Monospecific antibodies reveal details of U2 snRNP structure and interaction between U1 and U2 snRNPs

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Monospecific antibodies directed against several U small nuclear ribonucleoprotein (U snRNP) particle proteins were affinity purified from a patient's anti-(U1,U2)RNP serum. These were used to demonstrate that: (i) proteins equivalent to the mammalian U2 snRNP-specific A' and B'' proteins are present in *Xenopus laevis* oocytes; (ii) both proteins A' and B'' have the same structural requirements for binding to U2 snRNA; (iii) proteins B, B' and D have the same structural requirement for binding to U2 snRNA; (iv) using very high specific activity RNA probes it is possible to detect a fraction of either U1 or U2 snRNA precipitable by antibodies directed against proteins specific for the other U snRNP, indicating an interaction between U1 and U2 snRNPs. The structural requirements of this interaction were studied for the U2 snRNP. All changes made to U2 snRNA or snRNP structure resulted in loss of the interaction with U1 snRNP.

Key words: small nuclear ribonucleoproteins/monospecific antibodies/protein structure

Introduction

Since the discovery by Lerner and Steitz (1979) that sera from certain patients suffering from autoimmune disorders contain antibodies directed against ribonucleoprotein particles, these antibodies have been extensively used in studies of the structure and function of these RNPs (for reviews see Lerner and Steitz, 1981; Busch *et al.*, 1983; Mattaj, 1984; Brunel *et al.*, 1986). The most extensively studied of the RNPs have been the U snRNPs. There is now strong evidence for an involvement of U1, U2 and U7 snRNPs in the processing of mRNA precursors (Hernandez and Keller, 1983; Padgett *et al.*, 1983; Bozzoni *et al.*, 1984; Krämer *et al.*, 1984; Krainer and Maniatis, 1985; Black *et al.*, 1985; Galli *et al.*, 1983; Strub *et al.*, 1984) which lends additional interest to the study of the structure of the RNP particles themselves.

Studies on the particle proteins have been complicated by the difficulty in purifying the particles by standard biochemical fractionation methods, and by the number, at least 11, of different proteins associated with the U snRNAs (Kinlaw *et al.*, 1982, 1983; Hinterberger *et al.*, 1983; Pettersson *et al.*, 1984; Mimori *et al.*, 1984). Recently developed purification methods based on the use of antibodies to the unique trimethyl cap structures of the U snRNAs have enabled purification in good yields of pure U snRNPs for the first time (Bringmann *et al.*, 1983, 1984), and this in turn has enabled the preparation of monospecific antibodies to various U snRNPs by affinity purification from serum (Habets *et al.*, 1985a).

A complementary method of analysis of U snRNP structure, based on the analysis of mutant U2 snRNAs, their interaction with U2 snRNP proteins and the correlation of these interactions with nuclear accumulation of the RNAs (De Robertis *et al.*, 1982; Zeller *et al.*, 1983) has recently been developed (Mattaj and De Robertis, 1985). We have now combined the two methods to further analyse the RNA–protein interactions involved in U2 snRNP structure. This analysis has led us to the conclusion that epitopes on the U2 snRNP-specific proteins A' and B'' are highly conserved in evolution, as are other U snRNA-associated proteins, and that a small fraction of the U2 snRNPs exist in a complex with U1 snRNPs.

Results

U2 snRNP-specific proteins in Xenopus oocytes

By a variety of methods two U2 snRNP-specific proteins, named A' and B'', have been identified in mammalian cells. In *Xenopus* oocytes it has been possible to detect, by immunoblotting and immunoprecipitation, proteins equivalent to the common U snRNP proteins B, D, E, F and G and the U1 snRNP-specific proteins 70 K, A and C and even putative tissue-specific variants of some of these proteins (Zeller *et al.*, 1983; Fritz, 1983; Fritz *et al.*, 1984). It has not been possible, however, to identify unequivocally U2 snRNP-specific proteins in *Xenopus* by these methods (Fritz, 1983), although their existence can be inferred since anti-(U1,U2)RNP sera specifically immunoprecipitate U1 and U2 snRNPs from *Xenopus* oocytes (Mattaj and De Robertis, 1985).

