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In vitro assembly of U1 snRNPs

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An efficient system for the *in vitro* assembly of U1 snRNPs is described. RNA–protein interactions in a series of U1 snRNA mutants assembled both *in vivo* and *in vitro* were studied in order to verify the accuracy of the system. Two discrete protein binding sites are defined by immunoprecipitation with antibodies against different protein components of the U1 snRNP and a newly developed protein sequestering assay. The U1 snRNP-specific proteins 70K and A require only the 5′-most stem–loop structure of U1 snRNA for binding, the common U snRNP proteins require the conserved Sm binding site (AU_nG). Interactions between these two groups of proteins are detected. These results are combined to derive a model of the U1 snRNP structure. The potential use of the *in vitro* system in the functional analysis of U1 snRNP proteins is discussed.

Key words: U1 snRNPs/stem–loop structure/protein binding sites

Introduction

The U1 snRNP (small nuclear ribonucleoprotein particle) is the most abundant of the U snRNPs involved in the splicing process (reviewed in Sharp, 1987; Maniatis and Reed, 1987). It was the first U snRNP shown to be required for splicing (Krämer *et al.*, 1984) and evidence for direct base-pairing interactions between U1 snRNA and 5′ splice sites *in vivo* has recently been presented (Zhuang and Weiner, 1986). The protein components of the U1 snRNP can be divided into two classes, U1-specific proteins (70K, A, C) and common U snRNP proteins (B′, B, D, E, F, G). Although a role for the proteins in the binding of the U1 snRNP to 5′ splice sites has been demonstrated (Mount *et al.*, 1983) and the genes for two of the proteins, 70K and E, have been cloned (Theissen *et al.*, 1986; Wieben *et al.*, 1985), the exact function of these proteins is unknown.

As a first step in setting up assays for the function of U1 snRNP proteins an efficient *in vitro* assembly system has been developed. Previously the assembly of U snRNPs has been studied *in vitro* only on an analytical scale (Wieben *et al.*, 1983; Fisher *et al.*, 1983). We have made use of the fact that in *Xenopus laevis* the accumulation of the RNA and protein components of U snRNPs is not coordinated, leading to the presence of a large stockpile of U snRNP proteins in mature oocytes and eggs (De Robertis *et al.*, 1982; Zeller *et al.*, 1983; Fritz *et al.*, 1984). By synthesizing U1 snRNA *in vitro* with T7 RNA polymerase and combining it with *Xenopus* egg extracts it has been possible to obtain U1 snRNPs in microgram quantities.

RNA–protein interactions in U1 snRNPs assembled *in vivo* and *in vitro* were studied extensively and a structural model of the U1 snRNP is presented.

Results

Construction of mutant U1 genes

In order to identify protein binding sites in U1 snRNA and to assay for correct assembly of U1 snRNPs *in vitro* we wished to have a series of mutant U1 snRNAs which had altered protein binding properties.

Mutations were introduced, by site-directed mutagenesis (Kramer *et al.*, 1984), into a *X. laevis* U1 snRNA gene (Zeller *et al.*, 1984). We selected mutations carefully because we wanted to generate RNAs of predictable and stable secondary structure. Using available secondary structure models (Reddy and Busch, 1981) as well as computer derived energy calculations (D.Konings, personal communication) we hoped to delete single stem–loop structures without destroying adjacent ones. Where single-stranded regions were changed they were not altered in length and did not alter the predicted secondary structure of the RNA.

By this approach each of the three stem–loop structures at the 5′ end of the U1 molecule was deleted separately (ΔA–ΔC),

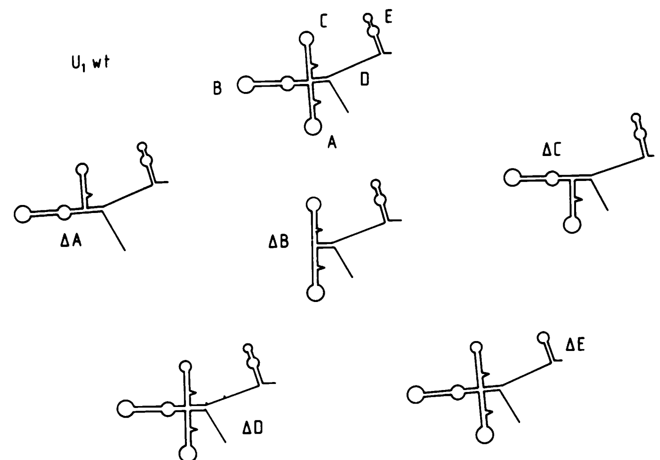

Fig. 1. Design of the U1 mutants. Deletions and substitutions were introduced into a *X. laevis* U1 snRNA gene (Zeller *et al.*, 1984) by site-directed mutagenesis (Kramer *et al.*, 1984).

