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Review

The special Sm core structure of the U7 snRNP: far-reaching significance of a small nuclear ribonucleoprotein

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Abstract. The polypeptide composition of the U7 small nuclear ribonucleoprotein (snRNP) involved in histone messenger RNA (mRNA) 3′ end formation has recently been elucidated. In contrast to spliceosomal snRNPs, which contain a ring-shaped assembly of seven so-called Sm proteins, in the U7 snRNP the Sm proteins D1 and D2 are replaced by U7-specific Sm-like proteins, Lsm10 and Lsm11. This polypeptide composition and the unusual structure of Lsm11, which plays a role in histone RNA processing, represent new themes in the biology of Sm/Lsm proteins. Moreover this structure has important consequences for snRNP assembly that is mediated by two complexes containing the PRMT5 methyltransferase and the SMN (survival of motor neurons) protein, respectively. Finally, the ability to alter this polypeptide composition by a small mutation in U7 snRNA forms the basis for using modified U7 snRNA derivatives to alter specific pre-mRNA splicing events, thereby opening up a new way for antisense gene therapy.

Key words. Histone RNA 3′ end processing; U7 small nuclear ribonucleoprotein; Sm-like proteins; SMN complex; symmetrical dimethyl arginine; Sm core assembly; Cajal bodies; antisense gene therapy; alternative splicing.

Introduction

The U7 small nuclear ribonucleoprotein (snRNP) is an essential factor for the maturation of animal replication-dependent histone messenger RNAs (mRNAs). As their name implies, these mRNAs are regulated during the cell cycle, with maximal levels being present during S phase. Features distinguishing them from all other mRNAs are their lack of introns and poly(A) tails. Their 3′ ends are formed by an endonucleolytic cleavage of longer pre-mRNAs that is mechanistically different from the cleavage/polyadenylation reaction generating all other mRNA 3′ ends. This 3′ cleavage is itself cell cycle regulated. Moreover, the mature 3′ end is involved in controlling later steps of the RNA's life cycle, such as translation and mRNA degradation, the latter process being cell cycle regulated as well.

Several reviews commenting these aspects of histone RNA metabolism and focussing on the mRNA 3′ end have been written [1–5]. The purpose of this paper is to discuss recent findings regarding the composition, function and cell biology of the U7 snRNP. These findings are not only important for understanding histone pre-mRNA and mRNA metabolism, but they are also relevant for other fields, in particular those of riboregulation, the cell biology of nuclear substructures and gene therapy.

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Role of the U7 snRNP in histone RNA processing

The histone RNA cleavage site is flanked by evolutionarily conserved sequences that interact with trans-acting processing factors (fig. 1). Upstream of the cleavage site is a highly conserved 26 nt sequence encompassing a hairpin structure. This RNA hairpin is recognised by the hairpin binding protein (HBP) [6] or stem-loop binding protein (SLBP) [7]. Because the hairpin sequence lies upstream of the cleavage site, HBP can remain bound to mature histone mRNA after 3′ processing. In fact, HBP is associated in stoichiometric amounts with histone mRNA on polysomes as well as in the nucleus [8, 9]. Moreover, it has been implicated as a key player in other steps of histone mRNA metabolism besides RNA 3′ end formation, such as nucleo-cytoplasmic export, translation and the cell cycle-specific degradation of histone mRNA at the end of S phase [10–12]. Furthermore, HBP is essential very early in development in both *Drosophila melanogaster* [13] and *Caenorhabditis eleg*ans [14, 15].

The human HBP is 270 amino acids long and contains a central RNA binding domain (RBD) of 75 amino acids that was defined by deletion and point mutations [7, 16–19]. It does not resemble other known RNA binding proteins. Similar to the cell cycle-specific regulation of histone mRNA, HBP itself is also cell cycle regulated. The protein accumulates to high levels in late G1 and during S phases, followed by low levels during the rest of the cell cycle. Both translational and posttranslational processes, such as phosphorylation of HBP, contribute to this regulation [20].

