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The determinants for Sm protein binding to *Xenopus* U1 and U5 snRNAs are complex and non-identical

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The Sm binding sites of different spliceosomal U small nuclear RNAs (snRNAs), the RNA structural elements required for interaction with common snRNP proteins, have been considered to be similar or identical. Here we show that this is not the case. Instead, structural and sequence features unique to U1 or U5 snRNAs that contribute to common protein binding are identified. The determinants of Sm protein binding in both RNAs are complex, consisting in U5 of minimally two and in U1 of minimally four separate structural elements. Even the most conserved features of the two RNAs, single-stranded regions whose generalized sequence is PuA(U)nGPu, are not functionally interchangeable in protein binding. At least one of the newly defined RNA elements functions in assembly with the common proteins, but is not required for their stable binding thereafter. U1, but not U5, snRNP requires a trimethyl guanosine cap structure for its transport to the nucleus. This is not a consequence of the differences in common snRNP binding to the two RNAs, but is due to structural features of U1 RNA that do not contribute to Sm protein binding.

Key words: RNA processing/RNA-protein interactions/U snRNAs/U snRNPs

Introduction

Small nuclear RNAs (snRNAs) are a class of stable, low mol. wt RNAs found in the cell nucleus (for reviews, see Guthrie and Patterson, 1988; Lührmann *et al.*, 1990; Zieve and Sauterer, 1990). The major nucleoplasmic snRNAs of multicellular eukaryotes exist in the form of ribonucleoproteins (snRNPs) that consist of one (U1, U2, U5) or two (U4/U6) snRNAs and several proteins. The snRNPs have been shown to be essential co-factors in pre-mRNA splicing (Steitz *et al.*, 1988; Lührmann *et al.*, 1990). The composition of the spliceosomal snRNPs has been most extensively studied in human (HeLa) cells. Highly purified U1, U2, U4/6, U5 and U4/6-U5 snRNPs have been isolated and their protein components shown to fall into two groups. Proteins specific to particular snRNPs (all the snRNPs obtained in this way except U4/U6 contain specific proteins) and those that appear to be common to all (reviewed by Lührmann *et al.*, 1990; Zieve and Sauterer, 1990). HeLa snRNPs have eight common proteins, called B′, B, D_1, D_2, D_3, E, F and G (Lührmann *et al.*, 1990). The common proteins are also known as Sm proteins because some of them contain epitopes recognized by antibodies of the Sm serotype found in patients suffering from systemic lupus erythematosus (Lerner and Steitz, 1979). The site of interaction with these proteins on the snRNA has therefore been called the Sm binding site.

Comparison of the structures of the RNAs which bind directly to the Sm proteins identified a conserved common motif [PuA(U)nGPu] flanked by two hairpin loops, which was called domain A and proposed to be the binding site of the common proteins (Branlant *et al.*, 1982). Experimental evidence in support of this was first obtained by nuclease protection experiments (Liautard *et al.*, 1982). Subsequent analysis in *Xenopus* oocytes of assembly with the common proteins of mutants of U2 snRNA and of artificial RNAs confirmed the essential role of the PuA(U)nGPu motif, which we will call the Sm core binding site in this paper, but did not provide support for a requirement for the flanking hairpin structures of domain A (Mattaj and De Robertis, 1985; Mattaj, 1986).

Studies of the snRNAs from the yeast *Saccharomyces cerevisiae* revealed that they also contained Sm core binding sites (reviewed by Guthrie and Patterson, 1988). Indeed, the yeast RNAs were found to be able to associate with common snRNP proteins in *Xenopus* oocytes (Riedel *et al.*, 1987; Tollervey and Mattaj, 1987). In *S.cerevisiae*, U1, U4 and one of the two forms of U5 snRNA terminate shortly after the Sm core sites and thus lack a 3′ flanking hairpin (Guthrie and Patterson, 1988). Thus the picture that emerged was that association of the common snRNP proteins was dependent only on the Sm core binding site. Although little is known about the common U snRNP proteins from yeast, it was therefore surprising when extensive mutagenesis of the Sm core site of *S.cerevisiae* U5 snRNA revealed that it was remarkably tolerant to mutation, indicating that this very stable protein-RNA interaction did not depend absolutely on more than a few particular nucleotide contacts within the Sm core site (Jones and Guthrie, 1990).

Another interesting aspect of the interaction between the common proteins and snRNAs is its role in nuclear targeting of the snRNPs (reviewed by Mattaj, 1988; Zieve and Sauterer, 1990). snRNAs transcribed by RNA polymerase II appear transiently in the cytoplasm where they bind to the common snRNPs and, dependent upon this binding, acquire a trimethyl guanosine (TMG) cap structure. After these steps, the snRNPs enter the nucleus. In the absence of interaction with snRNA, the Sm proteins remain in the cytoplasm (Zeller *et al.*, 1983). Similarly, snRNAs unable to bind the Sm proteins are unable to re-enter the nucleus and remain in the cytoplasm (Mattaj and De Robertis, 1985).

Studies of the signals targeting snRNPs to the nucleus in *Xenopus* oocytes have revealed a complex picture. For U1 and U2 snRNPs, both the TMG cap structure and the bound common proteins are required for nuclear migration (Mattaj and De Robertis, 1985; Fischer and Lührmann, 1990; Hamm *et al.*, 1990). In contrast, the nuclear localization of U5 snRNP is almost completely independent of the presence of