Mutant	Deleted (Δ) or substituted (S) nucleotides	Length
wt	–	165
ΔA	Δ 18–48	134
ΔB	Δ 51–92	123
ΔC	Δ 93–118	139
ΔD	S 125–130	165
	(TAATTT to CTCGAG)	
ΔE	Δ 145–149	155
	Δ 154–158	
	S 150–153	
	(TTCG to AGAA)	

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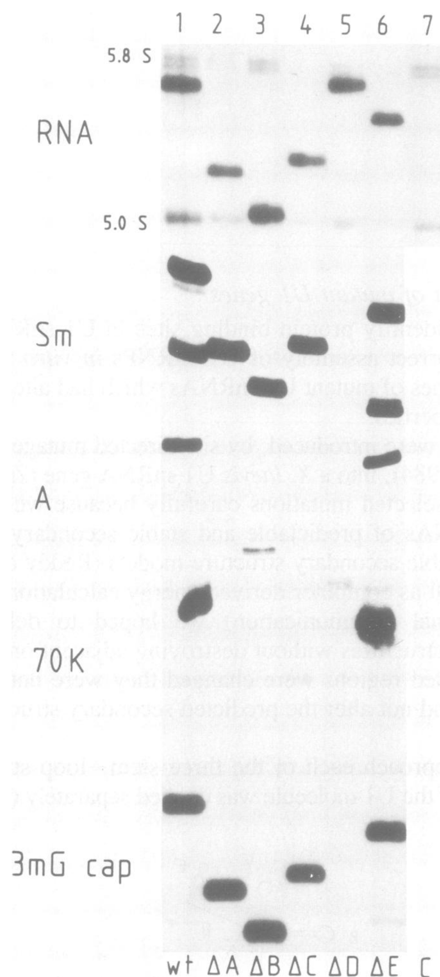

Fig. 2. Analysis of the *in vivo* assembled U1 snRNPs. **(a)** Transcripts of U1 genes (Figure 1) injected into the nucleus of *X. laevis* oocytes together with [α-^{32}P]GTP (**lane 1**, wt; **lane 2**, ΔA; **lane 3**, ΔB; **lane 4**, ΔC; **lane 5**, ΔD; **lane 6**, ΔE; **lane 7**, no DNA). RNAs were analysed on 8% polyacrylamide gels containing 7 M urea. **(b–d)** RNA immunoprecipitated from extracts of injected oocytes with antibodies against common U snRNP proteins (anti-Sm) or U1-specific proteins (anti-A, anti-70K). **(e)** RNA was extracted from injected oocytes and subsequently immunoprecipitated with anti-trimethyl-G cap antibodies (3mG).

the potential Sm binding site (Mattaj, 1986) was substituted (ΔD) or the 3'-most stem–loop was reduced in size while the sequence found conserved in this loop (Branlant *et al.*, 1982) was altered (ΔE). The predicted secondary structures of the mutant RNAs are shown in Figure 1.

Template construction for synthetic U1 snRNA

To synthesize U1 snRNA *in vitro* T7 RNA polymerase promoter sequences were inserted adjacent to the cap sites of the U1 mutant genes and sites for the restriction enzyme *Bam*HI were introduced downstream of the coding sequences. Three G residues were inserted to obtain efficient initiation of transcription. Transcription was initiated with a monomethyl-GpppG cap analogue. Due to these manipulations the synthetic U1 RNA contains three additional G residues at the 5' end and four additional nucleotides (GATC) at the 3' end. These extensions do not alter the predicted secondary structures (energy calculations, D.Konings). In addition to the U1 mutants within the transcription unit (Figure 1) double mutants (ΔAD, ΔBD, ΔDE) were constructed that carry the substitution of the Sm binding site plus the deletion of element A, B or E.

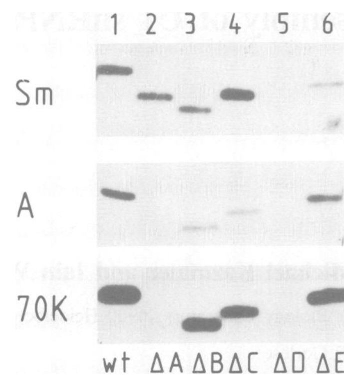

Fig. 3. Analysis of *in vitro* assembled U1 snRNPs. T7 U1 snRNA was incubated in egg extract for 60 min (Materials and methods) and immunoprecipitated with anti-Sm, anti-A or anti-70K antibodies (**lane 1**, wt; **lane 2**, ΔA; **lane 3**, ΔB; **lane 4**, ΔC; **lane 5**, ΔD; **lane 6**, ΔE).

Properties of in vivo assembled mutant U1 snRNPs

Wild-type or mutant U1 genes were injected into the nucleus of *Xenopus* oocytes and their transcripts were extracted and analysed (Figure 2a). The mutations in the coding sequence appear to affect neither the accuracy nor the efficiency of transcription. Wild-type and mutant genes are transcribed with equal efficiency when coinjected (data not shown). The transcripts are of the length expected and are stable.