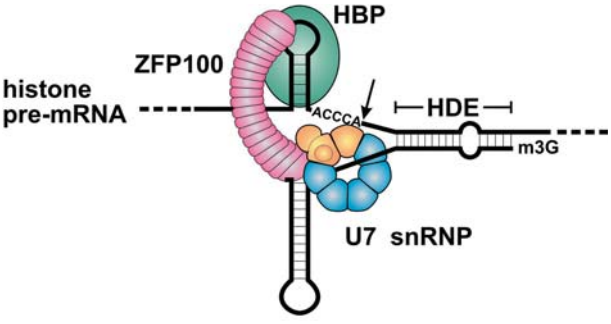

Figure 1. Molecular mechanism of histone pre-mRNA 3′ end processing. Animal replication-dependent histone pre-mRNAs contain all the cis-acting sequences necessary for 3′ end processing: a hairpin with a 6-bp stem and a 4-nucleotide loop that is bound by the hairpin-binding protein (HBP) and the histone downstream element (HDE) that tethers the m3G-capped U7 snRNA by base-pairing interactions. HBP functions by stabilising the association of the U7 snRNP with the pre-mRNA [21]. It is helped in this role by a 100-kDa zinc finger protein (ZFP100) [24] that forms a molecular bridge between HBP and the U7 snRNP [37]. The special Sm core structure of the U7 snRNP contains five Sm proteins that are also found in spliceosomal snRNPs (blue) and two U7-specific proteins, Lsm10 and Lsm11 (orange; see fig. 2) [36, 37]. The 3′ end of the mature histone mRNA is generated by an endonucleolytic cleavage at the extremity of the ACCCA sequence which immediately follows the hairpin. HBP remains bound to the mature mRNA and functions in several downstream events, such as nucleo-cytoplasmic export, translation and stability.

How does HBP enhance histone RNA 3′ end processing? Dominski et al. have determined that the minimal domain required for processing in vitro consists of the RBD plus the following 20 amino acids [21]. Furthermore, they found that HBP acts by stabilising the binding of the U7 snRNP to the second conserved sequence element, the purine-rich histone downstream element (HDE), confirming and extending previous work from our own group [22, 23]. Moreover, a 100-kDa zinc finger protein was identified that interacts with the HBP/RNA hairpin complex but not with the individual components of this complex [24]. This ZFP100 was shown to stabilise the interaction between the HBP-bound histone pre-mRNA and the U7 snRNP. Thus it seemed likely that ZFP100 might interact with one or more components of the U7 snRNP. Whether HBP and ZFP100, together, can account for a previously described, but poorly characterised, 'heat-labile factor' [25], or whether other processing factors are required, is presently unknown.

The second conserved sequence in the histone 3′ UTR, the purine-rich HDE, lies several nucleotides downstream of the cleavage site and interacts by base-pairing with the U7 small nuclear (sn)RNA component of the U7 snRNP. U7 snRNA is 57–71 nt long in the vertebrate and invertebrate species in which it has been characterised. Soon after its initial discovery in sea urchins [26, 27], it was found to have a trimethyl-guanosine cap structure and to interact with proteins recognised by anti-Sm antibodies [28]. These features characterised it as a member of the Sm-type snRNPs, along with most spliceosomal snRNPs. The U7 RNA structure is simple, consisting of a short 5′ end accessible to nucleases followed by a somewhat degenerate Sm binding site involved in snRNP assembly and a 3′-terminal hairpin required for stability [29]. The exposed 5′ end is complementary to the HDE in both vertebrate and invertebrate histone pre-mRNAs. The functional importance of this sequence complementarity for histone RNA 3′ end processing has been proven using complementary mutations in U7 and histone RNAs [23, 30, 31].

In addition to being necessary for RNA 3′ processing, U7 snRNA plays the role of a 'molecular ruler' that positions the cleavage activity close to the appropriate phosphodiester bond. When the distance between the HDE and the hairpin in histone pre-mRNA is increased, the processing site gets shifted in the 3′ direction by a corresponding number of nucleotides [32]. Moreover, when corresponding length insertions are made between the Sm binding site and the base-pairing region of U7 snRNA, the cleavage site can shift back as predicted by the ruler model, but only if the inserted sequences are complementary to those present in the histone pre-mRNA [33]. Additionally, the

cleavage efficiency at the selected position depends on an optimal phosphodiester bond (ideally 3′ of an adenosine) and on the appropriate juxtaposition between the hairpin/HBP and the U7 snRNP [33, 34].

After the cleavage has occurred, a 5′ exonuclease degrades the cut-off 3′ fragment of histone pre-mRNA and thereby helps to recycle the U7 snRNP [35].

After several unsuccessful attempts in different laboratories, we recently succeeded in purifying U7 snRNPs from HeLa cells in sufficient quantities to characterise the U7 snRNP-associated proteins. These studies revealed that U7 snRNPs contain all but two of the seven Sm proteins (fig. 2). In place of the missing Sm D1 and D2 proteins, additional polypeptides of 14 and 50 kDa are present. Microsequencing identified the 14-kDa polypeptide as a new Sm-like protein related to Sm D1 and D3, which we termed Lsm10 [36]. The 50-kDa polypeptide also proved to be a member of the Sm/Lsm protein family, albeit a highly unusual one, and it was consequently termed Lsm11 [37]. Its two Sm motifs are separated by 138 amino acids and preceded by an N-terminal extension of 170 residues (see below and fig. 3).

