Structure of the Small Nuclear RNP Particle U1: Identification of the Two Structural Protuberances with RNP-Antigens A and 70K

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Abstract. We have investigated the structure of the small nuclear RNP (snRNP) U1 by combining EM of complete and partially protein-deficient particles with immunoelectron microscopy employing mAbs against known components of the U1 snRNP. It was found that the two main protuberances of this particle can be identified with the U1-specific proteins A and 70K. The 70K protuberance is the one lying closer to the 5′ terminus of the snRNA, as identified by its 5′-terminal m3G cap. The round-shaped main body of U1 snRNP represents its core RNP domain containing the common snRNP proteins. Functional implications of these results are discussed. Our results may also point to the physical basis for the production of autoantibodies directed against specific groups of snRNP proteins. The physical grouping of the common proteins (Sm epitopes) and the specific proteins (RNP epitopes) could result in one or the other being presented to the immune system as is the case in patients suffering from SLE or MCTD, respectively.

U1 small nuclear RNP (snRNP) is one of the major snRNPs; the others are U2, U4/U6, and U5. All of these snRNPs are essential factors in the splicing of pre-mRNA; during the splicing process, several snRNPs unite to form the so-called spliceosome particle (for review see 24 and 41). Mammalian U1 snRNP consists of a single m3G-capped 165-nucleotide-long RNA molecule and at least 11 proteins. Eight of these (B′, B, D_1, D_2, D_3, E, F, and G) are common to all the snRNPs, while the proteins 70K, A, and C are contained only in the U1 particle (for review see 23).

The main task of the snRNPs is the recognition and removal of introns in the pre-RNA molecule. U1 snRNP plays an essential part in the recognition of the 5′ splice site, as the 5′ end of the RNA molecule of U1 base pairs with the consensus sequence at the 5′ splice site (38, 47). The proteins of U1 snRNP, especially protein C, are also involved in this recognition (16), and the other two U1-specific proteins A and 70K are also essential for the splicing function of U1 snRNP (12), although it is still unclear what role that they play. Apart from the splice site, the U1 snRNP also appears to recognize, directly or indirectly, a region of the intron in the neighborhood of the branch point. This was first observed in yeast (33, 36, 37) and has recently also been shown for the mammalian system (2).

The common proteins play an important part in the morphogenesis of the U1 snRNP and the other m_3G-capped snRNPs. Their binding to the U1 RNA in the cytoplasm is essential for the trimethylation of the 5′-terminal cap (25) and for the transport of the U1 snRNPs into the cell nucleus (9, 11). It is still unknown to what extent the common proteins are involved in the splicing reaction itself.

Experiments utilizing RNase digestion, chemical modification, and reconstitution assays in vivo and in vitro have revealed much concerning the interaction between RNA and proteins in the U1 snRNP. The common proteins, some in the form of a pre-formed complex, bind to a conserved sequence motif of the U1 snRNA. This motif, termed domain A, is also contained in the snRNAs U2, U4, and U5; it consists of a single-stranded region that is rich in U bases and is flanked by two hairpin loops (stem/loops III and IV) and occurs in the 3′ half of U1 snRNA (5, 20). The specific proteins 70K and A each possess a binding site on the U1 snRNA. Protein 70K binds primarily to the 5′ cap proximal hairpin loop I and protein A to hairpin loop II (1, 22, 27, 28, 30, 34). The binding site of protein C on U1 snRNP has not yet been determined; it is probable that this protein does not bind directly to the U1 RNA. In reconstitution experiments on oocyte extracts from *Xenopus laevis*, the binding of proteins A, 70K, and C was found to be cooperative (13, 14).

Interest in the RNP proteins of U1 is not restricted to their cell and molecular biology, as they have important clinical and immunological aspects. Patients suffering from inflammatory rheumatic disorders, such as systemic lupus erythematosus (SLE) or mixed connective tissue disease (MCTD), frequently produce antibodies against U1 snRNP proteins (reviewed in 43). The presence of antibodies against the common proteins, in particular B′, B, D_1, and D_3 (the Sm antigens) is regarded as diagnostic of SLE, and autoantibodies against the U1-specific proteins A, C, and especially 70K (RNP antigens) are diagnostic of MCTD. The cause of the production of the autoantibodies in such patients is largely unexplained. A variety of immunological abnormalities, such as B- and T-cell hyperreactivity, has been described for human SLE as well as for SLE-prone mice (45). However, it is becoming increasingly clear that the production of anti-

snRNP autoantibodies is antigen dependent (31, 43). This raises the perplexing question of why autoantibodies are produced only against 70K, A, and C in the one condition (MCTD) and against B'/B and D_1-D_3 in the other (SLE). A possible explanation is that the two antigen groups are distributed in a systematic, perhaps a polar, manner on the surface of the U1 snRNP particle, which could lead to the one or the other group being exposed to the immune system and eliciting an immune response.