To identify sites required for protein binding, RNA was immunoprecipitated from extracts of injected oocytes with antibodies against different protein components of the U1 snRNP. Substitution of the conserved sequence AU_nG (Branlant *et al.*, 1982) in ΔD results in the loss of precipitability with anti-Sm antibodies (Figure 2b). This is in agreement with studies of U2 mutants where alteration of this region also interfered with binding of the common U snRNP proteins recognized by this antiserum (Mattaj and De Robertis, 1985).

Antibodies against the U1-specific proteins A or 70K demonstrate the importance of the hairpin structures at the 5' end of U1 for binding these proteins. The mutant ΔE remains precipitable with these antibodies but only trace amounts of the mutants ΔA–ΔC are precipitated, while ΔD is not detectably precipitated (Figure 2c,d). This indicates that, under these immunoprecipitation conditions, stable binding of proteins 70K and A requires not only the three stem–loop structures at the 5' end of the molecule but also the association of the common U snRNP proteins with the Sm binding site. Similar results were obtained using several different antibodies (either patient sera or monoclonal antibodies) of anti-Sm, anti-A, or anti-70K specificity (data not shown). The specificity was determined by Western blotting and the immunoprecipitation assay shown in Figure 9.

The generation of the U snRNA-specific trimethyl-G cap structure of U2 and an artificial RNA has been shown to be correlated with the ability to bind the common U snRNP proteins (Mattaj, 1986). This is also true for U1 snRNA: all mutant RNAs except ΔD are immunoprecipitable with anti-3mG antibodies (Figure 2e).

In vitro assembly of U1 snRNPs

U1 snRNAs transcribed *in vitro* by T7 RNA polymerase (T7 U1 snRNA) were injected into the cytoplasm of oocytes and the assembled U1 snRNPs analysed by immunoprecipitation with

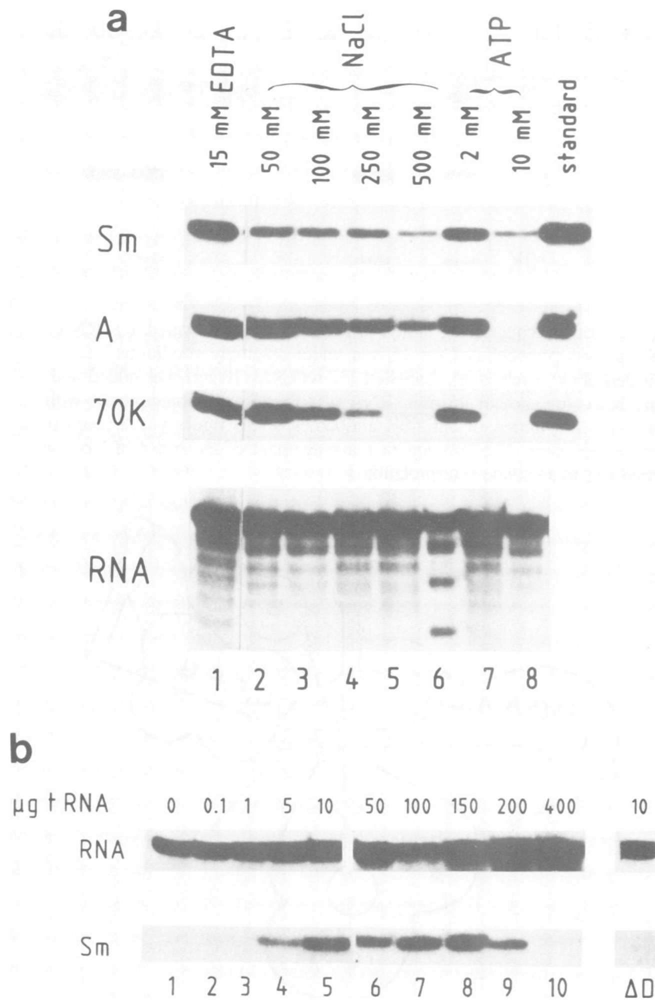

Fig. 4. Assembly conditions. **(a)** Standard conditions [25 mM Tris pH 7.4, 2 mM $MgCl_2$, 0.2 mM EDTA, 0.25 mM DTT, 40 mM $(NH_4)_2SO_4$] were modified prior to assembly. Four aliquots were made and either total RNA was extracted or RNA was immunoprecipitated (anti-Sm, anti-A, anti-70K) after assembly. **(b)** Titration of the quantity of the unspecific competittor tRNA (indicated in the top lane) added prior to assembly required to optimize the yield of U1 snRNPs assembled on 10 ng T7 U1 snRNA in egg extracts containing 30 µg of protein (to verify specificity mutant ΔD was incubated under the same conditions).

anti-Sm, anti-A, anti-70K or anti-3mG antibodies. The behaviour of the T7 RNAs closely resembled that of the *in vivo* transcripts shown in Figure 2 (data not shown). This demonstrated that the additional nucleotides in the T7 transcripts do not influence protein binding.