We used the anti-(U1,U2)RNP serum V26 (Habets *et al.*, 1985a) to try to detect proteins equivalent to A' and B'' in oocyte extracts, in the experiment whose results are shown in Figure 1A. In lanes 1 and 5 the reaction of V26 with proteins 70 K, A, A' and B'' in HeLa nuclear extracts is shown. The lack of obvious equivalent bands in oocyte extracts can be seen in lanes 2–4. To increase the sensitivity of detection we next purified U snRNPs from oocytes using anti-trimethyl cap antiserum (Bringmann *et al.*, 1982, 1984), and stained these with whole and fractionated V26 serum. V26 was fractionated into monospecific antibodies by elution of antibodies bound to specific bands on Western blots of HeLa extracts (Habets *et al.*, 1985a).

Figure 1B, lanes 1 and 2, shows the staining patterns obtained using the anti-(U1,U2)RNP sera B25, which recognises the HeLa A and B'' proteins, and V26, which recognises HeLa 70 K, A, A' and B'' proteins (Habets *et al.*, 1985a). Reaction of these antisera with the purified *Xenopus* oocyte U snRNP proteins allows the identification of equivalents to protein A (33 000 daltons), A' (32 500 daltons) and a triplet of proteins of ~26 000–28 000 daltons equivalent to B''. It is not clear whether these three bands correspond to different proteins or are modification or degradation products of a single protein. We did not detect a band equivalent to the HeLa 70 K protein. This must be due to lack of sensitivity since monospecific anti-70 K antibodies immunoprecipitate *Xenopus* oocyte U1 snRNP (see below).

Staining of the purified U snRNP proteins with monospecific

Fig. 1. Immunoblot analysis of *Xenopus* proteins with anti-(U1,U2)RNP sera. **Panel A.** Analysis of total *Xenopus* oocyte 12 000 g supernatant with V26 serum. **Lanes 1 and 5;** HeLa cell nuclear extract. The U snRNP proteins 70 K, A, A' and B'' are indicated. **Lanes 2, 3 and 4;** *Xenopus* oocyte 12 000 g supernatant. The approximate amounts of protein loaded were 40, 20 and 10 μ g. In comparison with the HeLa controls, there is no evidence for specific staining of particular U snRNP proteins. **Panel B.** Analysis of *Xenopus* oocyte U snRNPs purified with anti-trimethyl cap antiserum. The antisera and monospecific antibodies used were: **Lane 1;** serum B25, **lane 2;** serum V26, **lane 3;** affinity-purified anti-70 K, **lane 4;** affinity-purified anti-A, **lane 5;** affinity-purified anti-A', **lane 6;** affinity-purified anti-B'', **lane 7;** anti-Sm serum, **lane 8;** non-immune control serum. The positions of the stained bands equivalent to HeLa A, A' and B'' are indicated.

antibodies (Figure 1B, lanes 3–6) shows that the cross-reactivity between antibodies to proteins A and B'', already observed in HeLa extracts (Habets *et al.*, 1985a) is also present in *Xenopus*. The common epitope on these proteins has thus been conserved in evolution. The lack of cross-reactivity of antibodies to A' and 70 K allow monospecific anti-(U2)RNP and anti-(U1)RNP antibodies to be prepared.

An anti-Sm serum which recognises proteins B and B' as well as D in HeLa extracts (Habets *et al.*, 1985b) stains a single band in the region of protein B (27 000 daltons) in *Xenopus* U snRNPs (Figure 1B, lane 7). A single band of this size has previously been observed using other anti-Sm antisera to stain *Xenopus* oocyte extracts (Fritz *et al.*, 1984). It is not clear whether this band is a single protein or two proteins which are not resolved. The lack of reactivity of anti-Sm serum with any of the triplet of B'' bands underlines the difference between these different 'B' proteins.

In conclusion these results demonstrate the existence in *Xenopus* oocyte U snRNPs of proteins equivalent to the U2 snRNP-specific A' and B''. The patterns of immunological cross-reactivity, A and B'' cross-reacting while A and A' and B and B'' do not cross-react, is identical to that observed in mammalian cell U snRNPs.

Binding requirements of proteins B and B' on U2 snRNA

A method for analysing protein–RNA interactions in RNPs has 998

recently been developed and used to analyse U2 snRNPs. Genes coding for wild-type *X. laevis* U2 snRNA (Mattaj and Zeller, 1983) or for a series of mutants called ΔA – ΔE in which portions of the RNA sequence have been changed (Mattaj and De Robertis, 1985) were injected into *Xenopus* oocyte nuclei. The immunoprecipitability of these RNAs was then tested with various antibodies. In this way the RNA sequences required for the binding of particular antigens could be determined.

