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REVIEW

Pseudouridines in spliceosomal snRNAs

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ABSTRACT

Spliceosomal RNAs are a family of small nuclear RNAs (snRNAs) that are essential for pre-mRNA splicing. All vertebrate spliceosomal snRNAs are extensively pseudouridylated after transcription. Pseudouridines in spliceosomal snRNAs are generally clustered in regions that are functionally important during splicing. Many of these modified nucleotides are conserved across species lines. Recent studies have demonstrated that spliceosomal snRNA pseudouridylation is catalyzed by two different mechanisms: an RNA-dependent mechanism and an RNA-independent mechanism. The functions of the pseudouridines in spliceosomal snRNAs (U2 snRNA in particular) have also been extensively studied. Experimental data indicate that virtually all pseudouridines in U2 snRNA are functionally important. Besides the currently known pseudouridines (constitutive modifications), recent work has also indicated that pseudouridylation can be induced at novel positions under stress conditions, thus strongly suggesting that pseudouridylation is also a regulatory modification.

KEYWORDS small nuclear RNAs (snRNAs), pseudouridine, spliceosome, small nuclear ribonucleoproteins (snRNPs)

INTRODUCTION

In most eukaryotes, mRNA does not begin its life mature, with coding potential. Instead, it is first transcribed in the form of pre-mRNA, composed of both coding exons and non-coding introns (Berget et al., 1977; Chow et al., 1977). Therefore, before it is shipped to the cytoplasm to direct protein synthesis, a chemical reaction, termed splicing, must take place to excise the introns and join the exons into a mature mRNA (Staley and Guthrie, 1998; Burge et al., 1999; Yu et al., 1999; Karijolich and Yu, 2008; Will and Lührmann, 2011).

Splicing is catalyzed by the spliceosome, a large, dynamic RNA-protein complex that takes on various forms through the different steps of splicing (Konarska and Sharp, 1986; Cheng and Abelson, 1987; Nilsen, 1994; Burge et al., 1999; Yu et al., 1999; Karijolich and Yu, 2008; Will and Lührmann, 2011). Spliceosome activity depends on a large number of protein components, as well as five "uridyl-rich" snRNAs—U1, U2, U4, U5, and U6. The U snRNAs exist and function, along with proteins, as RNA-protein complexes known as snRNPs (small nuclear ribonucleoproteins) (Yu et al., 1999). These snRNPs coordinate spliceosome assembly in a stepwise fashion when observed *in vitro* (Fig. 1).

Early in the assembly process, the U1 snRNP (and perhaps other protein factors as well) recognizes the 5' splice site (Zhuang and Weiner, 1986). This recognition involves a base-pairing interaction between the 5' end region of U1 and the 5' splice site of the pre-mRNA. The U2 snRNP then recognizes the branch site through a base-pairing interaction involving the branch site recognition region of U2, thereby bulging out the branch point adenosine of the pre-mRNA (Parker et al., 1987; Zhuang and Weiner, 1989). The joining of U1 and U2 results in the formation of a pre-splicing complex (complex A). At this point, the tri-snRNP (a complex of U4 snRNP, U6 snRNP and U5 snRNP) is recruited, creating a fully assembled spliceosome (complex B1). In the newly formed spliceosome, a specific sequence of U5 interacts with the exon sequences at the 5' and 3' splice sites (Newman and Norman, 1991; Newman and Norman, 1992; Wyatt et al., 1992; Cortes et al., 1993; Sontheimer and Steitz, 1993), and other sequences of U4 and U6 base-pair with each other. Then, before the first step of splicing occurs, the spliceosome undergoes dynamic changes, resulting in the departure of U1 and U4, and the formation of new duplexes, including those between U2 and U6, and between U6 and the 5' splice site (Hausner et al., 1990; Datta and Weiner, 1991; Wu and Manley, 1991; Yean and Lin, 1991; Madhani and Guthrie,

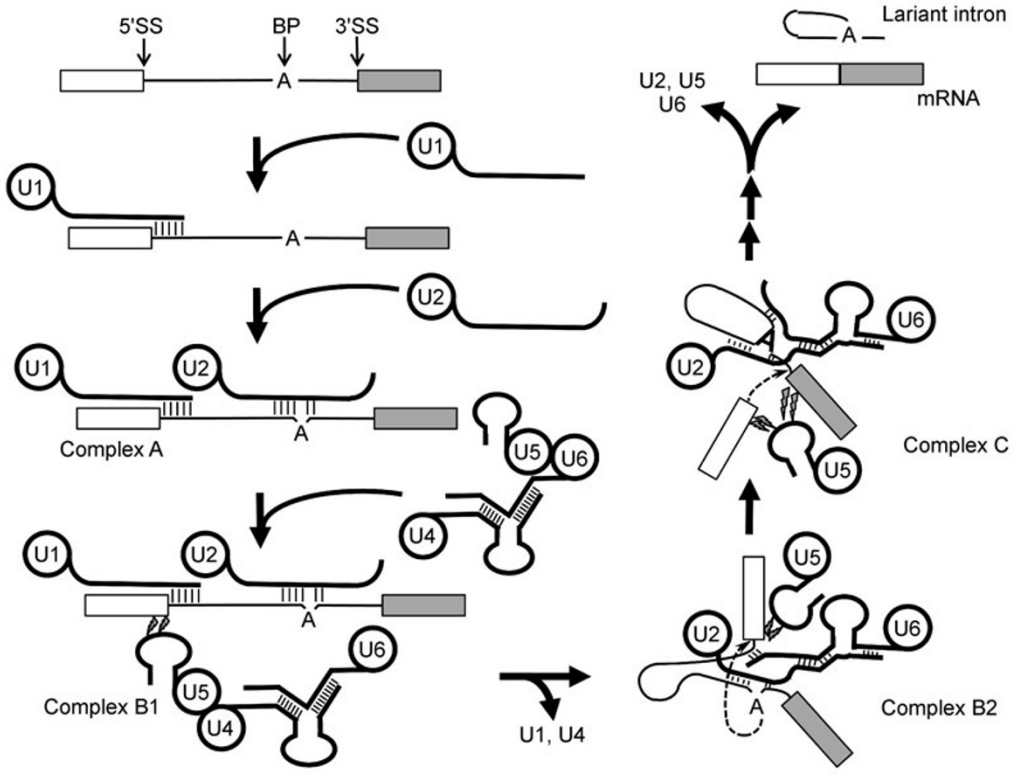

Figure 1. Spliceosome (Pre-mRNA splicing) cycle. The 5′ splice site (5′SS), the 3′ splice site (3′SS) and the branch point adenosine (BP) are indicated. The 5 spliceosomal snRNPs, U1, U2, U4, U5, and U6 are also schematically represented. Short lines depict base pairing interactions. The lightning bolts indicate the interactions/contacts between U5 and the 5′ splice site (and the 3′ splice site). The dashed arrows represent the two steps of splicing (transesterification reactions). Splicing complexes (complexes A, B1, B2 and C) at different stages of the spliceosome assembly cycle are also indicated.