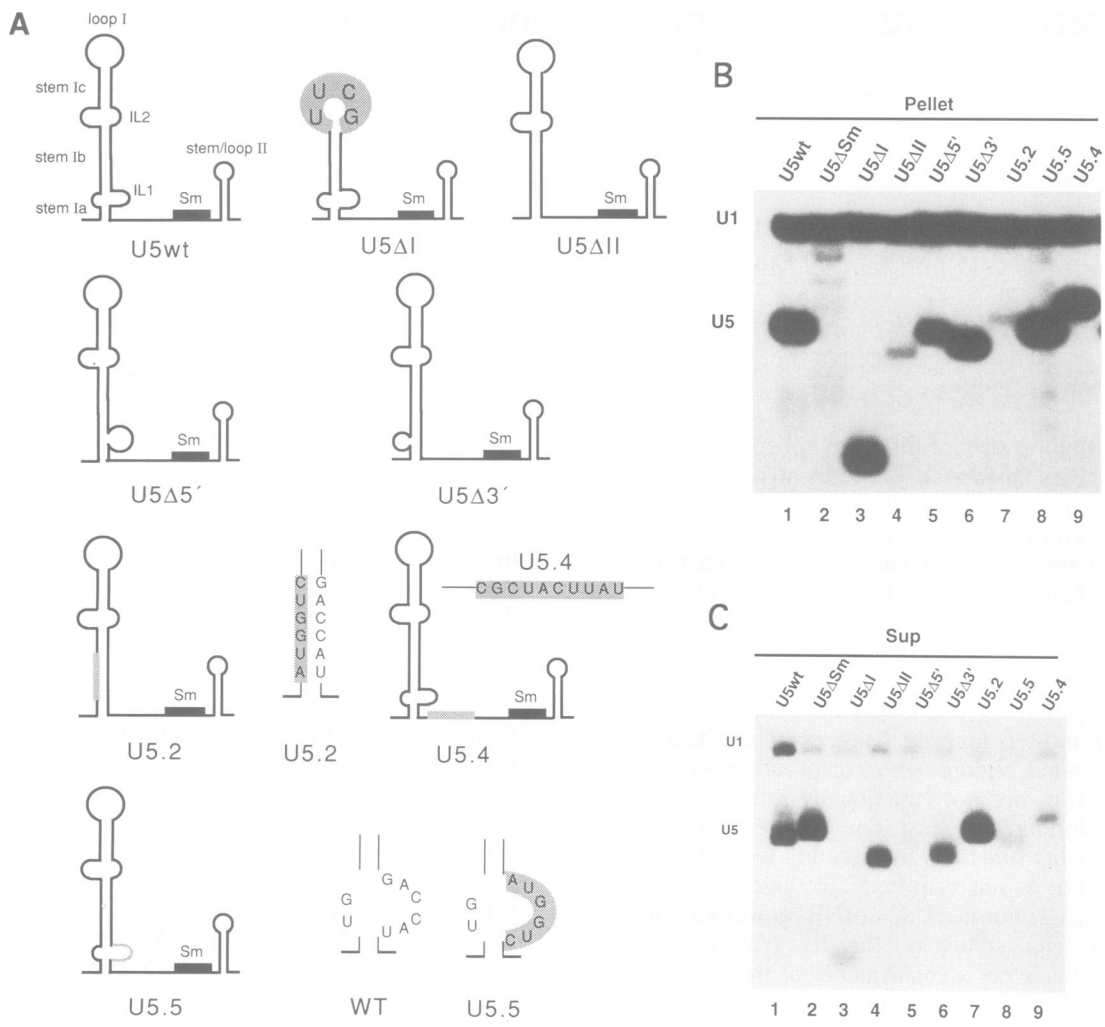

Fig. 1. Immunoprecipitation of U5 snRNA mutants with Y12 antibodies. (**A**) Diagrams of wild-type U5 and the U5 mutants: U5 snRNA sequence is marked in black, positions of inserted sequences in U5ΔI, U5.2, U5.5 and U5.4 are boxed grey, and the sequence changes indicated. (**B**) and (**C**) *In vitro* transcribed U5 wt or various U5 mutants whose structures are diagrammed in (A) were injected into the cytoplasm of oocytes. U1 wt snRNA was used as an internal control. After 3 h the injected oocytes were homogenized and snRNPs immunoprecipitated with Y12 anti-Sm antibodies. RNA extracted from immunoprecipitates (B) and supernatants (C) was analysed on 7 M urea−8% polyacrylamide gels.

a TMG cap structure, while U4 snRNP displays intermediate behaviour (Fischer *et al.*, 1991). In the present study we show that the accepted picture of the Sm binding site is incomplete, and that in fact the determinants of Sm protein binding to U1 and U5 snRNAs are quite different in composition and structure. Nevertheless, the differential TMG requirements for nuclear transport of U1 and U5 are not a consequence of this, but rather are due to the presence of structural elements in U1 snRNA that do not appear to influence the binding of the common proteins.

Results

A U5-specific element required for efficient binding of the common snRNP proteins

The predicted structure of the 5′ hairpin of U5 RNA contains three conserved loops called internal loop 1 (IL1), IL2 and loop 1 in order from the base to the top of the hairpin, as drawn in Figure 1A (Guthrie and Patterson, 1988). Chemical and nuclease accessibility experiments have implicated IL2 and the stems on either side of it in the binding of U5-specific proteins (Black and Pinto, 1989; Bach and Lührmann, 1991). A mutant lacking IL2 and the rest of the upper part of the 5′ hairpin (U5ΔI, Figure 1A) was therefore constructed. A second mutant, U5ΔII (Figure 1A), from which IL1 had been deleted, was made. *In vitro* transcripts of these two U5 mutants were co-injected with U1 snRNA into the cytoplasm of *Xenopus* oocytes. Wild-type (wt) U5 and U5ΔSm, in which the core of the Sm binding site had been changed from AAUUUUUUGA to AAUGGGUUGA, were included as positive and negative controls.

In order to assay assembly of the RNAs with the common U snRNP proteins, immunoprecipitation was carried out with the anti-Sm monoclonal antibody Y12 (Lerner *et al.*, 1981). Wt U5 and U5ΔI were efficiently precipitated, while U5ΔSm was not, suggesting that the absence of the proposed site of interaction with the U5-specific proteins had no effect on the binding of the common proteins (Figure 1B and C, lanes 1−3. Note that the presence of significant amounts of both wt U1 and U5 in the supernatant in Figure 1C, lane 1, indicates that, in this particular sample, saturating levels of anti-Sm antibodies had not been added.). Surprisingly, however, U5ΔII was immunoprecipitated to a very limited extent (Figure 1B and C, lane 4) indicating that IL1 was required either for efficient assembly with, or stable binding to, the common U snRNP proteins. Since the Y12 antibody

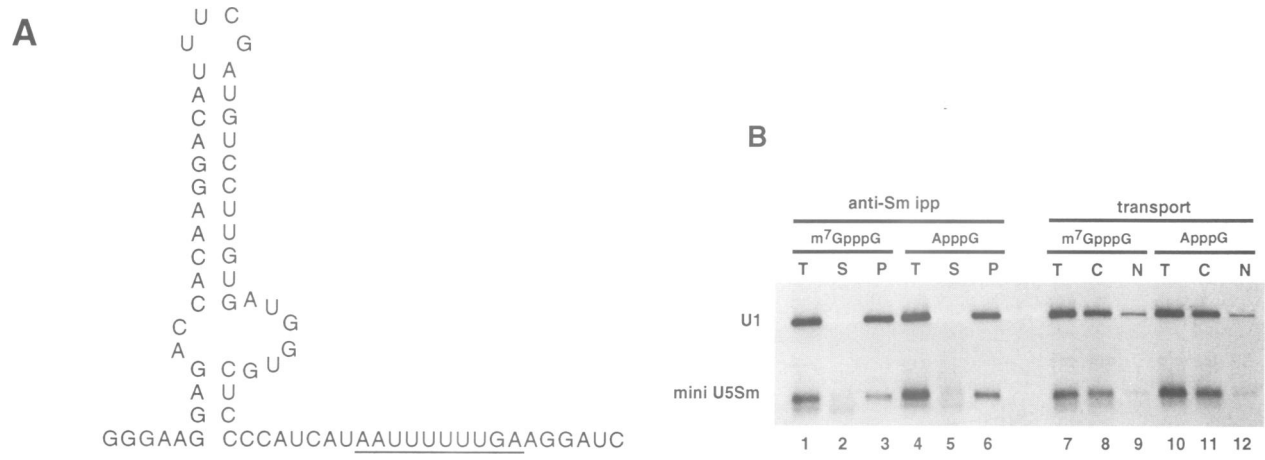

Fig. 2. The assembly and nucleocytoplasmic transport of the minimal U5 Sm RNA. (**A**) The sequence of the RNA is diagrammed according to the wt U5 structure in Figure 1A. The underlined core Sm binding site is the only sequence conserved from wt U5. (**B**) Oocytes were injected with *in vitro* transcribed minimal U5 Sm RNA capped with either ApppG or m^7GpppG. U1 wt snRNA capped with m^7GpppG was used in the experiment as an internal control. Five injected oocytes were homogenized and Y12 anti-Sm immunoprecipitation was performed. Lanes 1–3 and 4–6: anti-Sm immunoprecipitation of snRNPs from oocytes injected with m^7GpppG or ApppG capped minimal U5 Sm RNA respectively. T: total RNA extracted from injected oocytes; S: RNA extracted from supernatants; P: immunoprecipitated RNA. Additionally, RNA was extracted from either total oocytes (T) or oocytes dissected into cytoplasmic (C) and nuclear (N) fractions. Lanes 7–9: nuclear accumulation of m^7GpppG capped minimal U5 SmRNA. Lanes 10–12: nuclear accumulation of ApppG capped minimal U5 SmRNA.

recognizes several common proteins (B′, B, D_1, D_3 and E in HeLa snRNPs; Lehmeier *et al.*, 1990), it is very unlikely that changes in U snRNA structure would result in masking of all the epitopes recognized by Y12.