Previous experiments have shown that proteins D, E, F and G protect a short region of the U2 snRNP against micrococcal nucleus digestion (Liautard *et al.*, 1982) and that this region of U2 snRNA is required in order that the RNA be immunoprecipitated by anti-Sm antisera (Mattaj and De Robertis, 1985). Since most anti-Sm antisera recognise at least proteins B, B' and D it was not possible to determine what were the separate binding requirements of proteins B, B' and D.

Recently an anti-Sm antiserum called M13 which recognises proteins B and B' but not D has been described (Habets *et al.*, 1985b). We compared the immunoprecipitation of the U2 mutants with this serum with their immunoprecipitation by antiserum K22, which recognises proteins B, B' and D, as well as the U1 snRNP-specific A and C proteins (Habets *et al.*, 1985b). The transcripts of the wild-type and mutant genes are shown in Figure 2, panel A. Their immunoprecipitation by K22 antiserum is shown in Figure 2, panel B. This confirms that an anti-Sm antiserum

Fig. 2. Immunoprecipitation of wild-type and mutant U2 snRNAs with anti-Sm antisera. The two antisera used are K22, which recognises U snRNP proteins B, B', D, A and C, and M13 which recognises only B and B' (Habets *et al.*, 1985b). The wild-type U2 and mutants $\Delta A - \Delta E$ have been described (Mattaj and Zeller, 1983; Mattaj and De Robertis, 1985). *Xenopus* oocytes were microinjected with [α - 32 P]GTP alone (lanes G) or in combination with either wild-type U2 DNA (lanes U2) or one of the mutant DNAs $\Delta A - \Delta E$ (lanes A-E). **Panel A.** Total transcription products from microinjected oocytes. The positions of 8S, U2, 5.8S and 5S RNAs are indicated. Note that, on this gel, ΔB transcripts co-migrated with 5.8S RNA. **Panel B.** RNA precipitated by K22 antiserum. **Panel C.** RNA precipitated by M13 antiserum.

recognising B, B' and D immunoprecipitates wild-type U2 and all the mutants with the exception of ΔC , from which at least part of the Sm binding site has been deleted (Mattaj and De Robertis, 1985).

M13 antiserum immunoprecipitates exactly the same U2 mutants as K22 (Figure 2, compare panels B and C). Since this antiserum recognises only B and B' this indicates that these two proteins require the Sm binding site to bind to U2 snRNA. Since we already know from the results of Liatard *et al.* (1982) that one or more of the proteins D, E, F and G interact directly with this region of U2, this indicates that B and B' most likely bind to the U2 snRNP through protein-protein, rather than protein-RNA, interactions. This will be discussed further below.

Binding requirements of A' and B'' on U2 snRNA

A previous analysis of binding both common U snRNP proteins and U2 snRNA-specific proteins (Mattaj and De Robertis, 1985), could not distinguish which of the two U2 snRNP proteins A' and B'' were being studied. The monospecific anti-A' and anti-B'' antibodies (described above) were therefore used to further the analysis of U2 snRNP structure.

The results presented in Figure 3 represent immunoprecipitated RNAs from control oocytes or from oocytes injected with either wild-type or a mutant U2 gene. The antibodies used are either whole V26 serum or monospecific antibodies prepared by elution from HeLa protein immunoblots. It is important to note that

the control used is serum eluted from part of the immunoblot showing no specific staining. In other words it shows the small but detectable amount of V26 serum sticking non-specifically to the nitrocellulose (Figure 3, compare panels 1 and 2).

The RNAs precipitable by V26 serum are shown in Figure 3, panel 2. Endogenous U1 and U2 RNA are precipitated, as are wild-type U2, and RNAs transcribed from the U2 mutants ΔA and ΔB . RNAs transcribed from mutants $\Delta C - \Delta E$ are not immunoprecipitated by this serum. This result is identical to that previously obtained using another anti-(U1,U2)RNP serum called Pick (Mattaj and De Robertis, 1985) or P21 (Habets *et al.*, 1985a) which apparently recognised protein B'' but not A' (Habets *et al.*, 1985a). To test which parts of the U2 sequence were specifically required for binding A' and B'' we next tested these two monospecific antibodies separately. The results (Figure 3, panels 3 and 4) show that both these proteins have the same structural requirements for binding to U2. They require the binding site for the Sm antigens, deleted in ΔC (Mattaj and De Robertis, 1985) and the sequences 3' to this site deleted in ΔD and ΔE . As expected from their immunological cross-reactivity, monospecific anti-A and anti-B'' antibodies behaved identically in these assays (not shown).

The structural information gleaned from these results is incorporated into the schematic model of U2 snRNP structure presented in Figure 5.

