to a detailed and systematic experimental analysis is U2 snRNA. Thus, whether modified nucleotides in U1, U4, U5 and U6 play any roles in splicing is an open question. In addition, it is our hope that as structural techniques continue to advance and become more powerful we will see detailed images of the spliceosome at defined functional stages. In fact, we are already beginning to see high-resolution electron microscopy images.[131] With the growing attention given to RNA modification and pre-mRNA splicing, we expect a clear

picture, regarding whether and how spliceosomal snRNA modifications contribute to function, to emerge soon.

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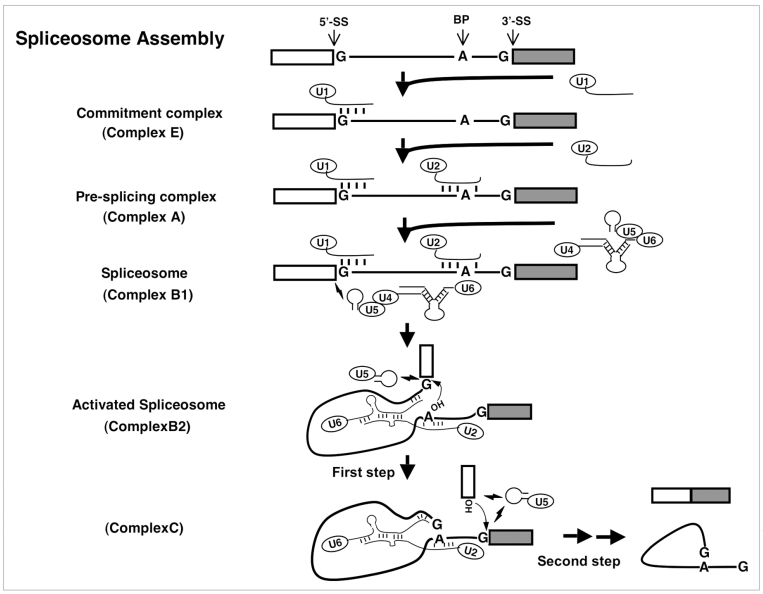

Figure 1.

Major spliceosome assembly and catalysis of pre-mRNA splicing. The thick lines represent the intron and the boxes are exons. The 5' splice site (5'-SS), the 3' splice site (3'-SS) and the branch point adenosine (BP) are indicated in the pre-mRNA. The conserved residues at the 5' and 3' splice sites and the branch site are shown. The headed thin lines are snRNAs with their names in the ellipses. The short thick lines between RNA strands represent Watson-Crick base-pairing interactions. The lightning symbols depict non-Watson-Crick base-pairing interactions. The 2'-OH groups of branch point adenosine and the cut-off 5' exon are pictured in the activated spliceosome. The small arrows near those 2'-OH group indicate the nucleophilic chemical reactions also known as trans-esterification reactions.

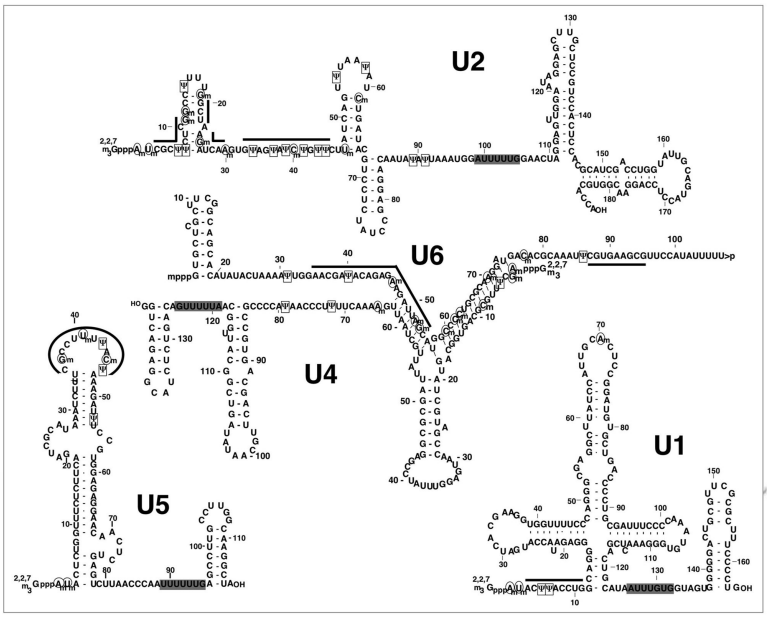

Figure 2.