1992; Sawa and Abelson, 1992; Wassarman and Steitz, 1992; Lesser and Guthrie, 1993; Nilsen, 1994). The resulting conformational changes lead to the formation of the active spliceosome (complex B2), triggering the first step of splicing, where the bulged-out branch point adenosine nucleophilically attacks the phosphate at the 5' splice site. As a result, a lariat 2/3 intermediate and a cut-off 5' exon intermediate are generated (Burge et al., 1999; Karijolich and Yu, 2008). After the first step of splicing, the spliceosome undergoes additional conformational changes, leading to the formation of complex C and the second step of splicing, which generates the mature mRNA and lariat intron products. The spliceosomal snRNPs eventually dissociate from the splicing products, and are free to enter a new round of spliceosome assembly and splicing.

It has been known for decades that all spliceosomal U snRNAs are posttranscriptionally modified, exhibiting extensive pseudouridylation and 2'-O-methylation, as well as other, more exotic modifications (Reddy and Busch, 1988). Importantly, these modified nucleotides (especially pseudouridines) are generally conserved across species and clustered in regions that are functionally significant (Reddy and Busch, 1988; Karijolich et al., 2009) (Fig. 2 and 3). For instance, there are a number of conserved pseudouridines in the aforementioned functionally important regions of spliceosomal snRNAs (sequences/regions involved in interacting with pre-mRNA and with each other during spliceosome assembly and splicing) (Fig. 1–3), thus suggesting strongly that spliceosomal snRNA modifications, including pseudouridylation, play a role in pre-mRNA splicing.

However, research into RNA modifications has historically lagged behind comparable areas, such as DNA and protein modification. About 15 years ago, though, several laboratories began to turn their attention to the mechanisms and functions of these modifications in spliceosomal snRNAs and rRNAs (Bachellerie et al., 1995; Cavaillé et al., 1996; KissLászló et al., 1996; Tycowski et al., 1996; Ganot et al., 1997; Ni et al., 1997; Smith and Steitz, 1997; Tycowski et al., 1998; Yu et al., 1998; Lowe and Eddy, 1999). Multiple effective assays and systems have since been developed for RNA modification research [reviewed in (Wu et al., 2011b)]. The advent of these assays and systems has helped to accelerate research into RNA modification, leading to some significant developments in this research area.

This review will give a brief overview of the history of modification research, followed by a breakdown of the two

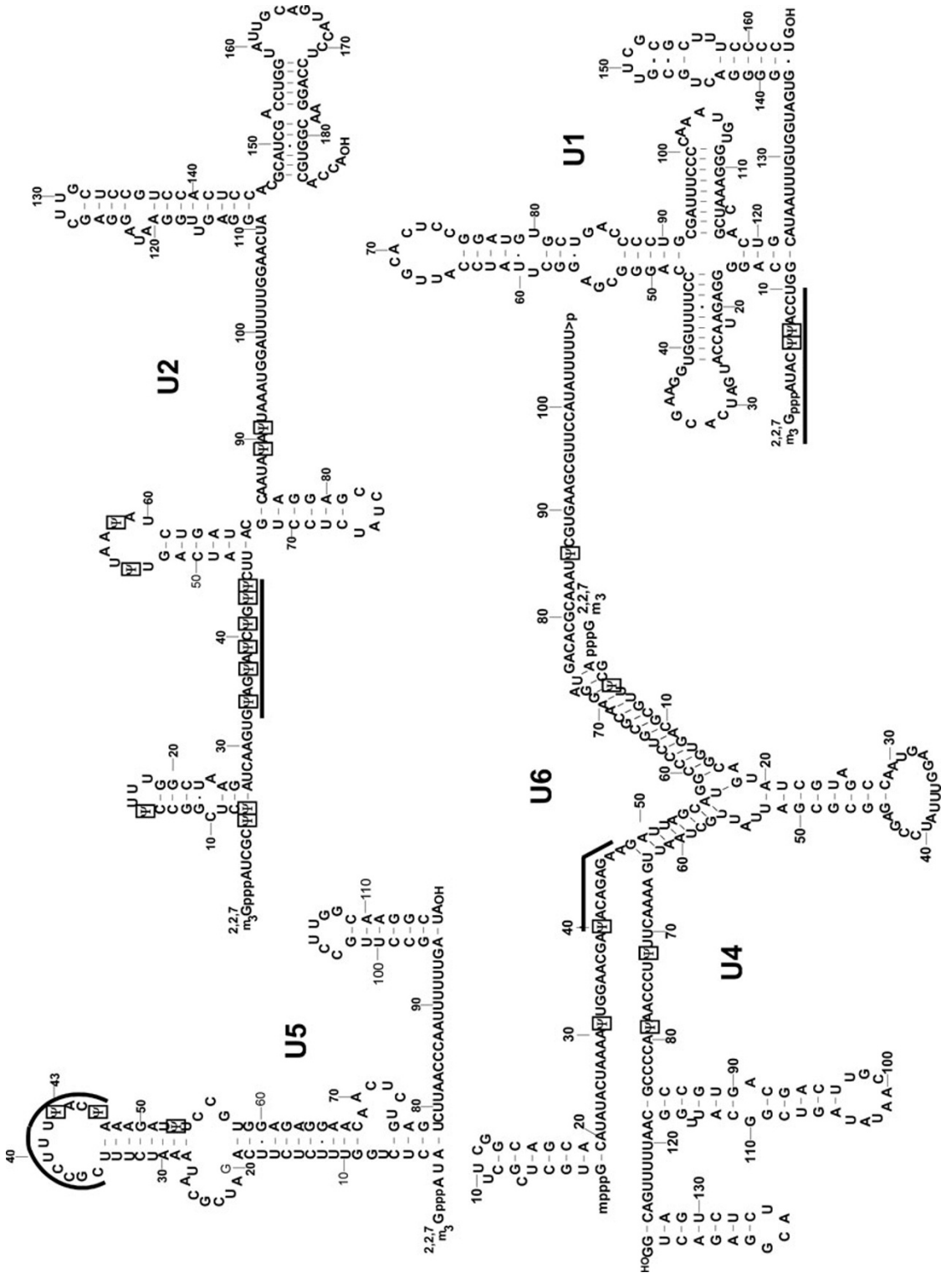

Figure 2. Vertebrate spliceosomal snRNAs. The primary sequences and secondary structures of all 5 spliceosomal snRNAs are shown. Pseudouridines (Ψ) are boxed. The thick lines indicate sequences/regions that are involved in base-pairing or come into contact with pre-mRNA (refer to Fig. 1).