We have recently examined the U1 snRNP particle by EM (17). Two morphologically distinct domain types were seen. One was a round main body, ~8 nm in diameter; the other consisted of two protuberances. Here we describe attempts to assign the U1 snRNP proteins to their morphological domains. For this purpose we used two different methods. First of all, we examined U1 snRNPs that were deficient in one or more of the U1-specific proteins 70K, A, and C. Secondly, we made use of immunoelectron microscopy with mAbs to define the position of the A and 70K proteins at the surface of U1 snRNP. Our results demonstrate that the two protuberances contain the A and the 70K protein, respectively, and that the round-shaped major domain represents the core RNP structure of U1 snRNP containing all the common proteins.

Materials and Methods

Preparation of U1 snRNPs

U1 snRNPs lacking either (*a*) the C protein, (*b*) both proteins C and A, or (*c*) all three specific proteins, C, A, and 70K, were isolated from HeLa cells as described in detail elsewhere (1). In summary, nuclear extracts were prepared according to Dignam et al. (8), and affinity chromatography with an anti-m_3G column and competitive elution by m^7G (3) was used to obtain a mixture of snRNPs U1 to U6. Pure U1 snRNPs containing the full set of specific proteins were isolated from this mixture by Mono Q chromatography at 4°C, while chromatography at elevated temperatures (20°C or 37°C) allowed the isolation of the protein-deficient U1 snRNPs (1). The identities of the snRNA and the protein constituents were determined by electrophoresis in 12% polyacrylamide gels as described by Bringmann et al. (6).

Chemical Cross-linking of U1 snRNPs

U1 snRNPs were purified in cross-linking buffer (20 mM triethanolamine at pH 8.5, 300 mM KCl, 1.5 mM $MgCl_2$) by 5–20% glycerol gradient centrifugation. Pooled fractions containing U1 snRNP (~140 μg protein per ml) were cross-linked with dithio(bis[succinimidyl]) propionate (DSP) by the following method, which follows Lamont and Fairbanks (21) and Walleczek et al. (46). A solution of DSP (Sigma Chemical Co., St. Louis, MO), 18 mM in DMSO was added slowly to the U1 snRNPs until the desired final DSP concentration (60 μM unless otherwise stated) was reached. After incubation at 0°C for 30 min, the reaction was terminated by the addition of glycinamide hydrochloride (3 M, pH 4.0, to give a final concentration of 50 mM) and a further incubation at 37°C for 40 min.

Immunocomplex Formation

Immunocomplexes were prepared with IgG purified from preparations of three mAbs with respective specificities for the A protein, the 70K protein, and the m_3G cap. The antibodies specific for A protein (D_5) and for m_3G (H20) have been described previously (3, 32). The 70K-specific mAb H111 was purified from the supernatant of the hybridoma cell line H111, derived originally from a C57Bl/6 mouse immunized with HeLa U1 snRNPs (Daser, A., R. Reuter, and R. Lührmann, unpublished results). The antibodies were incubated with U1 snRNPs overnight at 4°C; the U1 snRNPs used were either complete or else deficient in C or A protein. Both DSP-cross-linked U1 snRNPs and non-cross-linked U1 snRNPs were used. In a further experiment, the H111 antibody was preincubated (1 h, 4°C) with the pEE71 fusion protein before U1 snRNPs were added. This fusion protein, expressed in *E. coli*, contains the 33-kD NH_2-terminal part of the 70K protein fused to β-galactosidase (Hornig, H., K. Bark, A. Heyer, and R. Lührmann, unpublished observations).

Gradient Centrifugation

Immunocomplexes were separated from unreacted U1 snRNPs and antibodies by sucrose-gradient centrifugation (6–20% sucrose in cross-linking buffer) in a Beckman TLS55 rotor for 6 h at 50,000 rpm and 4°C. Gradients were fractionated from the bottom up, and aliquots of each fraction were analyzed for their snRNP and IgG content by microtitre ELISA as described previously (17). In summary, the cavities were coated with the sample and then saturated with BSA. The amount of U1 snRNP in the samples was then determined by reaction with a human anti-RNP serum followed by detection of the bound serum antibody complexes by an anti-(human IgG) antibody conjugated with phosphatase (Sigma Chemical Co.). The amount of mouse mAb in the samples was determined on a second ELISA plate by using a phosphatase-conjugated anti-(mouse IgG) antibody (Paesel).

EM

Negative staining with 2.5% uranyl formate was carried out by the double carbon film method as described by Kastner and Lührmann (17). The preparations were examined under a Zeiss EM109 electron microscope with an acceleration voltage of 80 kV, and electron micrographs were taken with magnification factors between 85,000 and 140,000.