To test further the requirements for binding of the common proteins to U5 snRNA, we separately deleted the two bulges comprising IL1 (Figure 1A, U5Δ5′ and U5Δ3′). Both RNAs were still able to bind Sm proteins, although the lower ratio of U5Δ3′ in the pellet and supernatant fractions compared to the U1 internal control was indicative of a reduction in assembly in the absence of the 3′ bulge (Figure 1B and C, lanes 5 and 6). We therefore replaced the 3′ bulge with an unrelated sequence (U5.5, Figure 1A). This change had no effect on immunoprecipitation (Figure 1B and C, lane 8). Together, these results suggested that a base paired stem in place of IL1 would not allow efficient assembly, but that the sequence or structure of the interruption in the stem was not highly critical.

To check this conclusion further, mutant U5.2 (Figure 1A) was constructed. In U5.2 the 5′ bulge is replaced by a six nucleotide sequence complementary to the 3′ bulge. This should induce stem formation at the base of the 5′ hairpin. This mutation abolished immunoprecipitation (Figure 1B and C, lane 7). A similar result was obtained when the 3′ bulge was replaced by bases complementary to the 5′ bulge (data not shown). Finally, insertion of 10 nucleotides between the 5′ stem and the core Sm binding site did not affect immunoprecipitation (U5.4, Figure 1B and C, lane 9). The above experiments define a new structural element, IL1, involved in the interaction between the common U snRNP proteins and U5 snRNA.

Based on the results described above and others which showed that the 3′ hairpin of U5 was not required for efficient immunoprecipitation (data not shown), a minimal U5 Sm snRNA that should assemble with the common snRNP proteins was designed. This RNA is shown in Figure 2A. It is 70 nucleotides long and resembles U5 in four respects: the core of the Sm binding site (AAUUUUUUGA), the size and position, but not the sequence, of IL1, and the length, but not the sequences, of the stems flanking IL1. A large fraction of this RNA was immunoprecipitated with Y12 antibodies (Figure 2B, lanes 1–3). These data show that there are two RNA elements in U5 required for Sm protein binding: the Sm core sequence and IL1. Note that there is no reason to postulate that IL1 must directly contact the common snRNP proteins. There are several alternative ways in which it could influence their binding (see Discussion). Nuclear transport of the minimal U5 Sm snRNA will be described later.

The Xenopus U1 and U5 core Sm sites are not equivalent

Since IL1 does not have an obvious sequence or structural counterpart in other Sm snRNAs, and since the core Sm binding sites of U1 and U5 are not identical in multicellular eukaryotes, we decided to test whether IL1 would cooperate with the U1 Sm core site to allow efficient binding of the common proteins. (U5 always has an uninterrupted stretch of five or six U residues. This is replaced in U1 either by UUUCU or UUUGU in these organisms; Guthrie and Patterson, 1988).

The RNAs used to test this possibility are shown in Figure 3A. In U5.7, the core Sm site was mutated to that of U1. In U5/U1, the 15 nucleotide (nt) unpaired region containing the U1 core site was transferred to U5. U5.8 was similar to U5/U1 except that in U5.8 the U5 core sequence had been introduced into the context of the 15 nt segment of U1. This mutant was made in order to distinguish between sequence-dependent and position-dependent effects.

In Figure 3B, lanes 3, 4, 8 and 9, it is shown that neither U5.7 nor U5/U1 were efficiently immunoprecipitated. Reintroduction of the U5 core sequence in an inappropriate position, in U5.8, caused a substantial increase in precipitability (lanes 5 and 10) albeit to a level lower than that of wt U5 (lanes 1 and 6). None of the Sm core regions tested allowed detectable precipitation by Y12 antibodies in the absence of IL1 (data not shown and Figure 5 below). Thus, the U1 Sm core sequence is unable to cooperate with IL1 to allow efficient interaction with the common snRNP proteins. The IL1–U5 Sm core cooperativity is shown by