Earlier studies from our laboratory had shown that converting the non-canonical Sm binding site of U7 snRNA (AAUUUGUCUAG; U7 Sm WT) into the consensus sequence derived from spliceosomal snRNPs (AAUUUUUGGAG; U7 Sm OPT; fig. 2) resulted in snRNP particles that accumulated more efficiently in the nucleus but were non-functional in histone RNA processing [38, 39]. Thus it seemed likely that the U7 Sm WT and U7 Sm OPT snRNPs would differ by the presence of Lsm10/11 and Sm D1/D2, respectively, and that at least one of the U7-specific Lsm proteins might be functionally involved in histone RNA 3′ processing. This hypothesis could be corroborated experimentally [37]. More specifically, we could ascribe an essential function in histone RNA processing to the N-terminal extension of Lsm11. At least part of this contribution appears to be mediated by an interaction between the N-terminus of Lsm11 and ZFP100. Thus, HBP, ZFP100 and Lsm11 form a sort of molecular bridge between the histone pre-mRNA and U7 snRNA (fig. 1). This not only stabilises this RNA:RNA interaction but also provides additional binding specificity.

What is still unknown is how the actual RNA cleavage occurs. Are the presently characterised processing factors sufficient or are additional components still awaiting their discovery? The low abundance of the factors involved and difficulties with expressing native recombinant proteins turns a full reconstitution of the system into a virtually insurmountable task. Most likely, a combination of genetic and biochemical approaches will be required to resolve this important question.

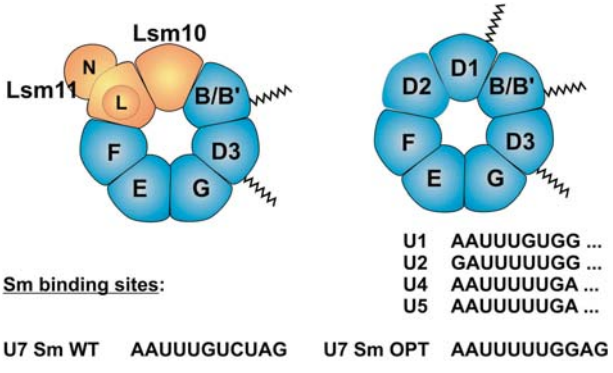

Figure 2. The unique Sm core structure of U7 snRNPs. Whereas spliceosomal snRNPs contain an Sm core structure consisting of the seven standard Sm proteins B/B′, D3, D1, D2, E, F and G (right) [41, 46, 98], the U7 snRNP (left) has two U7-specific Sm-like proteins, Lsm 10 and 11 (orange) replacing Sm D1 and D2 [36, 37]. In comparison with other Sm/Lsm proteins, Lsm11 has an extended N-terminus (N) and a long linker (L) between its two Sm motifs (see fig. 3). The N-terminus participates in the histone processing reaction by directly interacting with ZFP100 (see fig. 1). Converting the special U7 snRNA Sm binding site (U7 Sm WT) to a consensus derived from the spliceosomal snRNAs (U7 Sm OPT) leads to the formation of an Sm core containing D1/D2. Assembly of these two structures is mediated by distinct SMN complexes containing either of the two dimers, Sm D1/D2 or Lsm10/11 [37]. The zig-zag line extensions of B/B′, D1 and D3 denote Arg-Gly dipeptide repeats containing symmetrical dimethylarginine modifications introduced by the PRMT5 methyltransferase complex [78, 79].

A new type of Sm/Lsm protein structure involved in riboregulation

Originally, the Sm proteins were so named because they react with antibodies of the Sm serotype from patients affected by the autoimmune disease systemic lupus erythematosus [40]. The corresponding antigens are components of the spliceosomal snRNPs U1, U2, U4 U5, U11, U12 and U4atac. As these snRNPs were purified, they were each found to contain a set of seven small proteins which were termed Sm proteins B/B′ (alternative splicing products of the same gene), D1, D2, D3, E, F and G (reviewed in [41]). The widely used Y12 monoclonal anti-Sm antibody reacts to a variable extent with Sm B/B′, D1 and D3. Later it was revealed that these proteins share conserved amino acid residues that define a new family of proteins [42–44]. These residues form the Sm motifs 1 and 2, which are separated by a short, but variable linker (fig. 3). X-ray structures of co-crystals of either Sm B/D3 or D1/D2 showed identical folding patterns of all four proteins and identical subunit interfaces for the two heterodimers [45, 46]. Based on this structural similarity as well as on a host of other biochemical data, it was proposed that one molecule each of the seven Sm proteins form a seven-membered ring structure, the so-called Sm core (fig. 2). Additional evidence for such a structure was obtained from cryo-electron microscopy of purified U1 snRNPs [47] and from crystal structures of related complexes from Archeal proteins (see below).