Fig. 3. Immunoprecipitation of mutant and wild-type U2 snRNAs with anti-(U1,U2)RNP and monospecific antibodies. *Xenopus* oocytes were microinjected with [α - 32 P]GTP alone (lane G) or in combination with either wild-type U2 DNA (lane U2) or one of the mutant U2 DNAs ΔA - ΔE (lanes A-E). Extracts of oocytes were then immunoprecipitated with either whole V26 serum or with affinity-purified monospecific antibodies to 70 K, A' or B''. These were purified by elution of antibodies from immunoblots of HeLa nuclear proteins (Habets *et al.*, 1985a). As a control non-specifically sticking antibodies were eluted from a blank region of the immunoblot. **Panel 1**, control elution; **panel 3**, whole V26 serum, mock-eluted, endogenous U2 and U1 are indicated, **panel 3**, anti-A', endogenous U2 is indicated; **panel 4**, anti-B'', endogenous U2 is indicated; **panel 5**, anti-70 K, endogenous U1 is indicated. No endogenous U1 is detectable in anti-B'' [or anti-A (not shown)] immunoprecipitates in spite of the cross-reaction of these antibodies with A. This is probably due to the antibodies having a lower affinity for A than for B''.

Fig. 4. Immunoprecipitation of U1 RNA. *Xenopus* oocytes were injected either with [α - 32 P]GTP alone or together with DNA of the clone X.1.U1.3. Total RNA extracted from these oocytes is shown in panel A. Lane 1; GTP injection, lane 2; X.1.U1.3 injection. Extracts of the injected oocytes were then immunoprecipitated with monospecific anti-A' or anti-70 K antibodies eluted from immunoblots of HeLa nuclear extracts or with mock-eluted whole V26 serum or non-specifically sticking serum from a blank region of the immunoblot. Panel B, GTP-injected oocytes; panel C, X.1.U1.3 injected oocytes. Lanes 1; control elution, lanes 2; whole V26, lanes 3; anti-70 K, lanes 4; anti-A'.

Interaction between U1 and U2 snRNPs

An unexpected result was that monospecific anti-70 K antibodies immunoprecipitated not only endogenous U1 but also a significant amount of wild-type U2 RNA from oocytes injected with the wild-type gene (Figure 3, panel 5). That this immunoprecipitation of U2 is not due to unspecific binding is shown by comparison of control and anti-70 K precipitations (Figure 3, panels 1 and 5). The amount of mutants ΔA and ΔB precipitated by non-specifically bound V26 antiserum and by monospecific anti-70 K is similar. The amount of wild-type U2 precipitated by the anti-70 K antibodies is clearly above the control value, and indicates an indirect precipitation of wild-type U2 snRNPs through interaction with U1 snRNPs. We are able to detect this interaction due to the very high specific activity of U2 snRNA in oocytes injected with U2 genes. The specific activity of the endogenous RNA does not allow this detection (Figure 3, panel 5, lane G). The fact that none of the mutant U2 snRNAs is precipitable with anti-70 K antibodies indicates that all of the changes to U2 snRNA structure made in mutants $\Delta A - \Delta E$, and the resulting changes in the composition of the U2 snRNPs, prevent interaction with U1 snRNPs.

As a further check on the interaction between U1 and U2 we carried out the reciprocal experiment. X.1.U1.3, a minor U1 snRNA gene from *Xenopus* whose structure and sequence have been described (Zeller *et al.*, 1984; Mattaj *et al.*, 1985) was injected into oocytes in order to obtain high specific activity U1 snRNA (Figure 4, panel A). Monospecific antibodies were then used to analyse immunoprecipitable RNAs in control and injected oocytes.

Fig. 5. Schematic representation of the U2 snRNP. See text for discussion. The U2 snRNA secondary structure is based on data from Reddy *et al.* (1981), Branlant *et al.*, (1982), and Black *et al.* (1985). The shaded blocks represent the sequences altered in mutants $\Delta A - \Delta E$ (Mattaj and De Robertis, 1985).

The results from control oocytes show the expected pattern (Figure 4B). Whole V26 (lane 2) precipitates both U1 and U2, anti-70 K only U1 detectably (lane 3), and anti-A' only U2 detectably (lane 4). Anti-A and anti-B' precipitate, as expected, both U1 and U2 (not shown). When the specific activity of U1 is increased by gene injection, the immunoprecipitation pattern observed is that given in Figure 4, panel C). V26 precipitates endogenous U2 plus U1 (lane 2), anti-70 K only detectably U1 (lane 3), while anti-A' precipitates endogenous U2 and a small amount of U1 (lane 4). Comparison with the control (lane 1) indicates that the U1 precipitation is significant, arising from interaction with U2 snRNP rather than non-specific contamination with whole V26 serum.