Results

Specificity of the Antibodies

For the location of the proteins A and 70K on the U1 snRNP by immunoelectron microscopy, monoclonal IgG antibodies against these proteins were used. The antibody specific for the A protein (D5) has already been described in detail by Reuter et al. (32). On an immunoblot of U1 snRNP proteins, it reacts with the A protein only, and it does not react with protein-free U1 RNA (Fig. 1 *A*, lane *2*). In immunoblots of nuclear extracts and total snRNP proteins, antibody D5 also reacts with protein B″, which is present as a specific protein in U2 snRNP (32). Since in this work purified U1 snRNPs were used, the cross-reaction with B″ has no significance here.

The 70K-specific antibody H111 was obtained by immunizing mice with purified HeLa snRNPs. When allowed to react in immunoblot tests with mixtures of U1 snRNP proteins, either total U1 protein (Fig. 1 *A*, lane *3*) or proteins from nuclear extracts (not shown), this antibody reacts with the 70K protein only (7).

Chemical Cross-linking of Proteins A and 70K to U1 snRNP

The electron-microscopic investigation and subsequent location of the antibody-binding sites call for stable immune complexes that withstand the purification steps and the preparation for EM. A good measure of this is their stability during gradient centrifugation, which was used for the separation of immunocomplexes from unbound antibodies and U1 snRNPs (17). However, first experiments with the anti-A and anti-70K antibodies showed that none of the anti-A and at most only a small proportion of the anti-70K immunocomplexes survived the centrifugation. Investigation of the gradient fractions showed that it was not the antibody that dissociated from the antigen, but rather the antigen (A or 70K protein) that dissociated from the U1 snRNP after the antibody had been bound (data not shown). In an attempt to pre-

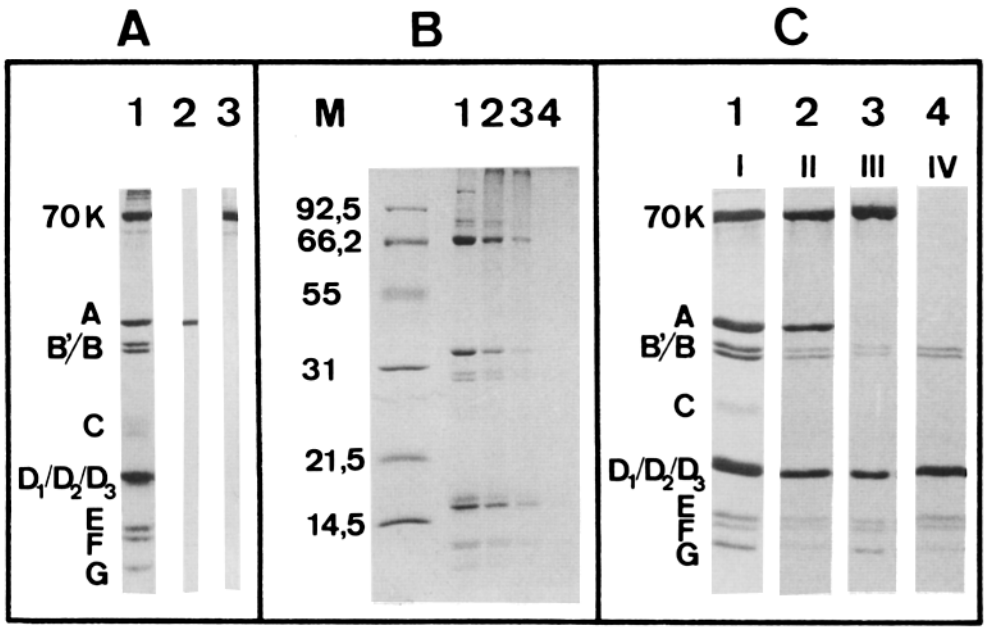

Figure 1. Protein analysis and immunoblots of U1 snRNPs using the mAbs D5 (against A protein) and H111 (against 70K). (*A*) Immunoblots with D5 and H111. U1 snRNPs proteins were separated by SDS-PAGE and immunoblotted with the A-protein-specific mAb D5 (lane *2*) or the 70K-protein-specific mAb H111 (lane *3*) by the method of Habets et al. (10). The Coomassie-stained U1 snRNP proteins are shown in lane 1. (*B*) SDS-PAGE of U1 snRNPs cross-linked with DSP. Purified U1 snRNPs were incubated without DSP (lane *1*), or with DSP at a concentration of 20 μM (lane *2*), 60 μM (lane *3*), or 180 μM (lane *4*). The proteins were then extracted and separated by electrophoresis. Proteins were made visible by Coomassie staining. *M*, Protein molecular mass standards are in kilodaltons. (*C*) Protein composition of U1 snRNPs gradually depleted of proteins C, A, and 70K. U1 snRNPs containing their full set of specific proteins (lane *1*), or lacking the C protein (lane *2*), both the C and the A proteins (lane *3*), or all three specific proteins A, C, and 70K (lane *4*) were analyzed by SDS-PAGE. The proteins were made visible by Coomassie staining. The Roman numerals refer to the corresponding panel in Fig. 6.

vent the loss of these two proteins from the RNP complex during the experiment, they were cross-linked covalently to the snRNP.