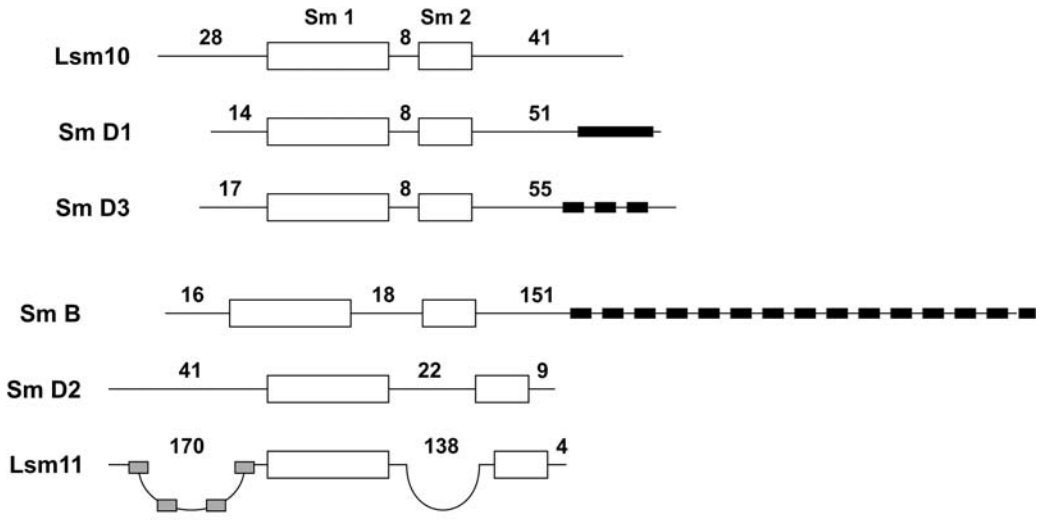

Figure 3. Lsm11 is an unusual member of the Sm/Lsm protein family. Sm and Lsm proteins share conserved amino acids defining the Sm motifs 1 and 2 that are separated by a usually short, variable linker [43, 44, 48, 49, 51]. Together, these form a defined structure – the Sm domain – consisting of an α-helix and five β-strands [45, 46]. The Sm proteins Sm D1, D3 and B/B′ have RG repeats (indicated by black bars; interrupted by non-RG sequences for D3 and B/B′). These repeats contain symmetrical dimethylarginine modifications introduced by the PRMT5 methyltransferase complex [78, 79]. Of the two U7-specific proteins, Lsm10 is most similar to Sm D1 and D3 but lacks RG repeats [36], while Lsm11 is closest to Sm D2, which has a moderately long N-terminus and a somewhat longer linker between its Sm motifs. Lsm11 stands apart from other Sm proteins with its unusually long linker and N-terminus. The N-terminus of Lsm11, which participates in histone processing contains four patches of amino acids that show conservation from vertebrates to invertebrates (grey boxes) [37, 67]. The numbers indicate the lengths of different segments in amino acids.

In addition to the standard Sm proteins, all eukaryotes have additional proteins containing the Sm domain, the so-called Sm-like or Lsm proteins [42, 44, 48–51, reviewed in 52]. Interestingly, these proteins exist in at least three structures that differ in function but overlap in their polypeptide composition. Thus, Lsm 2–8 form a heptameric ring around the 3′-terminal oligo-U stretch of U6 snRNA in the U6 mono-snRNP, the U4/U6 di-snRNP and the U4/U6 · U5 tri-snRNP [42, 44, 50]. In this context, these Lsm proteins are important for U6 snRNP recycling during pre-mRNA splicing [48, 50, 53, 54]. Unlike the Sm proteins, Lsm 2–8 can form a heptameric ring complex in the absence of U6 snRNA [48]. Most of these spliceosomal Lsm proteins have an identifiable closest relative among the standard Sm proteins, suggesting that the two heptameric units may have arisen by a coordinate gene duplication event. The second type of Lsm structure has Lsm1 replacing Lsm8: Lsm 1–7 form a cytoplasmic, rather than nuclear, complex that mediates mRNA decapping and 5′-exonucleolytic degradation [52, 55, 56]. Furthermore, Lsm 2–7 were recently shown to associate with snR5, a yeast box H/ACA small nucleolar (sno)RNA that functions to guide site-specific pseudouridylation of rRNA [57]. Additional functions for some Lsm proteins in the U8 snoRNP [58] and in transfer RNA (tRNA) maturation [59] have also been reported. Thus, by mixing and matching different proteins from this family, the cell can create different complexes with distinct functions.

Proteins of the Sm/Lsm family have also been identified in Archaea and in Eubacteria. Archaea usually have only one or two different Lsm proteins that form homoheptameric or -hexameric rings [60, 61]. In *Escherichia coli,* the long-known Hfq protein (host factor for Qβ) was recently shown to be an Lsm protein [62, 63]. Hfq associates with multiple short non-coding regulatory RNAs that modulate the translation or stability of target mRNAs via an antisense recognition mechanism. Moreover, like the archaeal Lsm proteins, Hfq has a very similar three-dimensional (3D) structure as the mammalian Sm proteins and assembles into homohexameric rings [62–64]. Thus the Sm/Lsm protein family is very ancient, and its members build similar structures and are involved in various mechanisms of riboregulation in all three domains of life. The U7 snRNP represents a new theme among these Sm/Lsm ring structures. In contrast to the structures containing Lsm 1–7, Lsm 2–8 or Lsm 2–7 where a single subunit is either replaced or missing, the U7 Sm core differs by two subunits compared to the spliceosomal snRNPs. The two Sm proteins missing from the U7 snRNP, D1 and D2, have been shown to form a heterodimer that acts as an intermediate of Sm core assembly in spliceosomal snRNPs [65, 66] (see below). A similar heterodimer is also formed by Sm D3 and B/B′, whereas Sm E, F and G form F/E/G trimers or $(F/E/G)_2$ hexamers. Interestingly, Lsm10 and the Sm domain of Lsm11, when co-expressed in *E. coli*, also form a heterodimeric complex [R.S.P. and D.S., unpublished re-