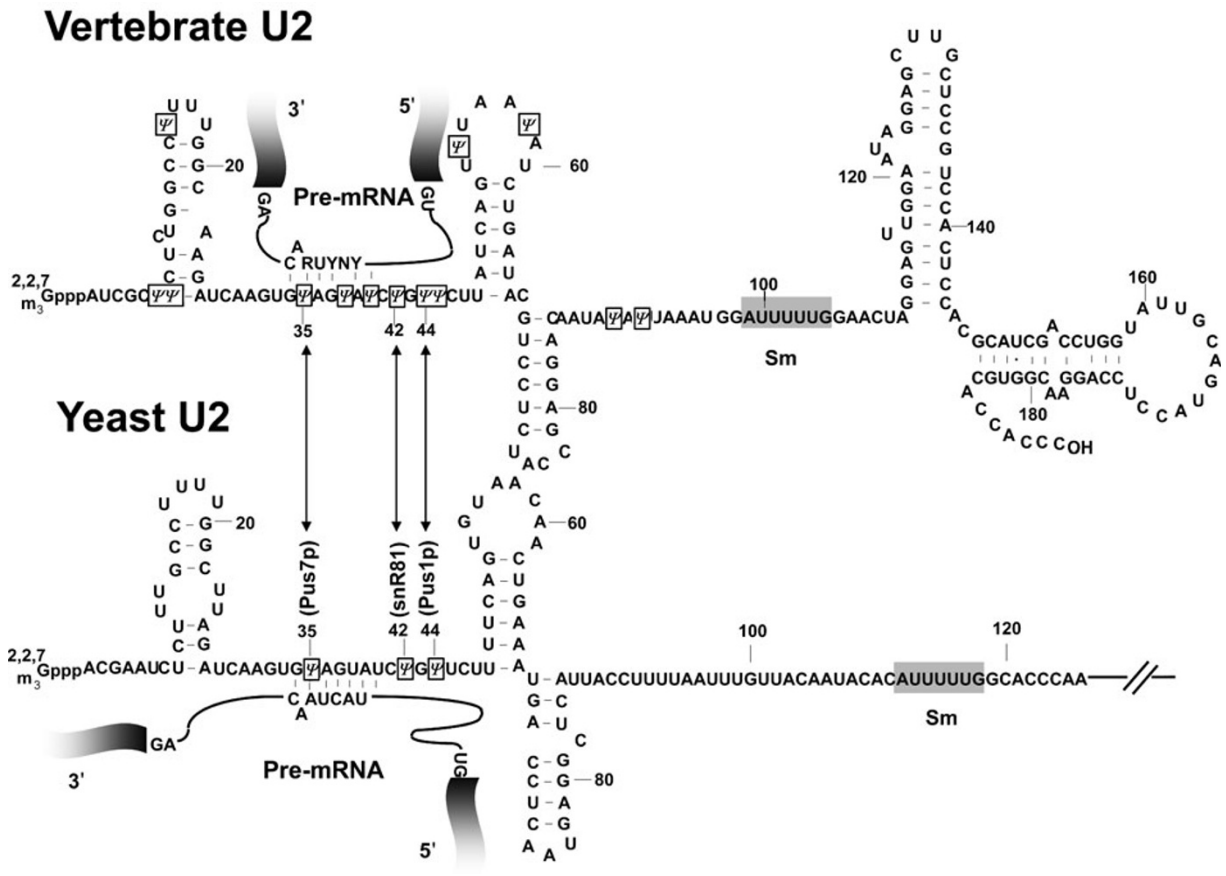

Figure 3. Vertebrate and *S. cerevisiae* U2 snRNA. The primary sequence and secondary structure of vertebrate U2 and partial primary sequence and secondary structure of yeast U2 are shown. Interactions between the pre-mRNA branch site and the U2 branch site recognition region are schematically depicted (N, any nucleotide; Y, pyrimidine; R, purine). Pseudouridines (Ψ) are boxed. The arrows indicate the three conserved pseudouridines, which are numbered. Enzymes responsible for yeast U2 pseudouridylation at the three sites are also indicated.

mechanisms (RNA-dependent and RNA-independent) that catalyze spliceosomal snRNA pseudouridylation, and then a discussion about the functions of this modification.

DISCOVERY OF PSEUDOURIDINES AND OTHER MODIFIED NUCLEOTIDES

Until the mid-late 1940s, nucleic acids were thought to consist solely of the five core nucleotides: adenosine (A), cytidine (C), guanosine G), thymidine (T), and uridine (U) (2′-deoxy form of A, C, G and T in DNA, and 2′-hydroxyl form of A, C, G and U in RNA) (Grosjean, 2009). The concept of “modified nucleotides” had not even begun to surface. Then, in 1948, the first non-canonical nucleotide—based on a cytosine residue—was isolated from DNA from calf thymus (Hotchkiss, 1948; Wyatt, 1950). However, modified nucleotides in DNA occur relatively infrequently. Of far more interest were the modified nucleotide residues in RNA.

In 1951, three years after the discovery of the first modified nucleotide, Cohn and colleagues used ion-exchange chromatography to analyze enzymatically digested calf liver RNA and found five compounds instead of the expected four (Cohn and Volkin, 1951). The first four strong signals were obviously the core nucleotides of A, C, G, and U, but what was the fifth one? Cohn designated it as “?”, and it was shown in the late 1950s to be a wholly new nucleotide: 5-ribosyluridine (Davis and Allen, 1957). We now know it as pseudouridine (Fig. 4).

The discovery of pseudouridine sparked a wave of interest in RNA modification, resulting in the discovery of—to date—anywhere between 110 to 119 modified nucleotides (the difference is accounted for by various ways of characterizing so-called “hypermodified” nucleotides) (Grosjean, 2009). However, deeper research into the mechanisms and functions of pseudouridines and other modified nucleotides would have to wait for the development of suitable assays, techniques and experimental systems, which would not come for another forty years or so.

Then, in the 1990s, after stagnating for about 40 years, the field of RNA modification began to pick up steam, as different

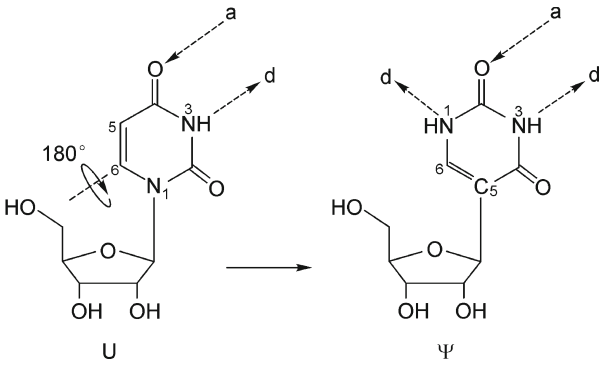

Figure 4. Pseudouridylation reaction. The isomerization reaction converting uridine (U) to pseudouridine (Ψ) is shown. The ring atoms of the bases (uracil and pseudouracil) are numbered.

labs began to develop novel techniques to research these non-canonical nucleotides (Bakin and Ofengand, 1993; Maden et al., 1995; Zhao and Yu, 2004a; Saikia et al., 2006; Dai et al., 2007). Powerful experimental systems, such as the *Xenopus* oocyte system and the yeast biochemistry and genetics systems, provided new ways to go about research into this area, and scientists took full advantage [reviewed in (Wu et al., 2011b)]. Now, we are beginning to develop a clearer picture as to how these modified nucleotides, especially pseudouridines, are formed, and what roles they play in the cell.

MECHANISMS OF SPLICEOSOMAL SNRNA PSEUDOURIDYLATION

Pseudouridylation is an isomerization reaction where the nitrogen–carbon (N1–C1′) bond linking the sugar and the uracil base (in uridine) is broken. The uracil base is then lifted up and turned 180° along the N3–C6 axis, leading to the formation of a new carbon–carbon (C5–C1′) bond. This new bond re-establishes the base-sugar linkage, creating pseudouridine (Fig. 4).