For this purpose, U1 snRNPs were incubated with various concentrations of the amino group–specific cross-linking reagent DSP, which possesses a cleavable disulphide bridge. The subsequent protein analysis of the U1 snRNP showed a decrease in intensity of the protein bands that increased with the quantity of DSP used (Fig. 1 *B*). At 180 μM DSP, the cross-linking is so extensive that protein bands can no longer be seen. The extended cross-linking presumably gives rise to protein complexes with such high molecular weight that they no longer can migrate into the gel (Fig. 1 *B*, lane *4*). However, the chemical nature of the bound molecules does not appear to change significantly, as shown by cleavage of the cross-link with mercaptoethanol, after which the bands of the U1 snRNP proteins again appear in their usual position. Only the migration rate of the D proteins retains a small alteration (not shown).

The reduction of the bands' intensity suggests that a sufficient degree of cross-linking of proteins 70K and A to the rest of the U1 snRNP is attained at a DSP concentration of 60 μM (Fig. 1 *B*, lane *3*). At this DSP concentration, the immunoreactivity of the U1 snRNPs with the A- and 70K-specific antibodies is only slightly reduced (analysis by ELISA; data not shown), and there is hardly any appearance of interparticulate cross-linking (analysis by density gradient centrifugation; data not shown); for these reasons the risk of artefacts of cross-linking may be neglected.

U1 snRNPs cross-linked with 60 μM DSP were examined under the electron microscope. Fig. 2 shows a selection of negatively contrasted cross-linked U1 snRNPs. The size, shape, and fine structure of these particles corresponded to those of untreated U1 snRNPs (17): globular main bodies, accumulation of stain at the center, and shapes with two adjacent protuberances can be seen clearly. We conclude from this that the introduction of the cross-linking reagent has not led to any significant change in the shapes of the particles. As previously discussed (17), the observed size variability of the protuberances might be due to differences in the orientation of the particle upon absorption to the carbon film.

Labeling of Cross-linked U1 snRNPs with U1-A-Protein-specific Antibodies

U1 snRNPs, cross-linked at a DSP concentration of 60 μM, were incubated with the antibody D5, which is specific for A protein. The immunocomplexes formed were separated from unbound U1 snRNPs and IgG molecules by gradient centrifugation. Fractions from the gradient were then analyzed by microtitre ELISA for their content of U1 snRNPs

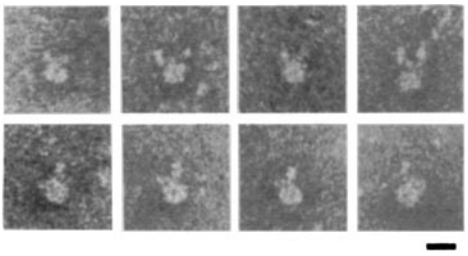

Figure 2. EM of U1 snRNPs cross-linked with DSP and negatively stained with uranyl formate. Selected micrographs of U1 snRNPs cross-linked with 60 μM DSP are shown. The first row shows images with roughly symmetrical protuberances, and the second row shows images in which one protuberance appears to predominate. The micrographs are oriented so that the protuberances point upwards. Bar, 10 nm.