sults]. Moreover, Lsm10 most closely resembles one of the proteins in the two other Sm heterodimers, i.e. Sm D1 and D3 [36]. By extension, it is also similar to the D1 and D3 'relatives' among the spliceosomal Lsm proteins, Lsm2 and Lsm4, respectively. Although Sm B/B′, D2 and Lsm11 share little more than a weak similarity in their Sm motifs and longer than average spacers between these, it can be speculated that the appearance of a heterodimer consisting of a D1-like and a D2-like protein may have been an early step in Sm/Lsm protein diversification in early eukaryotes. In this evolutionary scheme, each of the present pairs could have arisen by duplication of an existing dimer and by subsequent co-evolutionary diversification of both partners within a dimer. Presumably, Lsm10 and Lsm11 were added last, coincident with the advent of the U7-snRNP-dependent histone RNA 3′ end processing mechanism in metazoans.

Another novel aspect of the U7 Sm/Lsm core structure is the fact that Lsm11 contains long amino acid stretches at its N-terminus and between the two Sm motifs (fig. 3), and that the N-terminus plays a functional role in histone RNA processing (see above). Although the precise mechanism has not been elucidated, this functional contribution to a biochemical reaction is so far unique among the Sm/Lsm proteins. Bioinformatic analyses have revealed orthologs of Lsm10 and Lsm11 in most vertebrates, in tunicates and in arthropods, and the *Drosophila* orthologs have been characterised biochemically [67]. These studies also indicated that the N-terminus of Lsm11 contains four patches of evolutionarily conserved amino acid residues that are most likely parts of α-helices, whereas the remainder of the N-terminus is quite divergent (fig. 3). A detailed analysis of these conserved parts of the protein are likely to provide further insights into the function(s) of Lsm11.

Implications for snRNP assembly and subnuclear architecture

The challenge facing the cell in assembling Sm proteins onto the Sm binding site of U snRNAs, essentially a U-rich sequence, is daunting, considering that the chance of finding such a sequence in the transcriptome is quite high. In fact, in vitro assembly reactions performed with purified Sm proteins show that they assemble spontaneously not only with U snRNAs but also with other RNAs like tRNA and ribosomal RNA (rRNA) which are very abundant in the cell [68]. The special composition of the U7 Sm core and its functional importance for histone RNA processing pose a further specificity problem for the assembly of U snRNPs. The essential function of Lsm11 in histone RNA processing and the fact that U7 Sm OPT snRNPs are deficient in histone RNA processing (see above) [39] indicate that U7 snRNPs incorporating Sm D1 and D2 would be non-functional and might even be inhibitory for histone RNA processing. Conversely, Sm D1 and D2 might have specific functions in spliceosomal snRNPs. Thus, the cell should have effective means to prevent such potentially detrimental associations of 'wrong' Sm/Lsm proteins with a given snRNA.

The assembly of all snRNPs except for U6 follows a nucleo-cytoplasmic shuttling pathway (reviewed in [46]). The U snRNAs are transcribed by RNA polymerase II in the nucleus and then get exported to the cytoplasm where they assemble with stockpiled Sm proteins. Once the heptameric Sm core has been formed, the 7-methyl guanosine cap of the RNA is hypermethylated to 2,2,7-trimethyl guanosine. This trimethyl cap is recognised by the specialised transport adaptor snurportin, which cooperates with importin β [69], and the snRNP particle is re-imported into the nucleus.

As mentioned above, purified Sm proteins form specific hetero-oligomeric complexes consisting of B/B′-D3, D1-D2 and F-E-G, which can then spontaneously assemble onto the Sm site of a U snRNA [65, 66]. However, in vivo, Sm core formation is an ordered, protein-assisted process. An important factor regulating this assembly is the 'survival of motor neurons' (SMN) protein, which is mutated in the fatal neuromuscular disorder spinal muscular atrophy (SMA). SMN exists in the cell as a multiprotein complex along with several stably associated proteins called Gemins and varying amounts of the common Sm proteins (reviewed in [70, 71]). This SMN complex is both necessary and sufficient to assemble Sm cores on spliceosomal snRNAs in an ATP-dependent manner [68, 72, 73]. Recent evidence also suggests that the SMN complex may act as a specificity factor guarding against illicit association of Sm proteins with other RNAs [68]. Indeed, the specificity is attained only by Sm proteins that are already part of the SMN complex, and in vivo, Sm protein association with the SMN complex precedes their assembly onto U snRNAs [73]. To achieve the full specificity, the SMN complex interacts not only with the Sm proteins but also with the U snRNAs via specific SMN binding domains that may or may not overlap with the Sm binding site, depending on the particular snRNA involved [74, 75]. In vivo, the SMN complex is associated with the snRNAs throughout their cytoplasmic assembly phase [76].