While the description of this reaction is simple and straightforward, knowledge of the detailed mechanisms, in particular the enzymes responsible for this reaction, remained lacking for decades. In the 1990s, though, a major breakthrough occurred when a large number of box H/ACA guide RNAs were found to direct rRNA pseudouridylation (Balakin et al., 1996; Ganot et al., 1997; Ni et al., 1997; Smith and Steitz, 1997; Yu et al., 2005).

An RNA-dependent mechanism catalyzes spliceosomal snRNA pseudouridylation in vertebrates

In 1996, the Fournier group identified two major families of small RNAs in the nucleolus; one of these was the box H/ACA RNA family (Balakin et al., 1996). Due to their conserved structure and sequence elements, the box H/ACA RNA family was believed to function in guiding rRNA pseudouridylation (Fig. 5). In 1997, both the Fournier and the Kiss groups demonstrated experimentally that, indeed, rRNA pseudouridylation is guided by box H/ACA RNA (Ganot et al., 1997; Ni et al., 1997). In this RNA-guided scheme, the box H/ACA RNA folds into what is known as a “hairpin-hinge-hairpin-tail” structure, leaving an internal loop open in each hairpin. This loop (termed the pseudouridylation pocket) base-pairs with the target RNA in such a way that the target uridine is positioned at the base of the upper stem and pseudouridylated (Fig. 5).

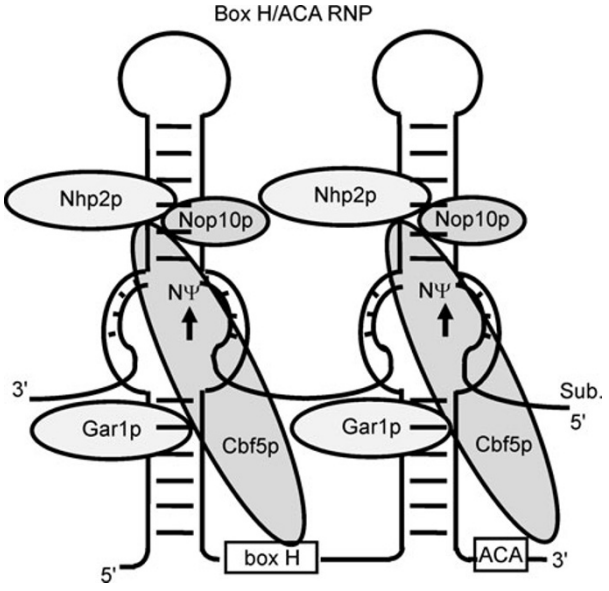

Figure 5. Box H/ACA RNP pseudouridylase. A complete box H/ACA RNP complex is diagrammed. The secondary structure of box H/ACA RNA (with two conserved boxes, box H and box ACA) and the 4 core proteins (Nop10, Nhp2, Gar1, and the enzymatic component Cbf5) are schematically represented. The substrate RNA (Sub.) and the target nucleotide (Ψ, pointed at by an arrow) are also indicated.

Box H/ACA RNAs do not function as naked RNAs in the cell. Instead, they associate with proteins to form RNA-protein complexes known as box H/ACA RNPs (Yu et al., 2005). Specifically, the box H/ACA RNPs are composed of a unique box H/ACA RNA and four core proteins [Cbf5 (dyskerin/NAP57), Nhp2, Gar1, and Nop10] (Meier and Blobel, 1994; Balakin et al., 1996; Ganot et al., 1997; Ni et al., 1997; Yu et al., 2005; Grozdanov and Meier, 2009). Cbf5 is the enzymatic component of the RNP that catalyzes, upon positioning of the target uridine, the U-to-Ψ isomerization reaction (Zebarjadian et al., 1999; Grozdanov and Meier, 2009) (Fig. 5).

Although this mechanism was first discovered in rRNA, it was believed that the same mechanism could be used to

pseudouridylate spliceosomal snRNAs as well. To test this idea, several labs focused their resources into a search for RNA guide sequences that matched those of spliceosomal snRNAs, and successfully found a number of candidate box H/ACA guide RNAs (Hüttenhofer et al., 2001; Jady and Kiss, 2001; Hüttenhofer et al., 2002; Zhao et al., 2002; Schattner et al., 2006). Detailed experimental analyses, using the mammalian system as well as the Xenopus oocyte microinjection system, confirmed the presence of guide activity stemming from a couple of the identified box H/ACA RNAs (Jády and Kiss, 2001; Zhao et al., 2002).

To date, the systematic research using a variety of sources, including computational predictions and experimental approaches, has led to the identification of an almost-complete library of box H/ACA guide RNAs corresponding to nearly all sites of pseudouridylation in higher eukaryotic rRNAs and snRNAs (Hüttenhofer et al., 2001; Schattner et al., 2006). These results suggest that, in higher eukaryotes, RNA-guided RNA modification is the main (if not the only) mechanism for the pseudouridylation of rRNAs and spliceosomal snRNAs.

Both RNA-dependent and RNA-independent mechanisms catalyze spliceosomal snRNA pseudouridylation in yeast

While computational analysis also identified a near-complete set of box H/ACA RNAs corresponding to almost all pseudouridines in yeast rRNA (Schattner et al., 2004), no box H/ACA RNAs were assigned to the known pseudouridylation sites in yeast spliceosomal snRNAs, despite the fact that there are far fewer pseudouridines in yeast (*S. cerevisiae*) spliceosomal snRNAs than in their higher eukaryotic counterparts (e.g. U2, see Fig. 3). However, yeast, as an effective experimental system, has been extensively used for studying the mechanisms of spliceosomal snRNA pseudouridylation. Interestingly, early efforts showed that pseudouridylation in yeast was fundamentally different from that in higher eukaryotes. Specifically, using both *in vivo* and *in vitro* assays, the Branlant lab indicated that Pus1, a single-peptide enzyme known to pseudouridylate yeast tRNA at various positions, also catalyzes pseudouridylation of yeast U2 at position 44 (Massenet et al., 1999).

Following up on this observation, Ma et al. screened the yeast GST-ORF (glutathione S-transferase-open reading frame) fusion protein library, and found that pseudouridylation of yeast U2 snRNA at position 35 was also catalyzed by a single-polypeptide enzyme, Pus7p (Ma et al., 2003). Pus7p isolated from *E. coli* was also found to be fully capable of catalyzing pseudouridylation at position 35 *in vitro*, demonstrating that it alone is the pseudouridylase responsible for Ψ35 formation (intriguingly, like Pus1p, Pus7p is also responsible for pseudouridylating tRNA at certain positions) (Behm-Ansmant et al., 2003). Thus, in two out of three instances (there are a total of three pseudouridines in yeast U2) (Fig. 3), yeast U2 pseudouridylation is catalyzed by an RNA-independent mechanism, suggesting that the RNA-independent mechanism might be a general mechanism that catalyzes spliceosomal snRNA pseudouridylation in yeast.