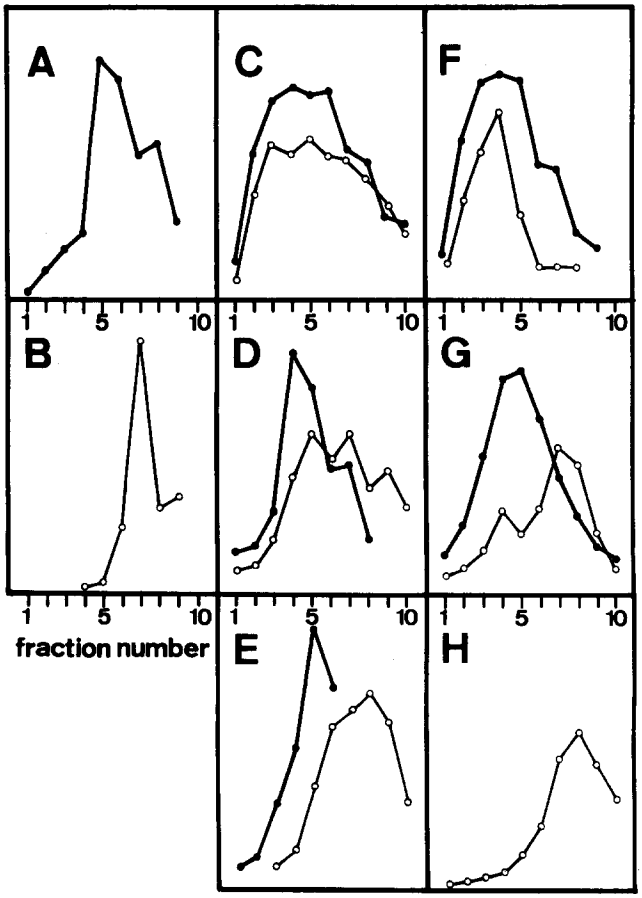

Figure 3. Immunocomplex formation monitored by density-gradient centrifugation. Incubation mixtures of U1 snRNP preparations and the A-protein-specific (*C–E*) or the 70K-protein-specific (*F–H*) antibody were centrifuged in a 6–20% sucrose gradient. The gradients were separated in 120-μl fractions from the bottom up. Direction of sedimentation was to the left. Aliquots of these fractions were then tested by the ELISA method, either (*a*) for their content of snRNP, with an anti-RNP serum followed by secondary antihuman antibody conjugated with alkaline phosphatase (*solid circles*), or (*b*) for their content of mouse anti-m3G antibody with an anti-(mouse IgG) antibody conjugated with phosphatase (*open circles*). (*First column*) in *A* only DSP-cross-linked U1 snRNPs and in *B* only IgG antibodies (*D5*) were loaded onto the gradient. These therefore indicate the positions to which the isolated U1 snRNPs and the IgG molecules sediment. (*Second column*) 1.3 μg of the A-protein-specific antibody (*D5*) was incubated with 3 μg of DSP-cross-linked U1 snRNPs (*C*), non-cross-linked U1 snRNPs (*D*) or DSP-cross-linked Δ[C,A]-U1 snRNPs (*E*), which lack the proteins C and A. (*Third column*) 1.6 μg of the 70K-protein-specific antibody (H111) was incubated with 3 μg of DSP-cross-linked (*F*) or non-cross-linked (*G* and *H*) U1 snRNPs before centrifugation. In *H*, the H111 antibody was first incubated with 0.3 μg of fusion protein pEE71 before U1 snRNPs were added. This protein contains the NH_2-terminal domain of the 70K protein fused to β-galactosidase. In this experiment, quantification of the U1 snRNP was not possible by the ELISA method used because of the strong cross-reaction of the anti-RNP serum used for detection of the U1 snRNPs with the fusion protein.

and IgG antibodies. In Fig. 3 (*A* and *B*), the sedimentation behavior of free U1 snRNPs and IgG antibodies in a 6–20% sucrose gradient is shown. The snRNPs (fractions 5 and 6) sedimented one to two fractions ahead of the IgG molecules (fraction 7).

Fig. 3 *C* shows the distribution of snRNPs and antibodies in the fractions after centrifugation of the incubation mixture of cross-linked U1 snRNPs with the A-protein-specific antibody D5. A shift of both the U1 snRNPs and the anti-A antibodies towards higher S values is seen clearly. This shows that most of the cross-linked U1 snRNPs and anti-A antibodies sediment as immunocomplexes. The cross-linking of the A protein to the rest of the snRNP thus overcame the problem of the instability of the immunocomplexes.

If the U1 snRNPs had not been pretreated by DSP cross-linking, stable immunocomplexes were not observed (Fig. 3 *D*). The slight shift of the U1 snRNPs and the antibodies toward higher S values suggests that immunocomplexes were formed in the incubation but dissociated during the centrifugation. That the immunocomplex formation in the case of the cross-linked U1 snRNPs indeed took place via the binding of the D5 antibody to the A protein was concluded from the following experiment. The A-protein-specific antibody D5 was incubated, under the same conditions as before, with cross-linked U1 snRNPs that lack the proteins C and A (Δ[C,A]-U1 snRNPs; see Materials and Methods). No immunocomplexes were observed on the density gradient (Fig. 3 *E*). The absence of the C protein was not responsible for the failure to react with anti-A antibodies, as the D5 antibodies showed just as little reactivity in an ELISA assay with U1 snRNPs that lack protein A only as they do with U1 snRNPs that lack both A and C; however, it reacted with complete particles, i.e., particles that contain A protein (data not shown).