Pseudouridines and 2'-*O*-methylated residues in human spliceosomal snRNAs. Primary and secondary structures of human major spliceosomal snRNAs (U1, U2, U4, U5 and U6) are shown. Pseudouridines (Ψ) are surrounded by rectangles; 2'-*O*-methylations are circled. The thick lines indicate the nucleotides participating in RNA-RNA interactions or involved in catalysis during pre-mRNA splicing. The gray boxes highlight the Sm-binding sites. The 5' caps (2,2,7 trimethylated guanosine cap for U1, U2, U4, U5 and γ-methylated guanosine cap for U6) are also depicted.

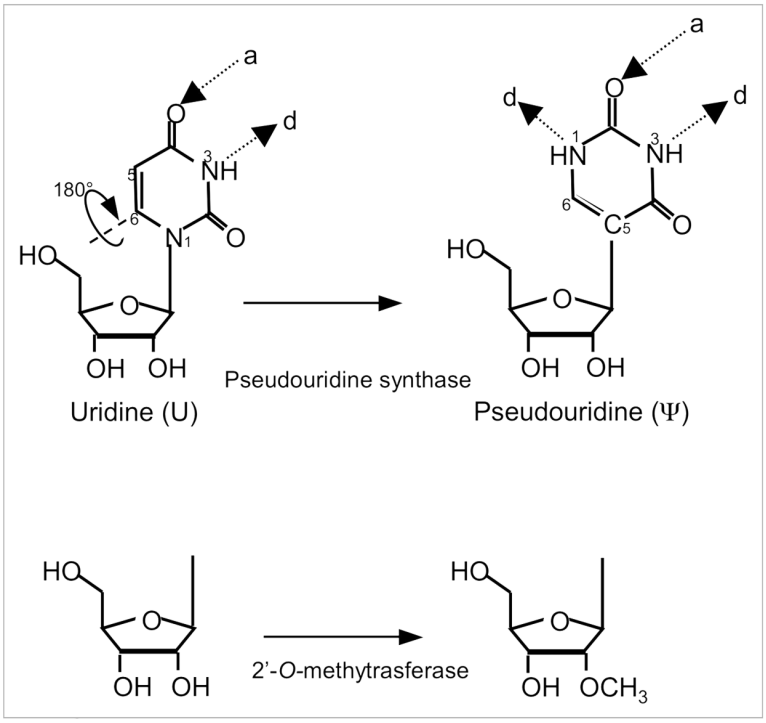

Figure 3.

Schematic depiction of the two most abundant modified nucleotides in spliceosomal snRNA. (Top) Pseudouridine is a rotational isomer of uridine, in which the N-C glycosidic bond is broken to form an C-C bond. This results in the presence of an extra hydrogen bond donor (d), while the number of hydrogen bond acceptors (a) is unchanged. (Bottom) Schematic representation of a 2'-*O*-methylated ribose.