Then, at a time when it was widely believed that spliceosomal snRNA pseudouridylation in yeast was completely different from the (RNA-dependent) system in higher eukaryotes, Ma et al. made a surprising finding (Ma et al., 2005). Using a singly radio-labeled U2 substrate, where the radiolabel was placed specifically at position 42 (the only pseudouridine site whose enzyme had not been assigned), they screened the yeast GST-ORF library and found that snR81, a box H/ACA RNP, catalyzed the U42-to-Ψ42 conversion. snR81 has two guide sequences (pseudouridylation pockets): one in the 5′ hairpin and the other in the 3′ hairpin. Interestingly, while the 5′ guide sequence directs the pseudouridylation of U2 snRNA at position 42, the 3′ pseudouridylation pocket catalyzes pseudouridylation of 25S rRNA at position 1051 (Fig. 6). Taken together, pseudouridylation of yeast U2 snRNA (and perhaps the other yeast spliceosomal snRNAs as well) is catalyzed by two completely different mechanisms: the RNA-independent mechanism (Pus7 and Pus1), which modifies U2 at positions 35 and 44 (Massenet et al., 1999; Ma et al., 2003), and the RNA-dependent mechanism (snR81) that pseudouridylates U2 at position 42 (Ma et al., 2005).

Yeast, then, seems to be a transitional phase in the evolution of the mechanisms of spliceosomal snRNA pseudouridylation, as it utilizes both the RNA-dependent and RNA-independent mechanisms to pseudouridylate nucleotides at different positions. Assuming that the RNA-dependent mechanism evolved from the RNA-independent mechanism (Lafontaine and Tollervey, 1998), the fact that snR81 pseudouridylates U2 snRNA at position 42 suggests that the box H/ACA RNP responsible for Ψ42 formation (RNA-dependent) has in fact evolved in yeast as well as in higher eukaryotes. In contrast, box H/ACA RNPs specific for Ψ35 and Ψ44 may have not yet evolved in yeast, leaving pseudouridylation at these positions to be catalyzed by the RNA-independent mechanism. Alternatively, it is possible that the box H/ACA RNPs for Ψ35 and Ψ44 did evolve in yeast, but were subsequently lost from the genome.

Spliceosomal snRNA pseudouridylation is induced at novel sites under stress conditions

Until recently, all known modifications in spliceosomal snRNAs (Fig. 2 and 3) were considered constitutive (they are introduced into the RNA soon after it is transcribed, and the modified nucleotides remain in the RNA over the course of its entire life). However, recent research suggests that pseudouridylation can be conditionally induced at novel sites (Wu et al., 2011a). Wu et al. looked into U2 snRNA of

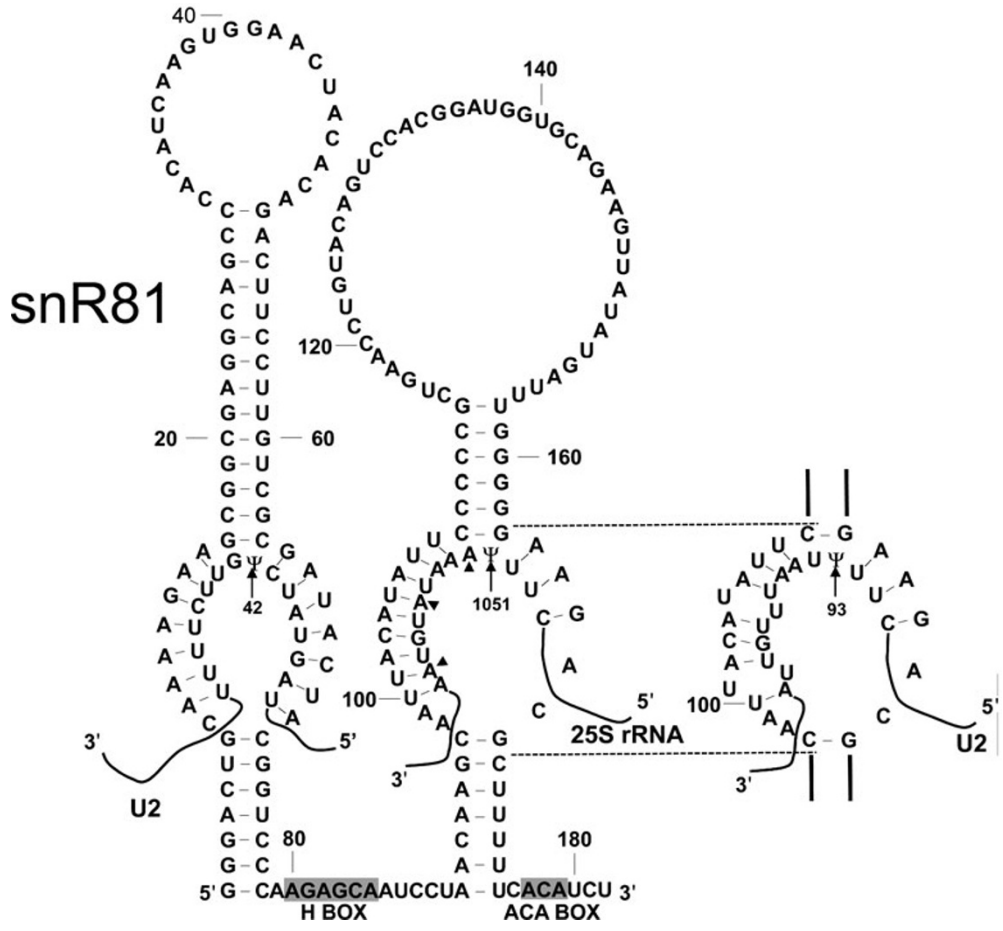

Figure 6. Base-pairing interactions between snR81 and its substrates. The primary sequence (along with box H and box ACA, indicated by shaded boxes) and the secondary structure of snR81 are shown. The substrates, a short sequence of U2 covering position 42 (pairing with the guide sequence at the 5′ pseudouridylation pocket) and a short sequence of 25S rRNA covering position 1051 (pairing with the guide sequence at the 3′ pseudouridylation pocket), are also shown. The base-pairing interactions between the inducible pseudouridylation substrate (the Ψ93 region of U2) and the guide sequence at the 3′ pseudouridylation pocket are depicted as well, on the far right. The targeted nucleotides are indicated by arrows.

S. cerevisiae to attempt to address a relatively old problem: DNA and proteins can be inducibly/reversibly modified to initiate regulatory functions, but RNA had never been shown to have this function—all modifications discovered in RNA, up to this point, had been constitutive (Wu et al., 2011a). To find novel inducible pseudouridines, Wu et al. subjected yeast to two stress conditions: nutrient deprivation and heat shock (Wu et al., 2011a). After applying these conditions, they isolated RNAs for pseudouridylation assays.

Intriguingly, when U2 snRNA derived from nutrient-deprived cells was analyzed, in addition to the three known constitutive modifications (Ψ35, Ψ42 and Ψ44), clear pseudouridine signals were also detected at two novel positions: Ψ56 and Ψ93, which had previously been identified as unmodified uridines (Massenet et al., 1998). Ψ56 can also be induced under stress conditions (Wu et al., 2011a). Detailed analysis has demonstrated that Pus7, a polypeptide enzyme that constitutively pseudouridylates U2 at position 35 and tRNA at several positions, is responsible for the induced formation of Ψ56, and snR81, a box H/ACA RNP that constitutively catalyzes pseudouridylation of U2 at position 42 and 25S rRNA at position 1051, is responsible for Ψ93 formation (Wu et al., 2011a).