Concerning the U7 snRNP, early studies using microinjected *Xenopus laevis* oocytes indicated that its assembly pathway is similar to the one for spliceosomal snRNPs [39]. Later it was found that U7 snRNP assembly in cell-free extracts from *Xenopus* eggs which yields functional U7 snRNPs [77] is ATP dependent and mediated by the SMN complex [37]. In this in vitro system, as in vivo, converting the U7 Sm binding site to Sm OPT, the consensus derived from spliceosomal snRNPs, results in assembly with the Sm proteins D1 and D2 instead of Lsm10 and Lsm11. Moreover, the resulting U7 Sm OPT snRNPs

are also non-functional in histone RNA processing. This implies that the SMN complex must be able to recognise and specifically combine the U7-specific Lsm10 and Lsm11 proteins and the Sm binding site of U7 snRNA. This could in principle be achieved by an SMN complex containing all Sm proteins as well as Lsm10 and Lsm11. However, the role of the SMN complex as a specificity chaperone would be facilitated by having separate preformed SMN complexes containing either of the two protein dimers SmD1/D2 or Lsm10/11 along with the other five Sm proteins. Indeed, our recent studies have provided evidence for two separate entities containing SMN and the five common Sm proteins, one containing Lsm10/Lsm11 and the other containing Sm D1/D2 [37]. Only the Lsm10/11-containing SMN complexes can mediate the assembly of the U7 snRNP, whereas complexes that contain the seven canonical Sm proteins fail in the same reaction, but assemble D1/D2-containing Sm core structures on U1 or U7 Sm OPT RNA. How these two mutually exclusive sets of Sm/Lsm proteins get incorporated into the SMN complex will be an interesting topic for further investigations.

Another complex participating in the assembly process, perhaps more at the level of its regulation, is the PRMT5 complex containing the PRMT5 protein methyltransferase, pICln, WD45 and possibly other protein subunits [78, 79]. This complex can convert the arginines in the C-terminal RG repeats of the Sm proteins B/B′, D1 and D3 (figs 2, 3) to symmetrical dimethyl arginines (sDMAs). Within the complex, the pICln subunit binds to the Sm domains of these substrate proteins, and PRMT5 performs the modification step. Clues to the functional consequence of these modifications came from the finding that their presence increases the affinity of Sm B/B′, D1 and D3 for the SMN complex. This suggests a sequence of events where the PRMT5 complex pre-assembles, modifies and presents these Sm proteins to the SMN complex, which then uses them for Sm core assembly. Additional known substrates for sDMA methylation, possibly also mediated by the PRMT5 complex, are Lsm4, myelin basic protein and coilin.

The PRMT5 complex may also play an additional role in snRNP assembly that is not related to methylation. This is at least suggested by our recent findings indicating that Lsm10 and Lsm11 interact with the PRMT5 complex in vivo and with pICln in vitro, although none of them has RG repeats and their binding to SMN is independent of methyltransferase activity [R.S.P. et al., unpublished results). Moreover, a larger complex containing all components of the PRMT5 and SMN complexes was recently isolated, and the presence of the PRMT5 complex components had an ATP-dependent stimulatory effect on the SMN-mediated snRNP assembly activity in vitro [73].

In the nucleus, the snRNPs are found concentrated in Cajal bodies (CBs), as well as diffusely distributed throughout the nucleoplasm, where they are stored in so-called interchromatin granules and used for splicing in perichromatin fibrils (reviewed in [80]). In fact, newly-assembled snRNPs target first to the CBs [81]. At least one function of CBs is the introduction of sugar 2′-O-methyl and pseudouridine base modifications on some U snRNAs, guided by small CB-specific RNAs (scaRNAs) [82]. Interestingly, SMN is also found in CBs in most cells. It is held there by a specific interaction with coilin, the marker protein for CBs. This interaction is dependent on sDMA modifications of RG repeats in coilin, and hypomethylation of these repeats leads to relocalisation of SMN in separate nuclear structures termed “gemini of CBs” or “gems” [83]. CBs are likely to have other functions which are still not fully understood. Also, components of snoRNPs and of polyadenylation complexes are transiently found associated with them (reviewed in [80]).