For EM of immunocomplexes between anti-A antibody and cross-linked U1 snRNPs, negatively contrasted preparations of fraction 4 from the gradient centrifugation (Fig. 3 *C*) were made. Fig. 4 *A* shows a general view of these immunocomplexes, in which free antibodies, free U1 snRNPs and U1 snRNP-immunocomplexes can all be recognized. In all the immunocomplexes, the antibody is seen bound to a protuberance of the U1 snRNP. Fig. 4 *B* shows a selection of typical immunocomplexes at higher magnification. In the 143 immunocomplexes examined in detail, about one-half of the U1 snRNPs appeared in a projection that allowed two protuberances to be recognized clearly (Fig. 4 *B*). The majority of the remaining immunocomplexes allowed only one protuberance to be recognized. The antibody-binding site was always located on a protuberance. The simultaneous binding of two antibodies, one at each protuberance, or two at one protuberance, was never observed.

Labeling of Cross-linked U1 snRNPs with 70K-Protein-specific Antibodies

U1 snRNPs cross-linked with 60 μM DSP were incubated together with the 70K-protein-specific antibody H111 and centrifuged in a sucrose gradient. The fractions were then analyzed by microtitre-ELISA for their content of U1 snRNP and IgG antibodies. Fig. 3 *F* shows the result: Almost the entire antibody population sedimented considerably more rapidly than free antibodies (compare Fig. 3 *B*). The antibodies also sedimented more rapidly than free U1 snRNPs. This is a clear indication that stable immunocomplexes were formed.

Non-cross-linked U1 snRNPs also formed immunocomplexes with the 70K-specific antibody H111. However, in this case the yield was lower; only a small number of the antibod-

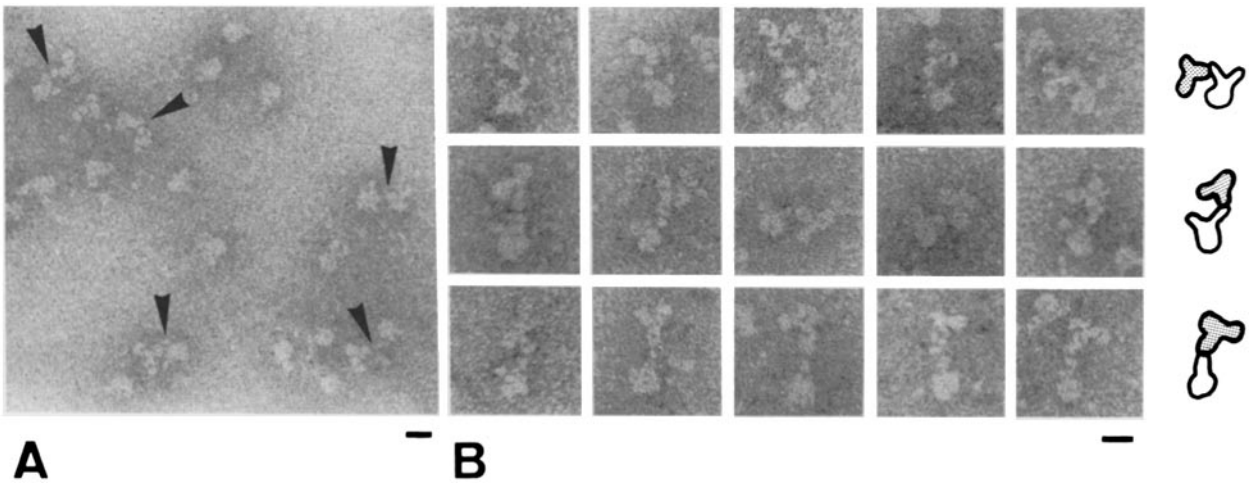

Figure 4. The binding of antibody against protein A to U1 snRNPs. (*A*) General view of the sucrose gradient fraction containing immunocomplexes formed by incubation of DSP-cross-linked U1 snRNPs with the A-protein-specific mAb D5. Arrowheads point to the antibody-binding sites. (*B*) Gallery of selected U1-(anti-A IgG) immunocomplexes. The U1 snRNPs in the complexes are oriented with the protuberances pointing upwards. The complex at the far right of each row is illustrated by an interpretative sketch. The stippled area marks the antibody. Bars, 10 nm.

ies appeared in the immunocomplex fraction (Fig. 3 *G*) in comparison with the experiment using cross-linked U1 snRNPs. If the H111 antibody is preincubated with the β-gal fusion protein pEE71, which carries the H111-reactive epitope (see Materials and Methods), the immunocomplex peak disappears completely (Fig. 3 *H*). This shows that the H111-antibody also recognizes the 70K protein in the intact particle. In agreement with this is the further observation that Δ[C,A,70K]-U1 snRNPs, which lack not only proteins A and C but also 70K (see below), likewise fail to form immunocomplexes (data not shown).