Remarkably, using a series mutagenesis analysis, Wu et al. further demonstrated that the imperfect substrate sequences surrounding positions 56 and 93 seem to be necessary for induction (Wu et al., 2011a). Specifically, the sequence flanking Ψ56 is similar, but not identical to, the sequence surrounding position 35 of U2, the target of Pus7. Likewise, the sequence surrounding position 93 is similar, but not identical to, the sequence surrounding position 1051 of 25S rRNA, the target of the 3′ guide sequence (3′ pocket) of snR81 (Fig. 6). Under stress conditions, then, it would seem that the normally strict requirements for matching sequences are loosened, resulting in the inclusion of the imperfect sequences.

Crystal structure analyses elucidate RNA-dependent and RNA-independent pseudouridylation

There are six main families of pseudouridylases: TruA, TruB, TruD, RsuA, RluA, and Pus10 (Mueller and Ferre-D'Amare, 2009). Their primary sequences are sometimes so divergent that they cannot be identified by sequence similarity alone, but recent breakthroughs in crystallography have succeeded in solving the structures of all six families in unbound form and/or with substrate attached (Foster et al., 2000; Sivaraman et al., 2002; Del Campo et al., 2004; Ericsson et al., 2004; Hoang and FerréD'Amaré, 2001, 2004; Kaya et al., 2004; Mizutani et al., 2004; Sivaraman et al., 2004; Hoang et al., 2006; McCleverty et al., 2007). Interestingly, all of the six families share a common catalytic domain structure despite their sequence differences (Mueller and Ferre-D'Amare, 2009). This domain structure is comprised mainly of anti-parallel β-sheets, with one face sporting two separated groups of α-helices and loops. These loops form a forefinger-thumb structure that pinches the target RNA, while a catalytic aspartate residue triggers the enzymatic reaction.

Box H/ACA RNPs rely on the enzymatic component Cbf5 for their activity. Cbf5 belongs to the TruB pseudouridylase family. Interestingly, it has been reported that archaeal Cbf5 alone, like TruB, can catalyze tRNA pseudouridylation at position 55 (Roovers et al., 2006). The complexity in substrate specificities revealed by Cbf5 (relative to TruB) has raised an interesting question: How is it that these two closely related pseudouridylases can modify tRNA without RNA guidance, but Cbf5, when pseudouridylating spliceosomal snRNAs and rRNAs, must be in a complex with the rest of a box H/ACA RNP? To address this question, a great deal of efforts have been expended to solve the crystal structures of archaeal box H/ACA snRNP complexes. As a result, the structures of various forms, from the initial complex of three core proteins to a complete, substrate-bound box H/ACA RNP, have been solved (Li and Ye, 2006; Manival et al., 2006; Rashid et al., 2006; Liang et al., 2007; Duan et al., 2009; Liang et al., 2009). A detailed picture of how this most complex pseudouridylase modifies its substrate has now become available.

By comparing the structure of tRNA substrate-bound TruB (Hoang and FerréD'Amaré, 2001) with the structure of substrate-bound box H/ACA RNP (Duan et al., 2009), the Ye group was able to identify the similarities and differences between TruB- and Cbf5-catalyzed pseudouridylation. While the two enzymes look strikingly similar in complex, there are a few key differences. One of these differences might explain, at least in part, why Cbf5 can act both independently of and in conjunction with box H/ACA RNA. In the tRNA-TruB complex, TruB caps the reverse Hoogsteen pair of its target tRNA, U54-A58, with a histidine residue, just as Cbf5 caps its corresponding Watson-Crick base-pair, U6′-A43 (numbered arbitrarily), in the complex of substrate-box H/ACA RNP. However, there is one main difference: the glycosidic bonds of the reverse Hoogsteen U54-A58 pair are in the *trans* orientation, but the bonds of the Watson-Crick U6′-A43 pair are in the *cis*. As such, although TruB caps its U54-A58 pair with its histidine residue H43, the corresponding histidine (H80) in Cbf5 does not stack over A43 of the U6′-A43 pair. Instead, Cbf5 exhibits a new histidine, H60, to cap its target pair. Thus, the structural data suggest that the capping systems differ in the two enzymes; they each cap their RNA substrates with different histidine residues. And because H63 and H80 are both invariant in Cbf5, it is conceivable that, like TruB, Cbf5 alone might be able to act, using H80, on the reverse Hoogsteen pair in its tRNA substrate (Roovers et al., 2006).

Another notable difference is the fact that the thumb loop of Cbf5 is 17 residues shorter than that of TruB (Hoang and FerréD'Amaré, 2001; Duan et al., 2009). Ye and colleagues suggest that, for the box H/ACA RNP-catalyzed reaction, the 17-residue deletion allows the 3′ arm of substrate RNA to be free of steric interference from what would otherwise be a longer loop. And yet, because the conformation of the shorter thumb loop is still similar to that of TruB′s, Cbf5, when acting alone, is still able to catalyze tRNA pseudouridylation (Duan et al., 2009). Also noticeable are the differences in the structural elements that each enzyme, TruB or Cbf5, needs to recognize its tRNA substrate. For instance, while TruB recognizes T stem loops of tRNA, Cbf5, due to its more elaborate PUA domain, may also recognize the 3′ CCA tail and acceptor stem of tRNA (Duan et al., 2009)

FUNCTIONS OF SPLICEOSOMAL snRNA PSEUDOURIDYLATION

Since the discovery of pseudouridine many decades ago, a number of labs have studied the functions of this modified nucleotide. Unfortunately, due primarily to the lack of effective assays and experimental systems, functional analysis of pseudouridines in spliceosomal snRNAs (and RNA in general) has proceeded slowly. In the late 1990s, though, several labs developed highly sensitive assays and systems for studying modified nucleotides in RNA, making it possible to dissect the function of pseudouridines in spliceosomal snRNAs during pre-mRNA splicing [reviewed in (Wu et al., 2011b)]. Since U2 contains the most pseudouridines when compared with all other spliceosomal snRNAs (Massenet et al., 1998; Yu et al., 1999), it has been the main focus of functional study.

Pseudouridylation of U2 is required for snRNP assembly

In 1998, Yu et al. developed a *Xenopus* oocyte reconstitution system to study U2 snRNA modification (Yu et al., 1998). In their work, an antisense U2 DNA oligonucleotide was injected into *Xenopus* oocytes to hybridize with endogenous U2

snRNA, thereby triggering endogenous RNase H activity that degraded the RNA strand (U2) of the RNA-DNA hybrid. *In vitro* transcribed U2 (unmodified), cellularly-derived U2 (completely modified), or a hybrid of the two (partially modified) was then injected into the U2-depleted oocytes. After a short reconstitution period (3.5 h), snRNP biogenesis and pre-mRNA splicing were analyzed, allowing a detailed assessment of the function of the injected U2 snRNA in these processes. U2 modifications occurred slowly in some regions (no modification or very light modification observed within 3.5 h), and thus the function (or lack thereof) of injected U2 in snRNP assembly and pre-mRNA splicing reflected the importance of modifications in these particular regions. Using this method, Yu et al. identified functionally important modified nucleotides within the first 27 nucleotides of U2 snRNA, including three pseudouridines (Yu et al., 1998) (Fig. 2 and 3).