This result, together with those presented above, suggests that a small fraction of U1 and U2 snRNPs exist in a co-precipitable form. The possible basis of this interaction will be discussed below.

Discussion

The results presented here, together with previous results obtained by the same method (Mattaj and De Robertis, 1985) can be combined with results from micrococcal nuclease digestion experiments in which the regions of U2 snRNA protected against nuclease in U2 snRNPs were determined (Liautard *et al.*, 1982; Reveillaud *et al.*, 1985) and results obtained from the study of assembly of U snRNP particles *in vivo* (Fisher *et al.*, 1985). In this way a consistent model of RNA-protein interactions in the U2 snRNP particles can be obtained. This is shown schematically in Figure 5. Proteins D, E, F and G, which associate with one another in the absence of RNA to form a 6S particle (Fisher *et al.*, 1985), bind to and protect a region near the centre of the U2 RNA molecule from micrococcal nuclease digestion (Liautard *et al.*, 1982). This region is required in order that the RNA be precipitated by anti-Sm antibodies which recognise proteins B, B' and D (Mattaj and De Robertis, 1985) or B and B' alone (this paper). The logical conclusion is that one or more of the proteins D, E, F and G bind directly to this region of U2, and that B and B' are attached to this particle through protein-protein interactions.

The U2 snRNP-specific proteins A' and B'' bind only to wild-type U2 and mutants ΔA and ΔB . In other words they require for binding not only that the Sm binding site be present, but they also require RNA structure 3' to this. They therefore appear to interact both with RNA in the 3' half of the U2 RNA molecule directly and with the proteins bound to the Sm binding site. The interaction of these proteins with the 3' half of the U2 RNA molecule is likely to be the cause of the protection of this region against micrococcal nuclease digestion of U2 snRNPs observed by Reveillaud *et al.* (1985). The results are combined in the model of U2 snRNP structure shown in Figure 5. The model has of course several simplifying assumptions, of which perhaps the most unjustified is that the proteins are all present in all particles in equal molar ratio. It may well be that several different U2 snRNPs exist which differ in their protein composition, and thus are structurally, or even functionally, distinct. For example in any given U2 snRNP only protein A' or B'' might be present.

The interaction of U1 and U2 snRNPs is of interest given that both of these RNAs are involved in the removal of introns from mRNA precursors in splicing (Hernandez and Keller, 1983; Padgett *et al.*, 1983; Bozzoni *et al.*, 1984; Krämer *et al.*, 1984; Krainer and Maniatis, 1985; Black *et al.*, 1985). This process occurs in a particle which has been called the spliceosome (Brody and Abelson, 1985; Grabowski *et al.*, 1985; Frendewey and Keller, 1985). The interaction which we see is, however, unlikely to represent the immunoprecipitation of splicing complexes since our immunoprecipitations are carried out in 0.5 M NaCl. At this ionic strength spliceosomes are at least partially dissociated (Grabowski *et al.*, 1985), and in fact earlier studies of U1 interaction with hnRNPs showed that U1 dissociates from hnRNP at ~0.15 M NaCl (Zieve and Penman, 1981). It is therefore more likely that we are studying a direct U1–U2 snRNP interaction, although this may well represent a sub-component of the spliceosome. It is interesting that none of the mutant U2 snRNAs is capable of forming an RNP which can interact with U1 RNP. This indicates that the interaction is not a simple one involving one binding site, but that it requires structural integrity of the U2 RNP. It will be of interest to study the entry of the mutant U2 snRNAs into splicing complexes, which may reveal whether the U1–U2 interaction is a step along the pathway to spliceosome formation.

Materials and methods

Immunoblotting and preparation of monospecific antisera

Whole oocyte extracts (Fritz *et al.*, 1984), HeLa nuclear extracts (Habets *et al.*, 1985a, 1985b) and oocyte U snRNPs purified by affinity chromatography on anti-trimethyl cap antibody columns (Bringmann *et al.*, 1983, 1984) were analysed as described (Habets *et al.*, 1985a). Monospecific antisera were eluted (Smith and Fischer, 1984) from high resolution immunoblots of HeLa nuclear extract (Habets *et al.*, 1985a, 1985b).

Oocyte microinjection and immunoprecipitation

Oocytes were injected, fractionated and extracted, and the extracted RNAs were analysed as described in Mattaj and De Robertis (1985).

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