For the electron microscopic location of the binding sites for antibodies, immunocomplexes of the anti-70K antibodies with cross-linked U1 snRNPs (see above) were investigated. After the centrifugation, on a gradient like that in Fig. 3 *F*, negatively contrasted preparations were made from fraction 4. Fig. 5 *A* shows a general view in which a number of immunocomplexes can be recognized. Again, without exception, the antibody-binding sites are all located on one of the protuberances. Fig. 5 *B* shows a selection of typical immunocomplexes at higher magnification. In the first three rows, both protuberances can be seen on the labeled U1 snRNP; this was the case for more than half of the 149 complexes examined in detail. The antibody always binds to one of the protuberances. The simultaneous binding of two antibodies, to both protuberances, or to one protuberance, was never observed.

The results of the two experiments involving labeling of U1 snRNPs with the antibodies against the A and 70K proteins showed that both the A and the 70K proteins are located in the U1 snRNP protuberances (Figs. 4 and 5). However, such experiments cannot reveal whether these proteins lie together in the same or in two separate protuberances. It also remains an open question whether the existence of the protuberances is attributable solely to the presence of these proteins. To study these questions, we investigated the shapes of protein-deficient U1 snRNPs under the electron microscope.

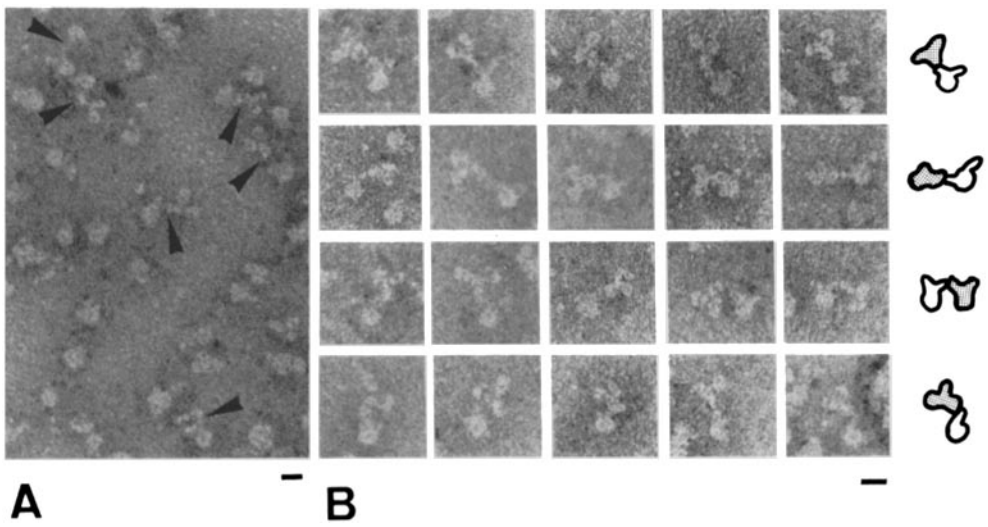

Figure 5. The binding of antibody against 70K protein to U1 snRNPs. (*A*) General view of a sucrose gradient fraction containing immunocomplexes formed by incubation of DSP-cross-linked U1 snRNPs with the 70K-specific mAb D5. Arrowheads point to the antibody-binding sites. (*B*) Gallery of selected U1-(anti-70K IgG) immunocomplexes. The U1 snRNPs in the complexes are oriented with the protuberances pointing upwards. The complex at the far right of each row is illustrated by an interpretative sketch. The stippled area marks the antibody. Bars, 10 nm.