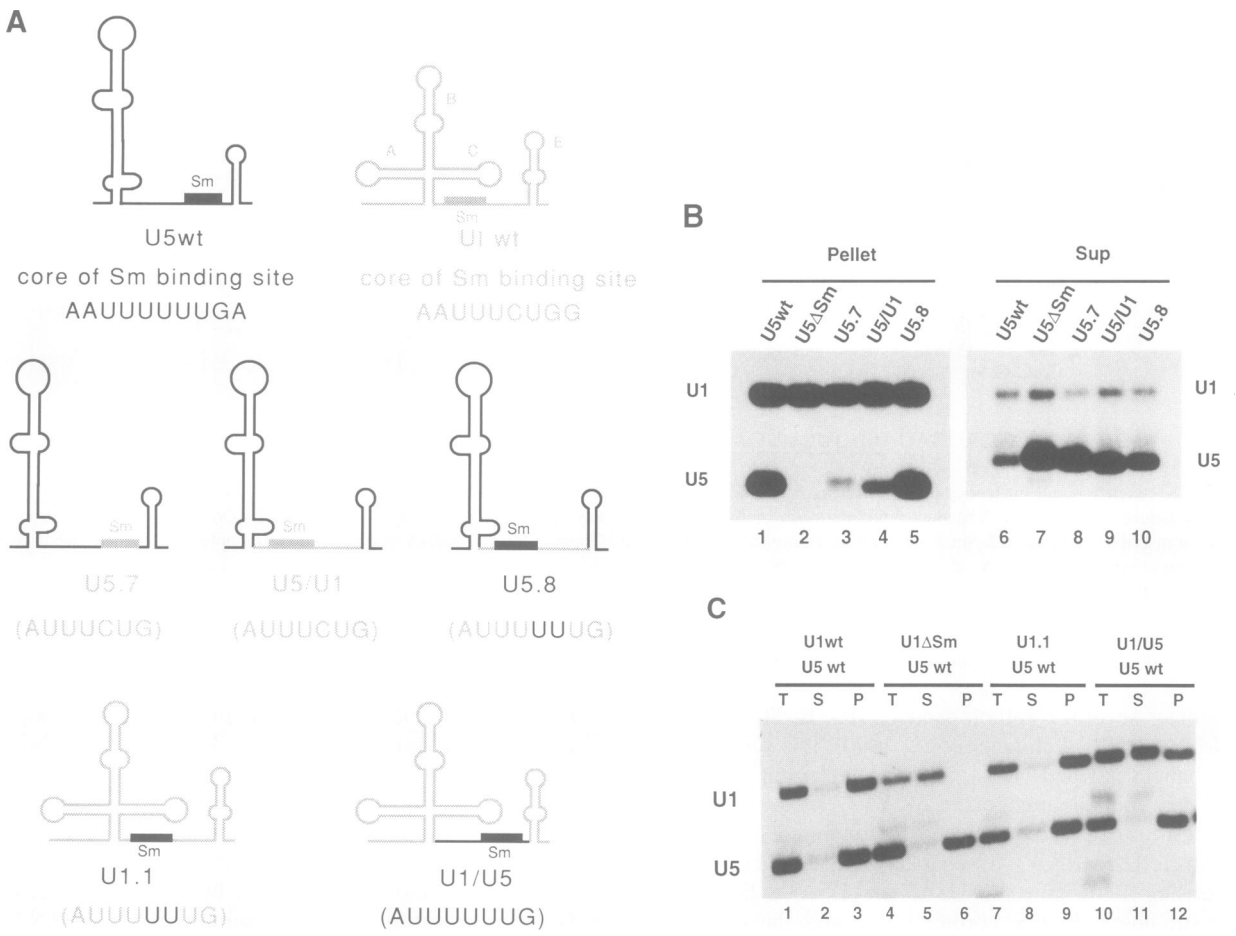

Fig. 3. Y12 immunoprecipitations of U5 and U1 chimaeric snRNAs. (**A**) Diagrams of the wild-type and mutant RNAs: U5 sequence marked in black, U1 sequence in grey. (**B**) *Xenopus* oocytes were injected with *in vitro* transcribed U5 wt, U5 ΔSm, U5.7, U5/U1 and U5.8 snRNAs. U1 wt snRNA was used as an internal control. After 3 h the oocytes were homogenized and snRNPs were immunoprecipitated with Y12 antibodies. RNA extracted from immunoprecipitates (left panel) and supernatants (right panel) was analysed on a 7 M urea−8% polyacrylamide gel. (**C**) *Xenopus* oocytes were injected with *in vitro* transcribed U1 wt, U1 ΔSm, U1.1 and U1/U5 snRNAs. U5 wt snRNA was used as an internal control. snRNPs from injected oocytes were immunoprecipitated with Y12 antibodies. RNA extracted from total oocytes (T), supernatants (S) or immunoprecipitates (P) was analysed as described in (B).

U5.8 to be to some extent dependent on either the relative positions of the two elements or on the sequences in U5 flanking the Sm core site.

The failure of the U1 Sm core sequence to function in U5.7 suggested strongly that U1 RNA must also contain structural elements able to cooperate with the Sm core region to enable efficient interaction with the common proteins. These elements are defined below. To determine whether the U5 core region could function in the context of the cooperating sequences of U1, two mutant RNAs were created. In U1.1 the Sm core sequence was changed to that of U5, while in U1/U5 the single-stranded central region of U5 was transferred to U1 (Figure 3A).

These RNAs were considerably more efficient in interaction with the common proteins than were U5/U1 and U5.7. Immunoprecipitation of U1.1 was similar to that of wt U1 or U5 (Figure 3C, lanes 7−9) and although U1/U5 was immunoprecipitated with reduced efficiency (lanes 10−12), the level was still considerably higher than in the converse experiment (U5/U1; Figure 3B, lanes 4 and 9). Thus, the U1 Sm core sequence does not function in the context of U5 snRNA, while the U5 Sm core sequence functions in common protein binding both in the context of U1 and of U5 snRNA.

Several structural elements of U1 RNA are required for common protein binding

It was previously shown that deletion of any one of the three 5′ hairpin loop structures of U1 RNA (called loops A, B and C, see Figure 3A) had no detectable effect on association with the common proteins (Hamm *et al.*, 1987). As a next step to characterizing the U1 structural elements involved in interaction with the common proteins two RNAs, U1ΔAB and U1ΔABΔ5′ (Figure 4A), were constructed. The ideas behind the experiment were: (i) to remove the junction between the A, B, C hairpins and the short closing stem which might have played a role in U1 similar to that of IL1 in U5 and (ii) to create U1-derived RNAs that resemble, at least superficially, the inactive U5ΔII, U5/U1 and U5.7 constructs.

U1ΔAB was immunoprecipitated similarly to wt U1 (Figure 4B, lanes 1−3). Thus, neither the A or B hairpins nor the structure of the four-way junction in the 5′ half of U1 are required for efficient association with the common proteins. Since the A and B hairpins are essential for the binding of the U1-specific proteins (Hamm *et al.*, 1990), this result also indicates that interaction with the U1-specific proteins does not influence common protein binding. In contrast, deletion of the single-stranded 5′ end from the ΔAB

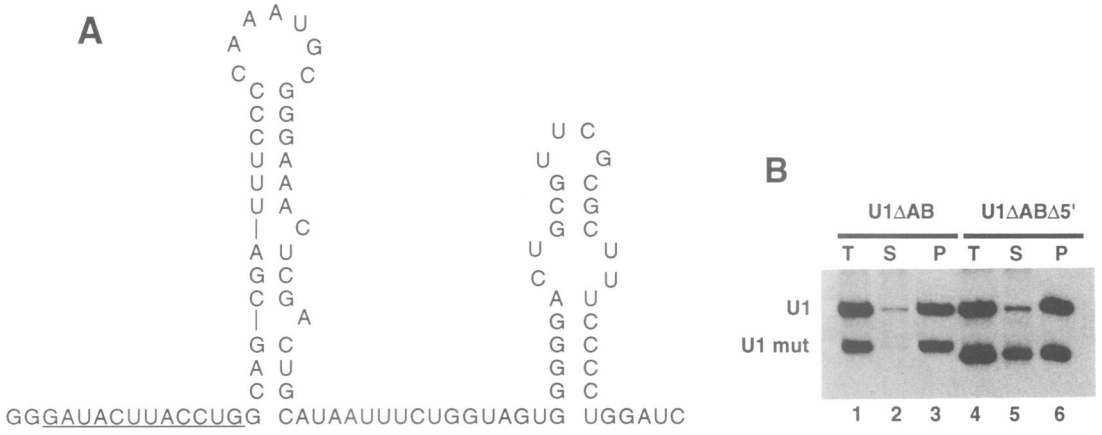

Fig. 4. The binding of Sm proteins to U1ΔAB and U1ΔABΔ5′ snRNAs. (**A**) The sequence of T7 U1ΔAB snRNA. In U1ΔABΔ5′ nucleotides 3–14 (underlined) were deleted. (**B**) U1ΔAB and U1ΔABΔ5′ were *in vitro* transcribed and injected into the cytoplasm of oocytes. U1 wt snRNA served as an internal control. After 3 h incubation the oocytes were homogenized and anti-Sm immunoprecipitation was carried out. Lanes 1–3: immunoprecipitation of U1ΔAB; lanes 4–6: immunoprecipitation of U1ΔABΔ5′; T,S, P: RNA extracted from total oocytes, supernatants and immunoprecipitates, respectively.