U1 snRNPs were therefore assembled *in vitro* by incubation of T7 U1 snRNA in egg extracts (Materials and methods), which contain large amounts of stockpiled snRNP proteins (Zeller *et al.*, 1983; Fritz *et al.*, 1984). As observed *in vivo*, anti-Sm antibodies precipitate all RNAs except ΔD (Figure 3, Sm). Antibodies against proteins A or 70K fail to precipitate both ΔA and ΔD (Figure 3; A, 70K). Compared with the wild-type the efficiency of precipitation of ΔB and ΔC is reduced with anti-A antibodies, but is similar with anti-70K antibodies.

In general the mutants assembled *in vitro* exhibit the same binding properties as in oocytes. Nevertheless there is a quantitatively different behaviour of ΔA−ΔC with respect to the binding of proteins A and 70K. Antibodies against these proteins precipitate ΔA (although with greatly reduced efficiency) from

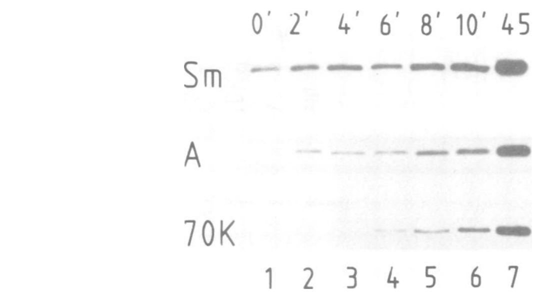

Fig. 5. Time course of *in vitro* assembly. Aliquots were removed from a single assembly mix over 45 min and diluted with IPP_{500} (Materials and methods). Aliquots were subdivided and RNA immunoprecipitated with anti-Sm, anti-A and anti-70K antibodies.

oocytes but not detectably *in vitro*. ΔB and ΔC are precipitated with anti-70K antibodies with high efficiency *in vitro* but with reduced efficiency from oocytes. Several explanations for these differences are possible, for example different RNA−protein ratios *in vivo* and *in vitro* or the vast excess of tRNA present during the *in vitro* incubation. However these explanations cannot be tested experimentally and we cannot currently explain the quantitative differences.

Several observations indicate that what we observe *in vitro* are U1 snRNP particles rather than interactions between RNA and single proteins. First, depending on the extract preparation used, 60−90% of the input RNA is immunoprecipitable with all three classes of antibodies. Secondly, the 70K and the A protein bind more stably in the presence of the common U1 snRNP proteins (see below). Finally, analysis of the mobility of T7 U1 snRNA on native gels after incubation under assembly conditions reveals a broad, ill-defined band with a much lower electrophoretic mobility than the free RNA (data not shown). Endogenous U1 snRNPs also run as a broad band on such gels (see for example Konarska and Sharp, 1987).

Factors affecting assembly in vitro

The requirement of assembly for energy in the form of ATP, divalent cations, and the sensitivity to salt concentrations was analysed. One of the parameters was changed before the addition of the RNA to the assembly reaction and the products were characterized by immunoprecipitation with anti-Sm, anti-A and anti-70K antibodies (Figure 4a). Increasing the salt concentration from 50 to 500 mM was found to affect binding of the 70K protein significantly but to have little effect on the ability of protein A or of the common U snRNP proteins to enter the particle. The yield of U1 snRNPs is sensitive to the addition of ATP, 10 mM ATP is sufficient to inhibit assembly significantly (Figure 4a, lane 7). This effect is not due to chelating of magnesium ions by ATP since the presence of 15 mM EDTA does not effect assembly (Figure 4a, lane 1). Although it is not possible to distinguish between direct and indirect effects on assembly neither energy in the form of ATP nor magnesium is essential. None of the factors analysed reduces the stability of particles once formed (data not shown).

To achieve efficient assembly, it was necessary to trap unspecific RNA binding proteins by the addition of tRNA prior to the U1 snRNAs. As much as 100 µg of tRNA is required to obtain optimal assembly in a quantity of extract containing 30 µg of protein (Figure 4b).