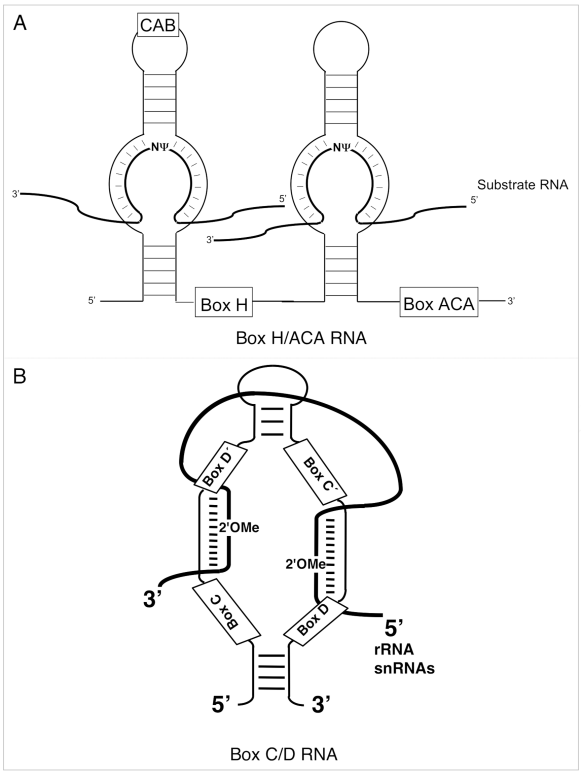

Figure 4.

Schematic depiction of box H/ACA and C/D RNAs. (A) Secondary structure of a eukaryotic pseudouridylation guide box H/ACA RNA. The RNA adapts a Hairpin-Hinge-Hairpin-Tail structure. Present within the hinge region is the box H (5'-ANANNA-3'), the box ACA (5'-ACA-3') motif typically lies three nucleotides from the 3'-end of the RNA. A CAB box (5'-ugAG-3'), responsible for Cajal body localization, may be present in the apical loop of either hairpin. Pseudouridylation is targeted to substrate RNAs by complementary base-pairing interactions between the internal loop (pseudouridylation pocket) and nucleotides adjacent to the target uridine. The thick lines denote substrate RNAs. (B) Secondary structure of a box C/D RNA. Boxes C, D, C' and D' are shown. The 2'*O*Me represents the target 2'-*O*-methylation site that is always the fifth nucleotide from box D or D'. The thick line represents substrate RNA.

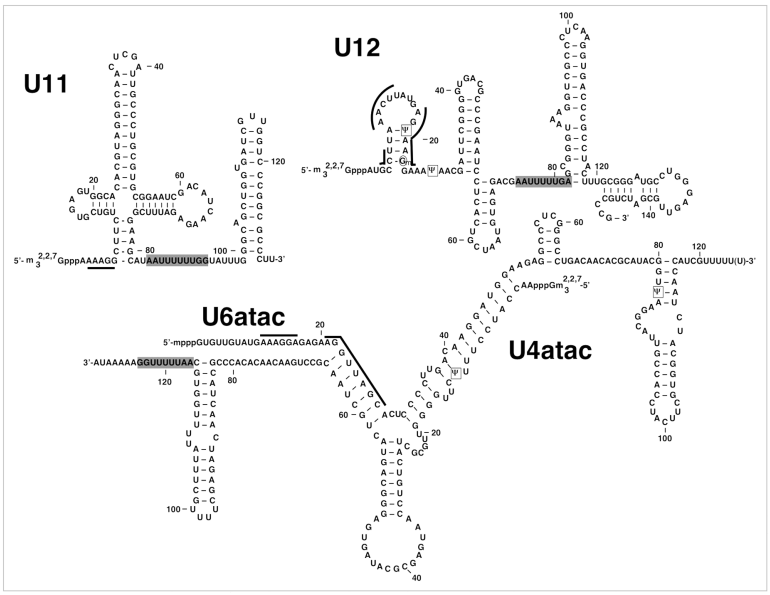

Figure 5.

Shown are primary and secondary structures of human minor spliceosomal snRNAs, U11, U12, U4atac and U6atac. U5 snRNA is shared by both the major and minor spliceosomes. Pseudouridines (Ψ) are surrounded by rectangles; 2'-*O*-methylations are circled. Pseudouridines within U12 and U4atac are believed to function analogously to their homologous modifications within U2 and U4 snRNAs, respectively. The thick lines indicate the nucleotides participating in RNA-RNA interactions or involved in catalysis during pre-mRNA splicing. The gray boxes highlight the Sm-binding sites.