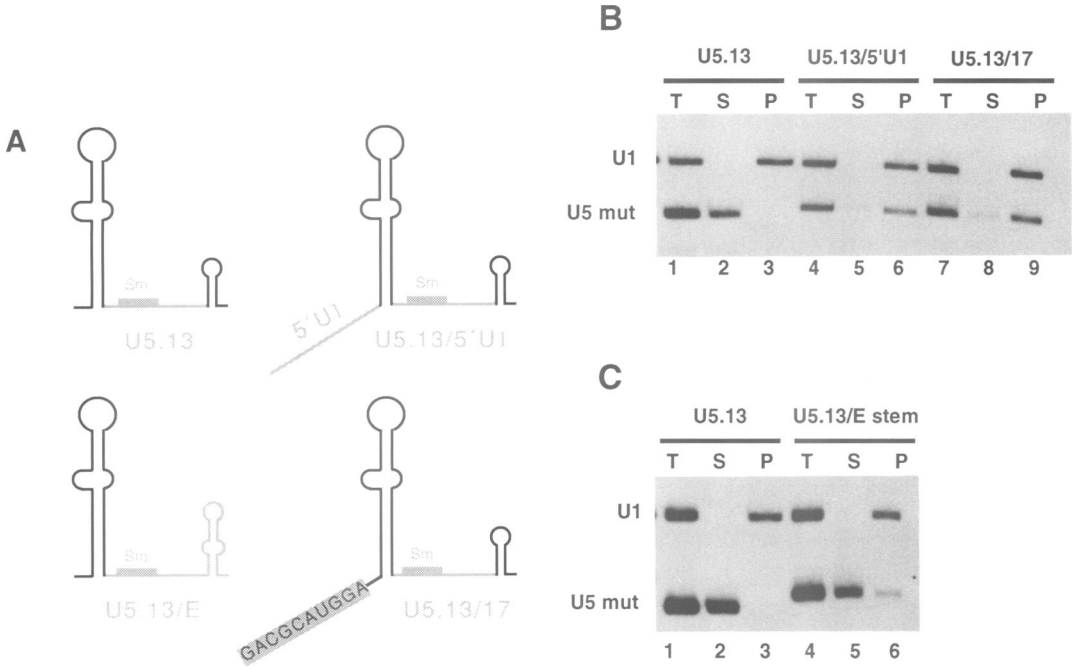

Fig. 5. The effect of 5′ and 3′ region of U1 snRNA on Sm protein binding. (**A**) Structures of U5.13, U5.13/5′U1, U5.13/17 and U5.13/E. U5 sequences are black, U1 sequences are grey. The unrelated sequence inserted into the U5.13/17 mutant is boxed. (**B**) *In vitro* transcribed U5.13, U5.13/5′U1 and U5.13/17 snRNAs were injected into the cytoplasm of oocytes together with U1 wt snRNAs as an internal control. The injected oocytes were homogenized and snRNPs were immunoprecipitated with Y12 antibodies. RNA extracted from total oocytes (T), supernatants (S) or immunoprecipitates (P) was analysed on 7 M urea–8% polyacrylamide gels. Lanes 1–3: immunoprecipitation of U5.13 snRNA; lanes 4–7: U5.13/5′U1; lanes 7–9: U5.13/17. (**C**) Oocytes were injected with U5.13 and U5.13/E snRNAs. U1 wt snRNA was used as an internal control. RNPs were immunoprecipitated and RNA analysed as described in (**B**). Lanes 1–3: Y12 immunoprecipitation of U5.13 snRNA; lanes 4–6: Y12 immunoprecipitation of U5.13/E snRNA.

construct, to create ΔABΔ5′, resulted in a reduction in immunoprecipitation (Figure 5B, lanes 4–6). This result suggested that the single-stranded 5′ end of U1 was involved in common protein association. Further, the difference between the immunoprecipitation efficiency of U1ΔABΔ5′ and U5/U1 and U5.7 (Figure 3B) implicated either the C or E hairpins of U1, or both, in common protein binding.

To obtain further evidence for the roles of these U1 elements, two series of experiments were carried out. In the first, elements of U1 were added to the inactive U5.13 construct (composed of the 15 nt Sm core-containing single-stranded region of U1 inserted into the two hairpins of U5ΔII, Figure 5A) to determine whether they could cooperate with the U1 Sm core sequence in protein binding. As shown in Figure 5B and C, the U1 5′ end strongly and the 3′ E hairpin weakly increased the immunoprecipitability of U5.13 (U5.13/5′ U1 and U5.13/E, lanes 1–6 of Figure 5B and C, respectively). It has recently been proposed that the single-stranded 5′ end of U1 can form base pairs with residues immediately adjacent to the U1 Sm core sequence (Sturchler *et al.*, 1992). The U5.13/17 mutant was made to determine whether the effect on common protein binding required this interaction (Figure 5A). In U5.13/17 the U1 5′ end was replaced by an unrelated sequence which was

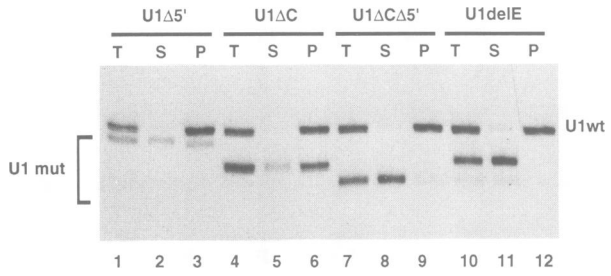

Fig. 6. The participation of different elements of U1 snRNA in Sm protein binding. Various deletion mutants of U1 snRNA were transcribed *in vitro* and injected into the cytoplasm of oocytes. U1 wt snRNA was injected as an internal control. The oocytes were homogenized and Y12 anti-Sm immunoprecipitation was performed. T, S, P: RNA extracted from total oocytes, supernatants and immunoprecipitates, respectively. Lanes 1–3: immunoprecipitation of U1ΔΔ5′; lanes 4–6: immunoprecipitation of U1ΔC; lanes 7–9: U1ΔCΔ5′; lanes 10–12: U1delE.

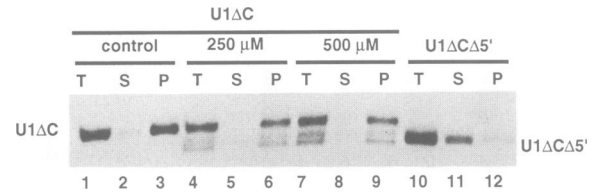

Fig. 7. The 5′ end of U1 snRNA is important for assembly with Sm proteins. *In vitro* transcribed U1ΔC and U1ΔCΔ5′ snRNAs were injected into the cytoplasm of oocytes. After overnight incubation, a DNA oligonucleotide complementary to the 5′ end of U1 snRNA was injected. The injected oocytes were homogenized 6 h later and snRNPs were immunoprecipitated with anti-Sm antibodies. Lanes 1–3: immunoprecipitation of U1ΔC snRNP, no oligonucleotide control; lanes 4–6: immunoprecipitation of U1ΔC snRNP after injection of the U1-5′ oligonucleotide at 250 μM; lanes 7, 8: immunoprecipitation of U1ΔC snRNP after injection of the U1-5′ oligonucleotide at 500 μM; lanes 9–12: immunoprecipitation of U1ΔCΔ5′ snRNP. T: RNA from total oocytes; S: RNA extracted from supernatants; P: RNA extracted from immunoprecipitates. The concentration of oligonucleotides in the oocytes is ~5–10% of the concentration injected.

not complementary to the Sm core region. This RNA was also efficiently immunoprecipitated by Y12 antibodies (Figure 5B, lanes 7–9). Thus, the presence of a free single-stranded 5′ end, rather than its sequence, was important for association with the common proteins.

The second series of experiments involved deletion of the three putative cooperating elements from U1 snRNA. Deletion of the 5′ single-stranded region (U1Δ5′) or the C hairpin (U1ΔC) resulted in a moderate decrease in immunoprecipitation (Figure 6, lanes 1–6). Note that while the presence of the U1 internal control validates these results, the level of immunoprecipitation of both U1Δ5′ and U1ΔC is lower in this experiment than in several other independent repetitions (see Figure 7). However, when both elements were deleted simultaneously (U1ΔCΔ5′), precipitation with Y12 antibodies reproducibly fell to low levels (Figure 6, lanes 7–9; Figure 7, lanes 10–12).

Removal of the 3′ E hairpin from U1 (U1delE) also caused a severe reduction in immunoprecipitation (Figure 6, lanes 10–12). Thus, four structural elements of U1 are involved in association with the Sm proteins. Alteration of the Sm core sequence or deletion of the 3′ hairpin have severe effects on common protein binding. The 5′ single-stranded region and the C hairpin are both also involved, but their roles in binding appear to be partially redundant. The minimal U1 RNA containing all the elements involved in association with the common proteins is therefore U1ΔAB (Figure 4A). The results in Figure 5 and previous work (Hamm *et al.*, 1987) indicate that the 5′ single-stranded region and E hairpin interactions are unlikely to be highly sequence or structure specific. However, the fact that the A and B hairpins cannot compensate for the loss of the C hairpin in U1ΔCΔ5′ suggests that the effect of the C hairpin on protein binding is, at least to some extent, specific.