Time course of assembly in vitro

A resolution of different assembly stages is not possible in undiluted extracts due to the rapidity of the reaction. After 10 min

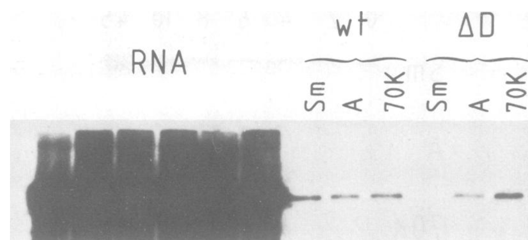

Fig. 6. Low stringency immunoprecipitation. U1 wt and U1 ΔD were immunoprecipitated from assembly mixes with anti-Sm, anti-A or anti-70K antibodies under low stringency conditions (0°C, 150 mM salt; Kurilla and Keene, 1983). This autoradiograph was exposed for 21 days. Those shown in Figures 1–5 and 7 were exposed for 12–30 h. The low efficiency is also revealed by comparing total and immunoprecipitated RNA in this figure and in Figure 4.

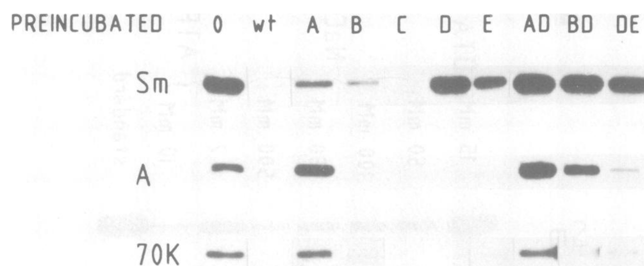

Fig. 7. Protein sequestering assay. Unlabelled wt or mutant U1 snRNA (300 ng) was preincubated for 15 min under assembly conditions (as indicated above each lane). Labelled U1 wtRNA (10 ng) was added and coincubated for another 45 min. RNA was then immunoprecipitated with either anti-Sm, anti-A or anti-70K antibodies. (This figure is the result of a single experiment. The subsequent rearrangement of the order of lanes was carried out to facilitate interpretation.)

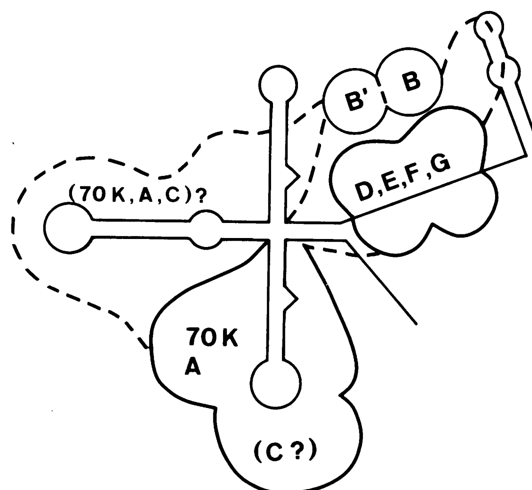

Fig. 8. Model of the U1 snRNP. This model summarizes the data on the protein distribution in the U1 snRNP. Proteins are shown as clouds. Regions containing essential RNA–protein contacts are shown with solid lines, weaker interactions by dotted lines.

the input RNA is completely complexed into particles (data not shown). However, in diluted extracts (1:5) the binding of proteins A and 70K is delayed with respect to that of the common RNP proteins, the order of binding being Sm proteins, A, then 70K (Figure 5).

This might have implied that the order of assembly is defined, and that binding of proteins A and 70K requires prior association of the Sm antigens. This was also suggested by the fact that mutant ΔD, which was not able to bind the common proteins, was not detectably precipitated with anti-A or anti-70K antibodies (Figures 2 and 3). Analogous results have been obtained with U2 snRNA, where mutants lacking the Sm binding site were not precipitated with antibodies against U2-specific proteins A′ and B″ (Mattaj and De Robertis, 1985; Mattaj *et al.*, 1986).

Recently, however, using a different immunoprecipitation protocol, Fresco *et al.* (1987) were able to precipitate U2 snRNA from vesicular stomatitis virus-infected cells with anti-A′ but not with anti-Sm antibodies, suggesting that A′ could associate with U2 snRNA in the absence of the Sm antigens. We used their protocol (which we have called low stringency precipitation since it is carried out on ice in the presence of low salt) to immunoprecipitate U1 wt and ΔD transcripts assembled into RNPs *in vitro*. In contrast to the results obtained with our normal method, U1 ΔD was detectably precipitated with anti-A and anti-70K antibodies (Figure 6). Only the wild-type was Sm precipitable; this finding demonstrates the ability of proteins A and 70K to associate with U1 snRNA in the absence of bound common proteins.

Identification of interactions by a protein sequestering assay

Immunoprecipitation as performed by the method which we have employed routinely is limited to the detection of strong interactions, because complexes have to survive several high salt washes at room temperature. The disadvantage of the low stringency protocol is its inefficiency. Extremely long exposure times are required to detect immunoprecipitated RNAs (Figure 6, legend). To overcome these and other restrictions, a more sensitive assay based on the template exclusion principle (Lassar *et al.*, 1983) was developed. This assay measures the ability to sequester proteins by binding, as detected by the subsequent addition of a competitor RNA.