Using native gel analysis, Yu et al. further demonstrated that upon injection, cellular U2 resulted in a full pattern of splicing complexes (A, B1, B2, and C; see Fig. 1) (Yu et al., 1998). This stood in stark contrast to the results when unmodified U2 was used, where no higher-order splicing complexes were detected after a 3.5 h reconstitution. Clearly, then, the modifications in U2 are necessary for spliceosome formation. Further analysis using oligonucleotide affinity purification and gradient centrifugation indicated that U2 snRNA modifications contributed to U2 snRNP assembly (Yu et al., 1998).

Later work carried out by the Luhrmann group looked into the role that pseudouridines of mammalian U2 snRNA play in pre-mRNA splicing in Hela cell extracts (Donmez et al., 2004). Their results confirmed that pseudouridines are required for the formation of early spliceosomal complexes; U2 lacking pseudouridine residues was found incompetent at forming complexes A, B1, B2, and C (Fig. 1).

Pseudouridines in the U2 branch site recognition region are also important for RNP assembly and splicing

To study those pseudouridines that are introduced rapidly (within 3.5 h) in Xenopus oocytes (e.g., the pseudouridines in the U2 branch site recognition region), Zhao and Yu utilized 5-fluorouridine-containing U2 to selectively block rapidly occurring U2 pseudouridylation at specific sites of interest, and identified the six pseudouridines in the U2 branch site recognition region as important for snRNP biogenesis and splicing (Zhao and Yu, 2004b).

The yeast genetics system was also used to study the function of spliceosomal snRNA modifications. Yang et al. used a genetic synthetic lethal screen to demonstrate that Ψ35, when coupled with a U2 point mutation at position 40, is required for pre-mRNA splicing in *S. cerevisiae* (Yang et al., 2005). This result is consistent with an NMR study showing that, when paired with the pre-mRNA branch site, Ψ35 is favored over uridine for maintaining the bulge of the branch point nucleotide adenosine for the nucleophilic attack in the first step of splicing (Newby and Greenbaum, 2002). However, it is worth mentioning a recent work by the Kielkopf group on the crystal structure of the pseudouridylated (Ψ35) U2 branch site recognition region duplexed with the pre-mRNA branch site (Lin and Kielkopf, 2008). Their results show that either the branch point adenosine or a preceding purine nucleotide bulges out from the duplex (Lin and Kielkopf, 2008). The importance of Ψ35 in splicing is further supported by the work of Valadkhan and Manley who have demonstrated that pseudouridylation at this position greatly enhances the production of X-RNA, a product generated by a splicing-related reaction in a cell- and protein-free system (Valadkhan and Manley, 2003).

In short, results from different experimental systems have established the functional importance of modifications in U2 snRNA (and perhaps the other spliceosomal snRNAs as well) in pre-mRNA splicing.

5-fluorouracil (5FU) functions as a pseudouridylation inhibitor blocking U2 function in splicing

During their studies of spliceosomal snRNA modifications, Zhao and Yu also established a clear link between 5FU and the inhibition of U2 snRNA pseudouridylation. U2 lacking proper pseudouridines then led to failure of splicing, further demonstrating the important role of pseudouridines in U2 function (Zhao and Yu, 2007).

5FU is an effective and widely used anti-cancer drug (Heidelberger et al., 1957). However, except for the postulation that it affects DNA metabolism, the mechanism of action of 5FU remained largely unclear (Parker and Cheng, 1990; Ghoshal and Jacob, 1997; Longley et al., 2003). Zhao and Yu applied 5FU to mammalian cell cultures, and found that 5FU was efficiently converted into 5-fluoroUTP, which was then readily incorporated into U2 snRNA at various positions (Zhao and Yu, 2007). The incorporation of 5FU into sites that were normally pseudouridylated effectively blocked their conversion into pseudouridines. Moreover, the 5-flurouridylated U2 snRNA also functioned as an inhibitor that blocked the pseudouridylation of newly synthesized U2 snRNA. As expected, U2 snRNA lacking proper pseudouridines failed to function in pre-mRNA splicing. Thus, Zhao and Yu concluded that 5FU, by incorporating into U2 and perhaps the other spliceosomal snRNAs, effectively blocked spliceosomal snRNA pseudouridylation and pre-mRNA splicing, thus contributing at least in part to the death of rapidly propagating cancer cells.

Pseudouridines have many intrinsic features that affect RNA structure and function

Pseudouridine differs from its counterpart uridine in several

ways, but most importantly, pseudouridine can, through its extra amine (-NH_2) group (Fig. 4), donate an extra hydrogen bond, resulting in many interesting characteristics which have been observed experimentally. For instance, pseudouridine can, using a water molecule as a mediator, facilitate hydrogen bonding to the phosphate oxygen of its own ribose backbone, thus making its RNA backbone more rigid (Charette and Gray, 2000). Work from the Steitz lab has shown that substitution of a specific uridine with pseudouridine in the U7 Sm binding site results in non-functional U7 snRNP (Kolev and Steitz, 2006). The Yu lab has also shown that one U-to-Ψ change at the polypyrimidine tract of a pre-mRNA leads to defects in binding to U2AF, an essential splicing factor, and consequently, failure to splice (Chen et al., 2010). Both instances suggest that pseudouridylation rigidifies the RNA backbone, which in turn compromises RNA function. Also, the ability of pseudouridine to alter RNA structure was directly observed in a tRNA crystal structure (Arnez and Steitz, 1994)

In addition, several lines of experimental evidence indicate that pseudouridylation of RNA leads to increased base stacking and more stabilized base-pairing. For instance, work by Davis, focused specifically on base stacking in the oligoribonucleotide sequences AAUA, AATA, and AAΨA, found that the AAΨA oligomer exhibited properties characteristic of better stacking compared with the oligomer AAUA (Davis, 1995). NMR measurements indicated that these effects then propagated throughout the helix, indicating that pseudouridine has an intrinsic ability to stabilize RNA-RNA base-pairing interactions. In the context of the base-pairing interaction between the pre-mRNA branch site and the U2 branch site recognition region, Newby and Greenbaum have shown that pseudouridine indeed enhances pairing affinity (Newby and Greenbaum, 2001). Taken together, it is conceivable that all these features of pseudouridine, including its ability to alter structure, increase base-stacking, and improve RNA-RNA base-pairing, contribute to the function of splicesomal snRNAs in splicing.

Induced pseudouridylation contributes to pre-mRNA splicing regulation

As described earlier, yeast U2 snRNA is inducibly pseudouridylated at novel positions under stress conditions; these positions include Ψ56 and Ψ93 (Fig. 7). Wu et al. dissected the function of Ψ93 using a well-established reporter system (ACT1-CUP1 fusion gene) where the expression level of the Cup1 fusion protein reflects or correlates with the efficiency of splicing of the fusion pre-mRNA (Wu et al., 2011a). They showed that splicing of an ACT1-CUP1 pre-mRNA was reduced when pseudouridylation was introduced into U2 snRNA at position 93. This result suggests that Ψ93 (and perhaps the other induced pseudouridines in spliceosomal snRNAs as well) indeed plays a role in pre-mRNA splicing under stress conditions.