Whereas mammalian CBs contain both U7 and spliceosomal snRNPs [84], a special situation prevails in *Xenopus* oocytes. There, the U7 snRNP is a major and perhaps the only snRNP component of the large CBs, which are also called spheres or C snurposomes [85]. In contrast, the spliceosomal snRNPs are found in structures lacking coilin that are termed B snurposomes. Overexpression of U7 snRNA leads to the formation of additional CBs [86]. Moreover, the non-canonical Sm binding sequence of U7 RNA was shown to be responsible for correct localisation; replacing it by an Sm binding sequence from U2 snRNA led to a failure in CB localisation [87]. Thus, in *Xenopus* oocytes, the U7-specific Lsm proteins might have a role in targeting the U7 snRNP to the spheres or C snurposomes, which often associate with the histone gene loci.

U7 snRNA derivatives as tools for nuclear antisense therapy

The above basic studies on the U7 snRNP, and in particular the properties of the U7 Sm OPT snRNA containing a consensus Sm binding sequence derived from spliceosomal snRNAs, engendered an idea to use modified U7 snRNA derivatives for a special antisense therapy approach. It has already been mentioned that U7 Sm OPT-derived snRNPs (which contain Sm D1/D2 instead of Lsm10/11) are deficient in histone RNA processing. In fact, such snRNPs were even found to exert a dominant negative effect in *Xenopus* oocytes that still contained functional, endogenous U7 snRNPs [B. Stefanovic and D.S., unpublished results]. This dominant negative effect was based on a competition for the HDE, as U7 Sm OPT derivatives with an altered base-pairing region were only deficient, but not dominant negative. We hypothesised that if U7 Sm OPT-derived snRNPs can compete with the histone 3′ processing machinery, they should also be able to block specific pre-mRNA splicing events which occur

in the same compartment, the nucleoplasm. Hence, U7 Sm OPT-derived RNAs equipped with antisense sequences targeting specific splice sites, with their exclusively nuclear location and their inability to cleave the target pre-mRNA, should be ideally suited to manipulate the splicing patterns of individual target genes.

This approach was modeled on a strategy pioneered by Kole et al., where antisense oligonucleotides are used to target specific splice sites in pre-mRNAs and, hence, to redirect alternative splicing decisions (reviewed in [88]). Oligonucleotide-based redirection of splicing can be beneficial for many inherited diseases such as β-thalassemias, muscular dystrophy and cystic fibrosis, or it can be used to affect the fine-tuning of the apoptotic response, e.g. in cancer cells. However, if these antisense sequences are expressed from an snRNA-encoding gene such as a modified U7 Sm OPT derivative, more sustained or even permanent nuclear antisense effects may be achieved. Work carried out in several different laboratories, including ours, has demonstrated that this is more than a remote possibility. Rather, U7-based approaches have proven to be effective in different systems and have been tested not only in tissue culture but also in in vivo situations.

Initial work, using a tissue culture model for β-thalassemia, confirmed the practicability of this approach [89–91]. Several mutations in the second intron of the human β-globin gene create 5′ splice sites (IVS2-654, -705 and -745) and activate a common cryptic 3′ splice site (ss) further upstream in the same intron. This results in the inclusion of an aberrant exon in β-globin mRNA and in the loss of β-globin protein production (fig. 4). Several U7 Sm OPT derivatives targeting the cryptic 3′ ss or the 5′ ss created by the mutations were able to mask these ss, resulting in the reappearance of correctly spliced β-globin mRNA and protein. However, although these effects were efficient for the IVS2-705 and -745 mutations, they were weak for the IVS2-654 mutation. An improved efficiency for all three mutations was obtained with U7 snRNA derivatives carrying two tandem antisense sequences, one targeting the branch point region upstream of the aberrant exon or the cryptic 3′ ss, and the other targeting the end of the exon, including its 5′ ss (fig. 4) [90]. This double-target approach has proven to be very effective as a means to induce exon skipping in the context of other transcription units such as the human [92] or mouse [93] dystrophin gene, the cyclophilin A gene [94] or the multiply spliced HIV-1 transcripts [G. Marti et al., unpublished results].

Other workers have also obtained efficient exon skipping when the U7 snRNA derivative carried a sequence complementary to exon-internal sequences [95]. In this case, the U7 may have acted by masking exonic splicing enhancer (ESE) sequences or by affecting the flexibility of the exon. We have also found a double-target construct targeting a bona fide ESE and the downstream 5′ ss to be very effective in the context of the human immunodeficiency virus-1 (HIV-1) transcription unit [G. Marti et al., unpublished results].