The (unlabelled) RNA to be analysed is preincubated for 15 min in assembly conditions in amounts which, if wt U1RNA is used, are sufficient to bind all the U snRNP proteins. After this preincubation, labelled wt U1RNA is coincubated for another 45 min and RNA is immunoprecipitated with anti-Sm, anti-A or anti-70K antibodies. Only if the unlabelled RNA in the preincubation is unable to bind a certain protein would the labelled wt RNA be precipitable with the corresponding antibody (compare lanes 0 and wt, Figure 7; U1 wt depletes all U1 snRNP proteins from the extract). Preincubation of the mutant RNAs demonstrates that all except ΔC have a reduced affinity for the Sm antigens. Those mutants in which the Sm binding site is substituted are competely unable to bind these proteins (compare lanes 0, D, AD, BD; Figure 7, Sm). In contrast only ΔA and ΔAD cannot sequester proteins A and 70K (Figure 7; A, 70K). Mutants ΔB–ΔD bind proteins A and 70K stably in the preincubation (but were only poorly or not at all precipitable with the corresponding antibodies, Figures 2 and 3). Due to the strong affinity of mutant ΔC for the common proteins it is not possible to assay its ability to sequester the U1-specific proteins, since immunoprecipitability with anti-A or anti-70K antibodies requires binding of the common proteins. We conclude that the 5′-most stem–loop structure is the only U1 snRNA element essential for binding of the U1-specific proteins.

These results also reveal the existence of protein–protein and RNA–protein interactions within the particle which were not detected by other methods. For example ΔB and ΔD both sequester protein A (although neither was efficiently precipitated by anti-A antibodies; Figures 2 and 3). The double mutant ΔBD

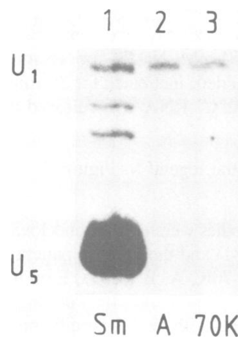

Fig. 9. Specificity of the anti-U1 snRNP antibodies. A U5 snRNA gene (Kazmaier *et al.*, 1987) was injected into oocytes together with [α-^{32}P]GTP and RNA immunoprecipitated with anti-Sm, anti-A or anti-70K antibodies. The exposure time was chosen such that transcripts of endogenous U1 snRNA genes are visible. This experiment verifies that the U1-specific sera exhibit no anti-Sm cross-reactivity and that the immunoprecipitation efficiency of the three sera is comparable.

does not sequester protein A. A possible explanation is that weak binding of protein A to ΔB is stabilized by interactions with proteins bound to the Sm binding site. The stabilization could be reciprocal since ΔA and ΔB sequester the Sm antigens less efficiently than wt U1, indicating that the weaker binding, or the lack of binding, of protein A to ΔA and ΔB affects the strength of Sm antigen binding.

Additionally, this assay demonstrates a role of the 3′-most stem–loop structure in the binding of the common proteins. Mutant ΔE has a much lower ability to sequester these proteins than wt U1 or mutants ΔA–ΔC.

Discussion

Structural studies

We have used a variety of assays to detect different classes of interactions within the U1 snRNP. These have allowed the characterization of sites of both strong and weak RNA–protein binding as well as of protein–protein interactions, and have led to the structural model of this particle proposed in Figure 8.

The binding of the common U snRNP proteins B′, B, D, E, F and G is dependent on the Sm binding site (element D, Figure 1). Mutation of this motif abolishes the binding of these proteins as analysed by immunoprecipitation (Figures 2 and 3) or the much more sensitive protein sequestering assay (Figure 7). The mutation of the 3′-most stem–loop structure (E, Figure 1) reveals an additional contact point for the common proteins, which helps to stabilize their binding. The RNA element E is essential neither for assembly nor for immunoprecipitability of U1 snRNPs (Figures 2, 3; lanes ΔE, Sm), but the mutation reduces the ability of ΔE to sequester these proteins (Figure 7). The loop sequence of element E (PyNPyG) and the structure of the Sm binding site (AU_nG) are conserved in U1, U2, U4 and U5 (Branlant *et al.*, 1982) and are protected against nucleases in RNPs but not in uncomplexed RNAs (Epstein *et al.*, 1981; Liautard *et al.*, 1982). The Sm binding site is apparently located in a single-stranded region of the RNAs and has been shown to be necessary and sufficient for the association of the common U snRNP proteins with U2 snRNA (Mattaj and De Robertis, 1985) and with an artificial RNA (Mattaj, 1986).