The U1 5′ end contributes to assembly with the common proteins

The U1 and U5 RNA elements defined above could conceivably contribute to either RNP assembly, stability or both. Although it would be experimentally difficult to distinguish these effects for most of the elements, the susceptibility of the single-stranded 5′ end of U1 snRNA to oligonucleotide-directed RNase H digestion in *Xenopus* oocytes (Pan and Prives, 1988) meant its role in Sm protein binding could be examined. U1ΔC transcripts were injected into oocytes and incubated overnight, to allow snRNP assembly. Oligonucleotides complementary to the U1 5′ end were then injected, and incubation continued for a further 6 h. Since the removal of the U1 5′ end occurs rapidly under these conditions (Pan and Prives, 1988), dissociation of the pre-assembled RNPs would be expected if the 5′ end were required for their stability. Conditions were chosen such that RNase H digestion was partial, to leave some intact U1ΔC to act as an internal control.

The controls for the experiment are U1ΔC and U1ΔCΔ5′, whose immunoprecipitation with Y12 antibodies is shown in Figure 7, lanes 1–3 and 10–12. The RNase H digestion products of U1ΔC, one of which is shorter than U1ΔCΔ5′ and thus presumably retains less of the 5′ single-stranded region, were immunoprecipitated similarly to U1ΔC (Figure 7, lanes 4–9). We conclude that the single-stranded 5′ end of U1 is not necessary to maintain the stability of a pre-assembled RNP, and must therefore be required for efficient assembly. In this context, we also determined that U1ΔC snRNPs were as stable as wt U1 snRNPs to high salt or urea concentrations (data not shown), indicating that the lack of the C hairpin might also have little effect on snRNP stability.

Transport of the minimal U1 and U5 Sm structures is cap independent

U1 and U5 snRNPs have differential requirements for the TMG cap in nuclear transport. Given the results obtained thus far, it was natural to ask whether this effect was related to the differences in Sm protein binding. U1 ΔAB (Figure 4A) and the U5 minimal structure (Figure 2A) were therefore transcribed *in vitro* with either an m^7GpppG cap (which is trimethylated upon binding of the common snRNP proteins; Mattaj, 1986) or with an ApppG cap, which cannot be trimethylated. Migration to the nucleus of wt U1 is prevented by ApppG cap incorporation (Figure 8, lanes 1–6), while U5 transport is not greatly affected (Fischer *et al.*, 1991). The U5 minimal structure and U1ΔAB (Figure 2B, lanes 7–12 and Figure 8, lanes 13–18) were both transported equally well with either cap structure. Thus, the differences in common protein binding described above were not the cause of the differential cap dependence. Rather, the presence of the A and B hairpins conferred cap dependence on U1 nuclear transport. Note that in these experiments the internal

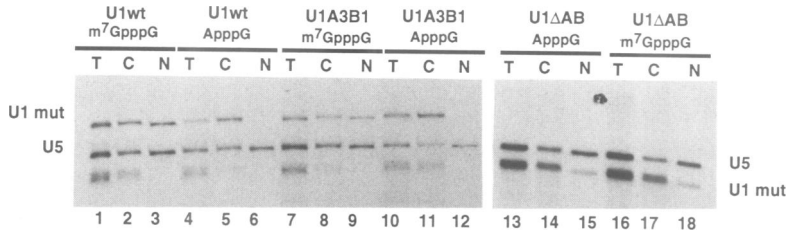

Fig. 8. Cap-independent nuclear accumulation of U1ΔAB snRNA. *In vitro* transcribed wt U1, U1A3B1 and U1ΔAB snRNAs were injected into the cytoplasm of oocytes. After 16 h oocytes were dissected into nuclear and cytoplasmic fractions. RNA was extracted from total oocytes (T), cytoplasmic (C) and nuclear (N) fractions. Nuclear accumulation of transcripts capped either with m^7GpppG or ApppG was compared. m^7GpppG capped wt U5 snRNA was used as an internal control for transport. Lanes 1–6: nuclear accumulation of U1 wt snRNA capped with m^7GpppG (1–3) or ApppG (4–6). Lanes 7–12: U1A3B1 RNA capped with m^7GpppG (7–9) or ApppG (10–12). Lanes 13–18: nuclear transport of U1ΔAB RNA capped either with ApppG (13–15) or m^7GpppG (16–18). The RNA fragments migrating below U5 snRNA in lanes 1–12 were degradation products of U1 wt and U1A3B1 snRNAs, as shown by analysing these RNAs in the absence of U5 snRNA (data not shown).

controls (U1 in Figure 2B, U5 in Figure 8) were always capped with m^7GpppG.

The A and B hairpins have two obvious effects on U1 snRNP. First, their absence or presence will greatly affect the structure of U1 RNA. Second, these hairpins are the sites through which the U1 snRNP-specific proteins U1 70K, U1A and U1C bind to U1 snRNA (Hamm *et al.*, 1990, and references therein). To differentiate whether the effects of the A and B hairpins on transport were due to protein binding or to RNA structure, we made use of the U1A3B1 mutant. This U1 derivative has clustered point mutations in the loops of the A and B hairpins which result in loss of U1-specific protein binding, but are unlikely to greatly affect U1 snRNA structure (Hamm *et al.*, 1990). As seen in Figure 8, lanes 7–12, transport of U1A3B1 to the nucleus requires a TMG cap structure. Thus, the cap dependence of U1 transport is not due to an effect of U1 snRNP-specific proteins, but rather to some aspect of the structure of U1 snRNA caused by the presence of the A and B hairpin loops. The TMG dependence does not, however, require an intact U1 snRNA structure since deletion of either the A, B or C hairpins individually did not affect TMG dependence (data not shown).

Discussion

The major finding of this work was the discovery that the RNA structural elements of U1 and U5 snRNAs required for the binding of the common U snRNP proteins are both complex and non-identical. The complex nature of the sites, although unexpected in the light of earlier studies which had suggested that the conserved PuA(U)nGPu sequence of the Sm core site might be sufficient for protein binding (see Introduction for details), may help to explain some previous observations.

The interaction between the common proteins and U snRNAs is remarkably stable. Core snRNPs (consisting of an snRNA and the common proteins) can survive treatment in 0.5 M CsCl/0.5% sarkosyl, 7 M urea or 2.5 M NaCl (Liautard *et al.*, 1982; Jones and Guthrie, 1990; A. Jarmolowski, unpublished observations). In fact, assembly of U1 snRNA with the common proteins can take place *in vitro* in solutions containing 750 mM NaCl (Hamm *et al.*, 1988). In spite of this, saturation point mutagenesis in combination with multiple point, deletion and insertion mutagenesis revealed that the *S.cerevisiae* U5 snRNA Sm core site was very insensitive to changes, as measured by the ability of the mutant U5 RNAs to support yeast growth (Jones and Guthrie, 1990). These results suggested that there were very few, if any, essential contacts between bases in the Sm core site and the common yeast proteins, and made the stability of the interaction difficult to understand. The data presented here suggest that the Sm proteins might contact more than one region of each U snRNA, and the existence of multiple RNA–common protein contacts could explain both the stability of the RNPs and the resistance to mutation of the Sm core site of yeast U5.