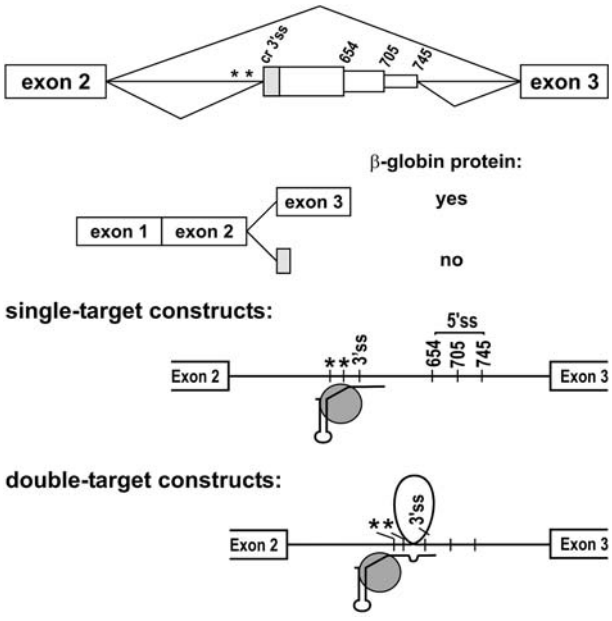

Figure 4. Modified U7 snRNPs as precision tools to affect splicing decisions. Several mutations in the second intron of the human β-globin gene cause β-thalassemia. These mutations create 5′ splice sites at positions 654, 705 or 745 of the second β-globin intron (top). This activates a cryptic 3′ splice site (cr 3′ ss) upstream in the same intron, resulting in the inclusion of an aberrant exon containing an early stop codon and, therefore, in the loss of β-globin protein production. U7 Sm OPT snRNAs modified to base-pair with either the cr 3′ ss or 5′ ss (single-target constructs) efficiently redirect splicing to generate functional β-globin mRNA and protein for the IVS-705 and -745 mutations, but inefficiently for IVS2-654 [89, 90]. U7 snRNAs that base-pair simultaneously to two regions upstream and downstream of the aberrant exon (double-target constructs), perhaps looping out the region between the two base-pairing sites, show an improved exon skipping efficiency, also for the IVS2-654 mutation.

In addition to these exon skipping strategies, a U7 Sm OPT derivative has also been used to enhance the inclusion of an exon that is normally skipped, probably due to the lack of an exonic splicing enhancer, in the survival of motor neurons 2 (SMN2) gene. This exon inclusion was achieved with a U7 snRNA that masks the 3′ ss of the subsequent exon [K. J. Hertel, personal communication]. Moreover, a U7 snRNA has been provided with an RNA decoy that will sequester an RNA binding protein responsible for high levels of collagen alpha1(I) mRNA in hepatic stellate cells [96].

Modified derivatives of the spliceosomal U1 snRNA have similarly been used to target nuclear splicing events. However, in direct comparisons, such U1 constructs were generally not more efficient than corresponding U7 constructs [92, 97]. Moreover, replacing the U7 promoter by

the U1 promoter also did not significantly increase the levels of the snRNA or the antisense effect on splicing [93, 97]. These findings are consistent with our earlier analyses, which showed that the ~100-fold difference in steady-state levels between endogenous U1 and U7 snRNAs can be accounted for by differences in functional gene copy number (~30-fold) and snRNP assembly (2–4-fold) [38]. In particular, exchanging promoters and 3′ regions or, in fact, most of the RNA coding regions between a *U1* and a *U7* gene had no significant effects on steady-state RNA levels. Rather, the 2–4-fold difference in RNA levels per gene was found to be primarily controlled by the respective Sm binding site, and replacing the U7 Sm binding site by the consensus Sm OPT sequence led to similar levels of RNA accumulation as from a *U1* gene [38]. Thus *U7* and *U1* genes have similar transcriptional activities, and U7 Sm OPT can be regarded as a well-expressed, 'generic' snRNA that is particularly well suited for the insertion of extended antisense sequences at its 5′ end. Nevertheless, it may be possible to improve the U7 promoter by design: in one study, the expression of an antisense U7 snRNA in cultured myoblasts was stimulated by the insertion of the muscle creatine kinase enhancer [93], indicating that the *U7* gene may respond to cell type-specific or even regulatable enhancer sequences.

For delivery into suspension cell lines, primary cells from patients or laboratory animals, standard DNA transfection protocols are usually not efficient. In this context, the small size of the U7 snRNA expression cassette (~600 bp) allows its insertion into virtually any vector, including viral ones that allow for stable DNA integration. Thus, U7 expression cassettes have been successfully introduced into a variety of cell types, including primary cells, by using retroviral [92], lentiviral [94, 95], [G. Marti et al., unpublished results], and adeno-associated viral vectors [L. Garcia, personal communication].

The modulation of splicing obtained with U7 snRNA constructs has generally been found to be very specific. Although ~1000 RNA copies can generally be expressed in most cells, toxic side effects have not been observed, and the desired antisense effect should only be exerted in those cells expressing the targeted pre-mRNA. For one U7 construct targeting thalassemic mutations in the human β-globin gene, transgenic mice have been generated that transmit and express the gene and that show no obvious defects [D. S., unpublished results]. All these features contribute to making antisense U7 snRNA an attractive tool for posttranscriptional gene modulation.

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