The U1-specific proteins A and 70K require only the 5′-most stem–loop structure of U1 snRNA (element A, Figure 1) for binding (Figure 7). Mutation of elements B or C (Figure 1), however, results in a decreased efficiency of immunoprecipitation with anti-A or anti-70K antibodies (Figures 2 and 3, lanes 3 and 4) suggesting a stabilizing effect of these elements on the binding of proteins A and 70K. Interaction with the common proteins strengthens at least the association of protein A, since mutants ΔB and ΔD are both able to sequester this protein efficiently while the double mutant ΔBD is not (Figure 7, lanes B, D and BD).

The reduced affinity of proteins A and 70K to the mutant U1 snRNAs ΔB and ΔC might be explained in several ways. These proteins might have in addition to the essential contacts in hairpin A weaker RNA contacts in hairpins B and C. Additionally, the U1-specific protein C could also be involved in stabilization of the RNP by binding to hairpins B or C. We were unable to investigate the interactions of protein C in U1 snRNPs due to the lack of a monospecific antibody against this protein.

It is also likely that the tertiary structure of the RNA is altered by the deletion of a complete stem–loop structure. This might have the result that two different protein binding sites on the RNA, although both still present, are in a different relative orientation in the mutant RNA. Because of this, interactions of proteins with these sites or with different proteins bound elsewhere in the RNP may be affected.

U1 snRNA is extensively modified *in vivo* (Busch *et al.*, 1982). The modifications appear inessential for protein binding because the synthetic U1 snRNAs are unmodified and it is unlikely that they are modified during the short time necessary for assembly.

The trimethyl-G cap structure is also not required for assembly *in vitro*. The T7 RNAs have a monomethyl-G cap which is trimethylated in the *in vitro* extracts at an efficiency bordering on the insignificant (data not shown). As expected (Mattaj, 1986), the caps of the T7 RNAs are trimethylated when injected into the cytoplasm of oocytes, provided they carry the Sm binding site (data not shown).

During assembly *in vitro*, binding of the common proteins can be detected prior to binding of the U1-specific proteins (Figure 5). Fisher *et al.* (1985) discovered an RNA-free 6S 'core' particle containing four of the common proteins (D, E, F, G) *in vivo*. Taken together, these findings suggest that the assembly is a multistep process with the 6S core particle being the first to enter the RNP. However, the sequestering assays and the low stringency immunoprecipitations demonstrate that binding of the U1-specific proteins and the common proteins can occur independently.

Fresco *et al.* (1987) showed recently that the U2-specific protein A′ can be associated with U2 snRNA in the absence of the common proteins in BHK cells infected with vesicular stomatitis virus. Our previous conclusion that prior binding of the common proteins was required for binding of A′ and B″ to U2 snRNA (Mattaj and De Robertis, 1985; Mattaj *et al.*, 1986) was based on immunoprecipitation studies carried out at high stringency. Since these conditions fail to detect the binding of the U1-specific proteins A and 70K to ΔD (compare Figure 2 with Figures 6 and 7), this conclusion is likely to be erroneous. The possibility remains, however, that assembly occurs in a defined order *in vivo*.

We cannot tell which proteins in the U1 snRNP have direct RNA contacts, but recent UV-induced cross-linking experiments result in the apparent cross-linking of the common protein D to U1 snRNA (data not shown). None of the U1-specific proteins were cross-linked in these experiments.

The rate of assembly in undiluted extracts is in good agreement with the cytoplasmic half-life of U1 snRNA (Eliceiri, 1974). After 10 min the input RNA is completely complexed with pro-

tein. This is the lifetime of cytoplasmic U1 snRNA precursors before their return to the nucleus.

Perspectives

The great advantage of our assembly system is the possible particle yield. In different experiments (data not shown) up to 0.2 μg of RNA was assembled into particles. In principle the method can be scaled up further, making possible structural studies requiring large amounts of U1 snRNPs.

However, the most direct use of this system is for the generation of U1 snRNPs of defined composition. These could be used to test the functions of single protein components of the U1 snRNP, for example in substrate binding or splicing. A first step in this direction is a functional test of the *in vitro* assembled U1 snRNPs. Although such experiments (in collaboration with A.Krämer) have so far been unsuccessful we are continuing with our efforts to demonstrate that the *in vitro* assembled U1 snRNPs are not only structurally correct but also functionally active.

Materials and methods

Site-directed mutagenesis

Mutations were introduced by the method of Kramer *et al.* (1984) into the *X. laevis* U1 snRNA gene XIU1.3 (Zeller *et al.*, 1984). To create mutants ΔA, ΔB and ΔC oligonucleotides complementary to 12–15 nucleotides on each site of the sequence to be deleted were used. ΔD was generated with a 23mer changing nucleotides 125–130 (indicated in Figure 1). ΔE was constructed with a 29mer and combined the deletion and substitution of nucleotides. The T7 promoter was created by inserting the sequence TAATACGACTCACTATAGGG (derived from Dunn and Studier, 1983) adjacent to the cap sites of the U1 snRNA mutant genes with a 50mer. The *Bam*HI site was constructed by deleting the first 23 nucleotides downstream of the coding sequence with a 20mer.