Aside from their complexity, the second surprising feature of the U1 and U5 Sm binding sites is their lack of identity. Within U5 RNA (Figure 3A) both the Sm core site and an interruption of the 5′ helix between stems Ia and Ib at the position of IL1 were shown to be required for efficient binding to the common proteins. In U1 (Figure 3A) the situation was more complex, and evidence for a role of the single-stranded 5′ end and the C and E hairpins, as well as the Sm core sequence, in common protein binding was obtained. It is important to note that these results do not necessarily imply direct interactions between these regions of U1 or U5 snRNA and the common proteins, at least in assembled RNPs. The data in Figure 7 show that the single-stranded 5′ end of U1, at least, plays a role in snRNP assembly, but has no detectable effect on snRNP stability once assembly has taken place. The assembly role could be, for example, in inducing an RNA conformation favourable for protein binding. Alternatively, this region of the RNA could, either directly or indirectly, induce an active RNA binding conformation in the protein or proteins that contact the Sm core sequence.

The structural differences between the regions of U1 and U5 necessary for Sm protein binding, in addition to the fact that the core Sm site of U1 was not able to functionally replace that of U5, makes it logical to ask whether the Sm proteins are indeed identical in different snRNPs, or whether differences in 'common protein' composition might in fact exist. The immunoprecipitation methods used here (and generally) to study common snRNP protein binding rely on the use of antibodies that recognize epitopes on several of the proteins, and are therefore not suited for the detection of possible differences in core snRNP composition. However, extensive studies of biochemically fractionated HeLa cell snRNPs by either SDS–PAGE or immunological methods have failed to reveal differences in the common proteins of different snRNPs (see e.g. Lehmeier *et al.*, 1990; reviewed by Lührmann *et al.*, 1990). If differences do exist, they are therefore likely to be relatively minor.

Why then are the U1 and U5 Sm core sites not interchangeable? The simplest explanation for the results

shown here would be that the U5 Sm core site binds more tightly to the common proteins than does the U1 site, such that the U5 Sm core−IL1 combination allows for a stronger binding than the U1 Sm core−IL1 pair. This may, in part, be true. However, other results suggest it is not the complete explanation. For example, the addition of the single-stranded 5′ end of U1 to U5.13 (which contains the U1 Sm core) allows efficient Sm protein binding (Figure 5). In contrast, the addition of the U1 5′ end to U5ΔII (Figure 1), which is like U5.13 except that it contains the U5 Sm core, results in inefficient Sm binding protein (data not shown). It therefore seems that the two Sm core regions cooperate specifically in protein binding with other elements of U1 or U5 RNA and are not interchangeable. This is likely to indicate that the common protein−Sm core site interaction affects the ability of the proteins to make subsidiary RNA contacts. This could be achieved if the different Sm core sequences bound alternative sites on one common protein, or to different proteins. Alternatively, binding to the transcript core sites might induce different conformational changes in the common proteins that would facilitate particular secondary RNA contacts and disfavour others. While it is not easy to design experiments to test these possibilities in detail, it has recently been shown that one of the common proteins, the G protein, can be cross-linked to the U1 Sm core site (Heinrichs *et al.*, 1992). It would be of interest to repeat this experiment with U5 snRNP to determine whether an identical result is obtained.

Of the characterized vertebrate U snRNAs, one other, U7 snRNA, has an unusual Sm core sequence. Like the previously characterized sea urchin U7 snRNA (Strub *et al.*, 1984), the characterized mammalian U7 snRNAs lack a sequence of the PuA(U)nGPu type and instead have AAUUUGUCUAG (Mowry and Steitz, 1987; Cotten *et al.*, 1988). Recent studies in which the Sm core site of the mouse U7 RNA was replaced by a canonical Sm core site, or in which the U7 core sequence was introduced into U1 snRNA, led to the conclusion that these two Sm core sites are neither identical nor interchangeable (C.Grimm and D.Schümperli, personal communication).

Other recent work has revealed that the Sm core binding sites of the spliced leader (SL) RNA and of U1 snRNA from the nematode *Ascaris lumbricoides* are also not functionally equivalent (T.Nilsen, personal communication). Thus, the differences observed here are not unique to the U1/U5 comparison, and may be generally expected when different U snRNPs are compared.

Relative to the vertebrate RNAs, the most divergent forms of U1 and U5 snRNAs thus far characterized are from yeasts. The sequences of the Sm core site of *S.cerevisiae* and vertebrate U5 RNAs are similar, both containing a run of six U residues (although not all characterized U5 RNAs share this feature; Guthrie and Patterson, 1988). Further, an internal loop at the position of IL1 is present in all characterized U5 RNAs (Guthrie and Patterson, 1988). Two forms of U5 RNA are present in *S.cerevisiae*, a long and a short form, which differ in that the short form lacks a 3′ hairpin structure and ends close to the Sm core site (Patterson and Guthrie, 1987). Thus, both forms of yeast U5 retain the two essential features of the U5 minimal Sm RNA defined here (Figure 2A): the Sm core sequence and IL1. The lack of a 3′ hairpin had at most a small effect on the immunoprecipitability of the minimal RNA with anti-Sm antibodies, and little or no effect on nuclear accumulation (Figure 2).

In contrast, the regions involved in Sm protein binding in U1 snRNP are very poorly conserved. U1 snRNAs from the budding yeasts *S.cerevisiae, S.uvarum* and *Kluyveromyces lactis* are much longer than vertebrate U1 (Kretzner *et al.*, 1987, 1990; Siliciano *et al.*, 1987) and are predicted to have several yeast-specific secondary structure features (Kretzner *et al.*, 1990). Of the U1 RNA elements shown here to be involved in Sm protein binding, only the single-stranded 5′ end is conserved and this region of the RNA has an essential function in base pairing with 5′ splice sites that adequately explains its conservation (Zhuang and Weiner, 1986; Séraphin *et al.*, 1988; Siliciano and Guthrie, 1988). The budding yeast U1 snRNAs lack a 3′ hairpin, have a yeast-specific structure in place of the vertebrate C hairpin and have, unlike the vertebrate U1 RNAs, an uninterrupted run of 5 U residues in their Sm core sequences. We would therefore predict that some of the yeast-specific sequence and structural elements (Kretzner *et al.*, 1990) replace the vertebrate C and E hairpins to allow efficient association with the common snRNP proteins.

Aside from their (presumably essential) role in allowing U snRNP accumulation in the nucleus, there is little evidence concerning other potential functions of the Sm proteins. Wersig and Bindereif (1992) showed that a human U4 snRNA mutant, whose Sm core site was mutated such as to abolish immunoprecipitation with anti-Sm antibodies, was active in an *in vitro* splicing complementation assay. This result suggests that the common proteins bound to U4 snRNA play no essential role in splicing *in vitro*. It could be that the situation is different *in vivo* or in other snRNPs. The results presented here suggest that the common snRNP proteins are likely to make more than one contact with RNA and that some of these interactions will be different in different snRNPs. Therefore, one or more potential RNA interaction surface will be unoccupied in each Sm snRNP. It is possible that these surfaces could play a role in stabilizing either snRNP−snRNP or snRNP−pre-mRNA interactions by interacting with other RNAs. Experiments to test this will, however, have to await better characterization of the common Sm snRNP proteins. In the immediate future it will be important to define in more detail the Sm binding sites of other major and minor snRNAs.

Materials and methods

Plasmids

The genes used in this paper were wild-type or mutant versions of *Xenopus* U5 snRNA (Kazmaier *et al.*, 1987) and *Xenopus* U1 snRNA (Zeller *et al.*, 1984) genes. Both genes were cloned into the M13 mp9 vector to carry out mutagenesis. The T7 promoter and unique restriction sites (*Bam*HI for U1 and *Eco*47III for U5) were introduced (Hamm *et al.*, 1987). The U1 ΔSm (ΔD), U1ΔC and U1A3B1 mutants have been described previously (Hamm *et al.*, 1987, 1988, 1990). The U5 ΔSm mutant has a substitution within the Sm core binding site ($T_{89}TTTTT_{94} \rightarrow T_{89}GGGTT_{94}$).