Table 1

Sites of pseudouridylation within yeast and human spliceosomal snRNAs

Organism	snRNA	Position	Mechanism	Enzyme	Verified/Predicted	Reference
Yeast	U1	Ψ5	Cbf5 dependent	Cbf5	NR	62, Unpublished data Yu Lab
		Ψ6	Cbf5 dependent	Cbf5	NR	62, Unpublished data Yu lab
	U2	Ψ35	Protein only	Pus 7	Verified	62, 65
		Ψ42	H/ACA RNP	snR81	Verified	62, 68
		Ψ44	Protein only	Pus 1	Verified	62
	U5	Ψ99	NR	NR	NR	62
Human	U1	Ψ5	H/ACA RNP	ACA47	Verified	132, 133
		Ψ6	H/ACA RNP	U109	Predicted	132, 134
	U2	Ψ6	NR	NR	NR	
		Ψ7	H/ACA RNP	U100	Predicted	135, 136
		Ψ15	NR	NR	NR	
		Ψ34	H/ACA RNP	U92	Predicted	54, 137
		Ψ37	H/ACA RNP	ACA45	Predicted	133, 137
		Ψ39	H/ACA RNP	ACA26	Predicted	133, 137
		Ψ41	H/ACA RNP	ACA26	Predicted	133, 137
		Ψ43	NR	NR	NR	
		Ψ44	H/ACA RNP	U92	Predicted	54, 137
		Ψ54	H/ACA RNP	U93	Predicted	29, 136-138
		Ψ88	NR	NR	NR	
		Ψ89	H/ACA RNP	ACA35	Predicted	133, 137
		Ψ91	NR	NR	NR	
	U4	Ψ4	NR	NR	NR	
		Ψ72	NR	NR	NR	
		Ψ79	NR	NR	NR	
	U5	Ψ43	H/ACA RNP	ACA57	Predicted	133, 139
		Ψ46	H/ACA RNP	U85	Verified	139, 140
		Ψ53	H/ACA RNP	U93	Predicted	29, 137, 138
	U6	Ψ31	H/ACA RNP	ACA65	Predicted	136
		Ψ40	H/ACA RNP	ACA12	Predicted	133, 141
				HBI-100	Predicted	135
		Ψ86	H/ACA RNP	ACA65	Predicted	135
	U4atac	Ψ12	NR	NR	NR	117
	U6atac	Ψ83	NR	NR	NR	117
	U12	Ψ19	H/ACA RNP	ACA68	Predicted	117, 136
		Ψ28	H/ACA RNP	ACA66	Predicted	117, 136

Note: NR is for Not Reported.

Table 2

Sites of 2'-0-methylation within human spliceosomal snRNAs

Organism	snRNA	Position	Mechanism	Enzyme	Verified/Predicted	Reference
Human	U1	A70	Box C/D RNP	U90	Predicted	54, 135
	U2	G11	Box C/D RNP	HBII-382	Predicted	54, 135
		G19	Box C/D RNP	mgU2-19/30	Predicted	54, 118
		G25	Box C/D RNP	mgU2-25/61	Predicted	118, 135
		A30	Box C/D RNP	mgU2-19/30	Predicted	54, 118
		C61	Box C/D RNP	mgU2-25/61	Predicted	118, 135
				HBII-382	Predicted	54, 135
	U4	C8	Box C/D RNP	mgU12-22/U4-8	Predicted	54, 118
				U91	Predicted	54, 118, 135
	U5	A65	Box C/D RNP	U87	Predicted	54, 142, 143
		U41	Box C/D RNP	U87	Predicted	54, 139, 143
				U88	Predicted	54, 139
		C45	Box C/D RNP	U85	Predicted	54, 55, 140
	U6	A47	Box C/D RNP	mgU6-47	Predicted	144
		A53	Box C/D RNP	mgU6-53	Predicted	145
				mgU6-53B	Predicted	145
		C60	Box C/D RNP	HBII-166	Predicted	135
		C62	Box C/D RNP	U94	Predicted	146
		C77	Box C/D RNP	mgU6-77	Predicted	144
	U12	G22	Box C/D RNP	mgU12-22/U4-8	Predicted	54, 118