Microinjection of oocytes

30–50 nl purified DNA (330 μg/ml) was injected into the nucleus of *X. laevis* oocytes together with [α-^{32}P]GTP (Nishikura *et al.*, 1982). 12–20 h later oocytes were homogenized in 10 mM Tris–HCl pH 8.0, 150 mM NaCl (10 oocytes/ml). After centrifugation for 10 min in an Eppendorf centrifuge the supernatant was removed and Nonidet-P40 added (final concentration 0.1%).

T7 RNA synthesis

Pulse-chase transcription was performed. 1 μg linearized (*Bam*HI) template (0.5 μg/μl) was incubated in a total volume of 10 μl (40 mM Tris–HCl pH 8.0, 8 mM $MgCl_2$, 2 mM spermidine, 50 mM NaCl, 30 mM DTT, 0.4 mM ATP/UTP/CTP, 10 units T7 polymerase – Stratagene, 0.25 A_{250} m7-GpppG, 20 units RNasin) at 37°C for 5 min in the presence of 10 μCi [α-^{32}P]GTP (10 μCi/μl). Unlabelled GTP was added (final concentration 0.4 mM) and the incubation continued for 10 min. RNA was phenol extracted, purified over spun columns and precipitated with 3 volumes ethanol/0.1 volume 3 M Na-acetate. 1–2 μg RNA were synthesized by this method (corresponding to $1-5 \times 10^6$ c.p.m.).

Unlabelled RNA was synthesized with the following alteration: instead of the [α-^{32}P]GTP unlabelled GTP was present during the whole incubation of 15 min.

In vitro assembly

Fresh *Xenopus* eggs were treated wtih 2% cysteine–NaOH (pH 7.8) for 5–10 min, washed several times with Barth medium and whole cell extracts prepared (Manley *et al.*, 1980; Sergeant *et al.*, 1984). The final extract conditions were 17% glycerol, 25 mM Tris–HCl (pH 7.4), 2 mM $MgCl_2$, 0.1 mM EDTA, 0.25 mM DTT, 40 mM $(NH_4)_2SO_4$.

8 μl egg extract (protein concentration 4 μg/μl), 1 μl tRNA (100 μg/μl) and 1 μl T7 RNA (10 ng/μl) were incubated for 45 min at 19°C.

Immunoprecipitation

2–10 μl serum (dependent on the antibody titre), 40 μl protein A–Sepharose CL-4B beads (0.1 g/ml in IPP_{500}; Pharmacia), 400 μl IPP_{500} (10 mM Tris–HCl pH 8.0, 500 mM NaCl, 0.1% Nonidet P-40, 0.1% sodium azide) were rolled for 2 h at room temperature and the beads washed three times with 1 ml IPP_{500}.

The extract of the homogenized oocytes or the *in vitro* assembly mix was added together with IPP_{500} to a total volume of 1 ml and rolled for 2 h at room temperature. The beads were washed three times for 10 min with 1 ml IPP_{500} and the precipitated RNA released by digestion with 400 μl homomedium (50 mM Tris–HCl pH 7.4, 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, precipitated wtih 3 volumes of ethanol and analysed on 8% acrylamide/7 M urea gels.

'Low stringency' immunoprecipitation

5 μl of serum and the *in vitro* assembly mix were incubated in a volume of 200 μl IPP_{150} (as IPP_{500}, but 150 mM NaCl) for 15 min at 0°C. 40 μl protein A–Sepharose beads were added, incubated for 30 min at 0°C and washed three times with 1 ml IPP_{150} at 0°C. RNA was released and analysed as above.

Protein sequestering assay

This assay is described in the legend to Figure 7.

Antibodies

Two different anti-Sm antibodies were used, monoclonal Y12 recognizing proteins B′, B, D (Lerner *et al.*, 1981) and the anti-Sm patient serum Küng (Fritz *et al.*, 1984) which stains proteins 70K, A, B′, B, D, E on immunodecorated Western blots (data not shown).

The anti-A serum P21 reacts with the U1-specific protein A and the U2-specific protein B″ (Habets *et al.*, 1985).

Two anti-70K antibodies were used: a monoclonal antibody (Billings *et al.*, 1982) and a patient serum B.K. having no anti-A activity (R.Lührmann, personal communication). The anti-3mG antibody (Bringmann *et al.*, 1983) was also a gift of R.Lührmann.

To verify that the anti-A (P21) and anti-70K (B.K.) sera contain no anti-Sm cross reactivity, a U5 snRNA gene (Kazmaier *et al.*, 1987) was injected into oocytes and the extract immunoprecipitated with these antibodies in addition to the anti-Sm serum (Figure 9). No U5 snRNA was precipitated, demonstrating that P21 and B.K. are true anti-U1/U2 and anti-U1 sera, respectively.

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