Other U1 and U5 mutants used in this paper contained the following alterations. U1ΔAB (U1 wt: Δ16−91); U1 ΔABΔ5′ (U1 wt: Δ1−12, Δ16−91); U1ΔCΔ5′ (U1 wt: Δ1−12, Δ92−117); U1delE (U1 wt: Δ139−164); U1Δ5′ (U1 wt: Δ1−12); U1/U5 (U1 wt: substitution $A_{123}-T_{137} \rightarrow$ TTCGTTCAATTTTTTGAA); U1.1 (U1 wt: substitution $T_{127}TTCT_{131} \rightarrow T_{127}TTTTT_{131}$); U5ΔI (U5 wt: Δ19−59, insertion $C_{18}\underline{TTCG}G_{60}$); U5ΔII (U5 wt: Δ7, 8 and 70−75); U5Δ5′ (U5 wt: Δ7 and 8); U5Δ3′ (U5 wt: Δ70−75); U5.2 (U5 wt: substitution $T_7G_8 \rightarrow$ ATGGTA); U5.5 (U5 wt: substitution $G_{70}ACCAT_{75} \rightarrow$ ATGGTC); U5.13 (U5 wt: Δ7, 8 and 70−75; substitution $T_{80}-A_{97} \rightarrow$ ATAATT-

TCTGGTAGT); U5.13/E (U5 wt: Δ7, 8 and 70–75; substitution all U5 sequences from T_{79} to 3′ end with U1 sequence from A123 to 3′ end); U5.13/5′U1 (U5.13: substitution A_1T_2 → ATACTTACCTG); U5.13/17 (U5.13: insertion G_{10}ACGCATGGA$_{11}$); U5/U1 (substitution U1 wt $T_{80}-A_{97}$ → ATAATTTCTGGTAGT); U5.7 (U5 wt: substitution $T_{89}-T_{94}$ → TTTCT (U1 Sm core); U5.8 (U5/U1: substitution U1 Sm core TTTCT → U5 Sm core TTTTTT); U5.4 (U5 wt: insertion T_{81}–CGCTACTTAT–C_{82}).

Mutagenesis

All mutations (except deletion of the 3′ hairpin of U1) were introduced using an oligonucleotide-directed *in vitro* mutagenesis system from Amersham. After mutagenesis, the entire coding region was checked by sequencing and recloned into the pUC19 vector.

Construction of U1delE

The deletion of the last 3′ stem of U1 was performed by polymerase chain reaction (PCR). Amplification was carried out in a volume of 100 μl in 20 mM Tris pH 8.5, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 1 ng of U1 DNA, 100 ng of each primer, 2 U Ampli Taq polymerase (Perkin Elmer), 0.2 mM of each dNTP (Pharmacia); 5′ primer: GGAATTCTAATACGACTCACTATAGGG; 3′ primer: CGGGATCCACTACCAGAAATTATGCAGTC; 25 cycles were performed (94°C, 1 min; 55°C, 1.5 min; 72°C, 1.5 min) followed by 5 min final incubation at 72°C using a Techne PHL-1 thermal cycler. The amplified fragment was digested with *Bam*HI and *Eco*RI, and cloned into pUC19 then sequenced.

Construction of the U5 Sm minimal structure

The following two oligonucleotides, A and B (2 μg) each, [A, CGAGAATTCTTAATACGACTCACTATAGGGAAGGAGACCACAAGGACATTTCGATGTCC; and B, GGTGGATCCTTCAAAAAATTATGATGGGGAGCACCATCACAAGGACATCGAAATGTCCTTGTGG] were mixed in 50 μl of 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM DTE, incubated at 65°C for 10 min and at 37°C for 30 min. dNTP mix (5 μl) (2.5 mM each; Pharmacia) was added. The filling in reaction was started by adding 10 U of Klenow polymerase (Boehringer) and incubated at room temperature for 1 h. The DNA fragment obtained was digested with *Bam*HI and used directly as a template for *in vitro* transcription with T7 RNA polymerase. Full-length transcripts were gel purified and used for microinjection.

In vitro transcription

One microgram of linearized plasmid (*Bam*HI for U1 and U5.13/E constructs, and *Eco*47III for other U5 mutants) was transcribed in a volume of 10 μl in 40 mM Tris–HCl, pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 10 U of T7 RNA polymerase (Stratagene), 30 mM DTT, 0.4 mM of each NTP (UTP, CTP, GTP, ATP), 10 μCi [α-^{32}P]UTP (800 Ci/mmol), 1 mM m^7GpppG or ApppG dinucleotides (Pharmacia) and 10 U RNasin (Promega). After 45 min incubation at 37°C, proteins were extracted with phenol/chloroform and unincorporated [α-^{32}P]UTP was removed on a spin column (Sephadex G-50). RNA was precipitated with ethanol and resuspended in 10 μl of water. Transcript (1 μl) was loaded on an 8% polyacrylamide–7 M urea gel. For microinjection, transcripts were diluted 10–20 times with water. T7 U snRNAs were mixed in ratios to obtain similar intensities of radioactive signals on autoradiographs.

Microinjection

In vitro generated transcripts were injected into the cytoplasm of *Xenopus* oocytes and later extracted from dissected oocytes as described previously (Mattaj and De Robertis, 1985).

Antibodies and immunoprecipitation

Y12 monoclonal anti-Sm antibody was used in this study (Lerner *et al.*, 1981); 40 μl of protein A–Sepharose CL4B beads (0.1 g/ml; Pharmacia) in Ipp 150 (10 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% Nonidet P-40, 0.1% sodium azide) were coupled with 5–10 μl of Y12-containing ascites fluid in 400 μl of Ipp 500 (as Ipp 150 but 500 mM NaCl) at 4°C for 2 h. The beads were washed four times with 1 ml of Ipp 500 at room temperature. Five injected oocytes were homogenized in 500 μl of homogenization buffer (10 mM Tris–HCl, pH 8.0, 150 mM NaCl) and the insoluble fraction (yolk and pigment) was removed by centrifugation for 10 min in an Eppendorf centrifuge. Then 5 μl of 10% Nonidet P-40 were added to each supernatant. The supernatants were transferred into tubes with beads coupled to Y12 antibodies and rotated for 2 h at 4°C. The beads were washed at room temperature three times for 10 min each with 1 ml of Ipp 500 and incubated with 400 μl of homo medium (50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 1.5% SDS, 300 mM NaCl, 1.5 mg/ml proteinase K) for 30 min at 37°C. RNA was extracted with phenol/chloroform and precipitated with 1 ml of cold ethanol using glycogen as a carrier. For total RNA lanes, five injected oocytes were homogenized directly in 400 μl of homo medium, incubated for 30 min at 37°C, extracted twice with phenol/chloroform and ethanol precipitated. In all experiments, 0.5 oocyte equivalents of RNA were loaded per lane on 8% polyacrylamide gels containing 7 M urea.

RNase H-mediated cleavage of the 5′ end of U1 snRNA

In vitro transcribed U1ΔC snRNA was injected into the cytoplasm of *Xenopus* oocytes (~0.5 ng/oocyte). After overnight incubation, the oocytes injected with U1ΔC snRNA were divided into three groups of 10 oocytes each. One group was left as a control (no oligonucleotide control), two others were injected with the U1-5′ oligonucleotide (TTCAGGTAAGTACTCA) at either 250 or 500 μM. After 6 h of further incubation, oocytes were homogenized and immunoprecipitation carried out as described above.

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