Mechanistically, how do induced pseudouridines affect pre-mRNA splicing? Recently, the Ares and Staley labs proposed a model reliant on the fact that U2 toggles between two mutually exclusive structures, stem-loop IIa and stem IIc, during splicing (Hilliker et al., 2007; Perriman and Ares, 2007) (Fig. 7). While stem-loop IIa favors U2-substrate interaction, stem IIc is necessary for catalysis (the chemical reaction of splicing) (refer to Fig. 1). Thus, toggling between the two structures is believed to be necessary to move splicing forward. Interestingly, both Ψ56 and Ψ93 fall into the regions of stem-loop IIa and stem IIc (Wu et al., 2011a). Wu et al. have thus hypothesized that the induced pseudouridines play an important role in regulating the switching between stem-loop IIa and stem IIc (Wu et al., 2011a). Because the Ψ-A pair is more stable than the U-A pair, such interactions might tip the thermostability balance in favor of stem IIc, thus negatively impacting pre-mRNA splicing (Fig. 7).

CONCLUDING REMARKS

Spliceosomal snRNAs exhibit pseudouridylation in functionally important regions—suggesting that this modification is functionally relevant. Indeed, functional analyses in a number of differing experimental systems have shown that pseudouridines in spliceosomal snRNAs contribute to and are required for splicing. Studies on the mechanisms of spliceosomal snRNA pseudouridylation have also yielded fruitful results over the past decade. Two disparate mechanisms have been identified, and crystal structures of various pseudouridylases, from single-polypeptide enzymes to complex box H/ACA RNPs, have been solved, leading to the emergence of a clearer picture of the mechanism of this reaction.

While research progress on spliceosomal snRNA pseudouridylation has been remarkable over the past decade or so, there are still a number of questions that remain to be addressed. In particular, the recent discovery of inducible spliceosomal snRNA pseudouridylation has raised a number of interesting questions. For instance, how abundant and widespread is inducible pseudouridylation? Does inducible pseudouridylation also occur in higher eukaryotic spliceosomal snRNAs? Are other types of post-transcriptional RNA modification (2′-O-methylation, various base modifications, etc.) also inducible? Are induced modifications reversible? What regulatory roles do inducible/reversible RNA modifications play? Undoubtedly, regulatory RNA modification, as a completely new research area, will draw a great deal of attention. With the new technologies currently available and being developed, we are confident that a clear picture of spliceosomal snRNA pseudouridylation (and RNA modification in general), both constitutive and inducible, will soon emerge.

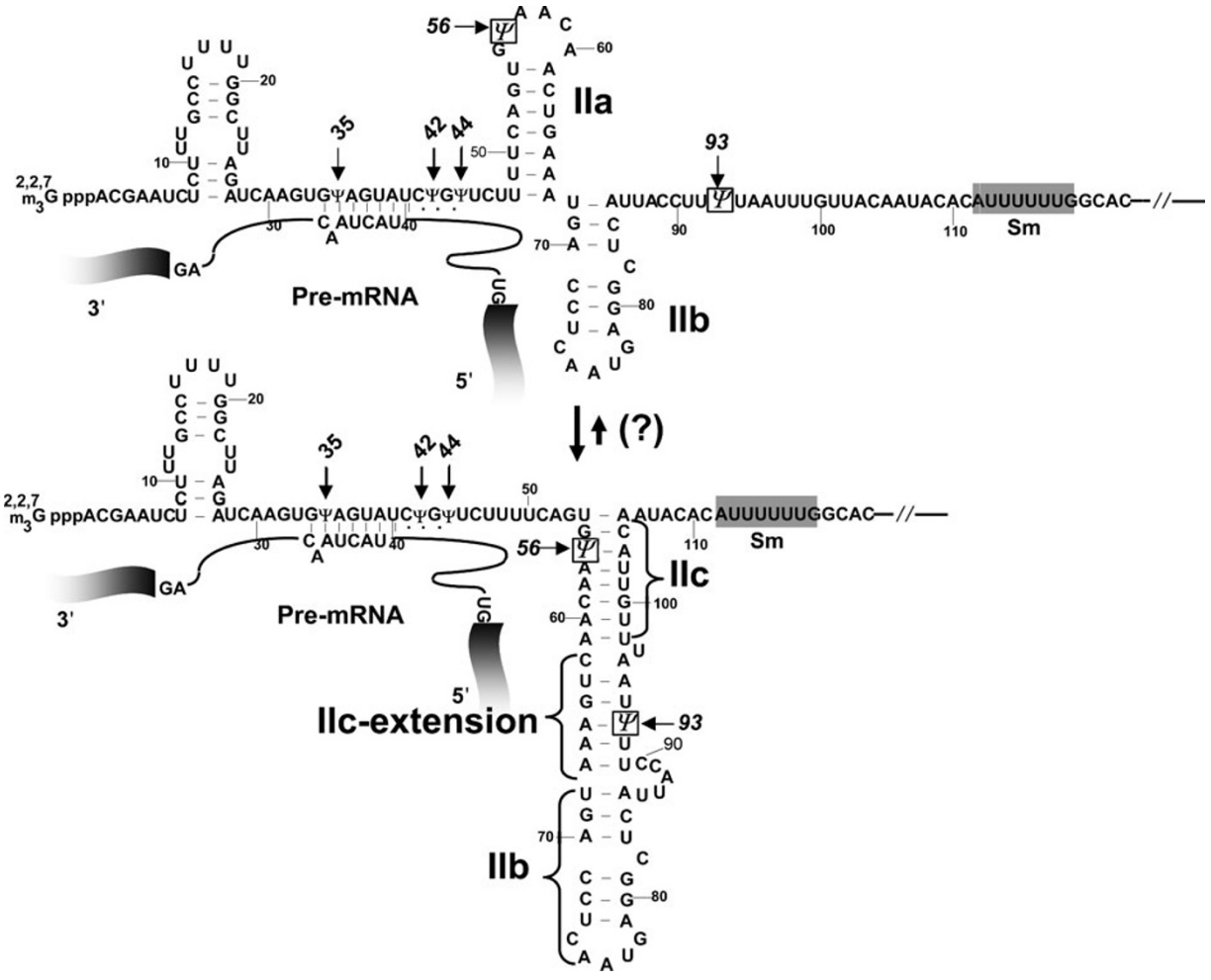

Figure 7. Toggling between two U2 structures. Parts of the primary sequence and secondary structure of yeast U2 snRNA are shown. Two competing structures (Hilliker et al., 2007; Perriman and Ares, 2007), stem-loop IIa (top) and stem IIc (bottom), are schematically represented. The base-pairing interactions between the U2 branch site recognition region and the pre-mRNA branch site are also schematically shown. The three constitutive pseudouridines in the U2 branch site recognition region (positions 35, 42, and 44), as well as the two inducible pseudouridines (positions 56 and 93), are indicated. Shaded boxes represent the Sm binding sites. The unbalanced conversion arrows (with a question mark) reflect the hypothesis that induced pseudouridylation at positions 56 and 93 might favor the stem IIc structure, tipping the balance in that direction.

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