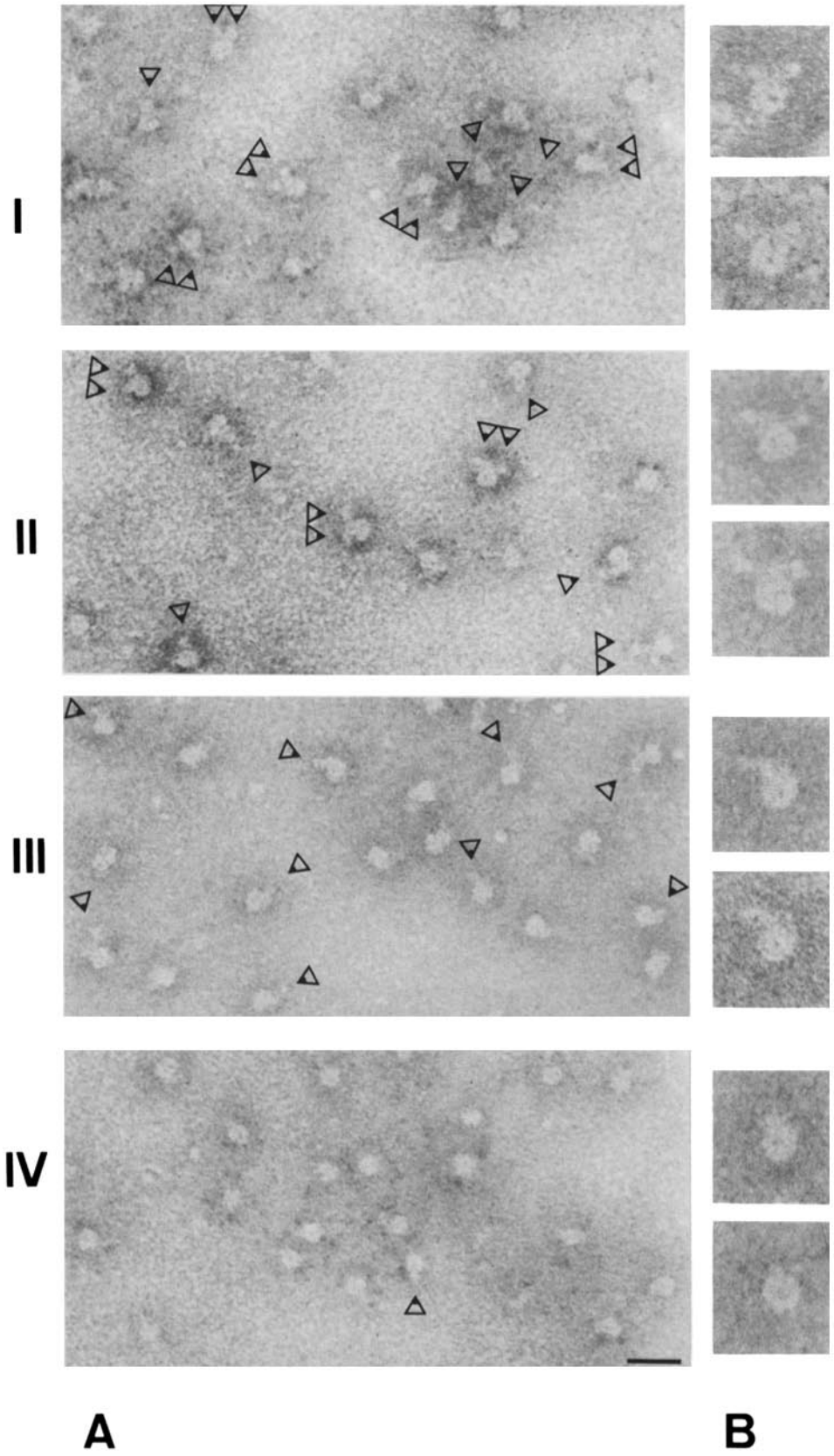

Figure 6. U1 snRNPs gradually depleted of their specific proteins. General views of negatively stained preparations (*A*) and selected images (*B*) of complete (*I*) and U1 snRNPs lacking protein C (*II*), both proteins C and A (*III*), or all three specific proteins C, A, and 70K (*IV*). In *A* protuberances are labeled with triangles. Bar, 20 nm. In *B* images are selected which show a high degree of structural similarity even if protuberances are missing.

EM of Protein-depleted U1 snRNPs

It has recently been shown that U1 snRNPs can be depleted gradually of their specific proteins C, A, and 70K by ion-exchange chromatography using Mono Q columns at elevated temperatures (1). With this method, U1 snRNPs selectively depleted of protein C (ΔC-U1), protein A (ΔA-U1), both proteins C and A (Δ[C,A]-U1), or of all three U1-specific proteins, C, A, and 70K (Δ[C,A,70K]-U1) can be isolated. The three U1-snRNP species Δ[C], Δ[C,A], and Δ[C,A,70K] (see Fig. 1 *C* for protein analysis) were purified in sufficient amounts to study their morphology by EM. Fig. 6 *A* shows general views of negatively stained preparations of each of the protein-deficient U1 snRNPs and, as well, complete U1 snRNPs containing the full set of particle-specific proteins. In all four preparations, the images contain a globular structure ~8 nm in diameter, similar to the main body described previously (17, 18). But there are substantial differences in the appearance of the protuberances in the images of the protein-depleted U1 snRNPs. In the preparation of the Δ[C]-U1 snRNPs (Fig. 6 II), the two protuberances can be seen clearly (marked with *double arrows* in Fig. 6), and they appear with about the same frequency as they do in the complete U1 snRNP (see Table I). Furthermore, the images with only one recognizable protuberance (marked by *single arrows* in Fig. 6) are present in the same proportion as in the control (Table I). The Δ[C,A]-U1 snRNPs also show images with one protuberance, but images with two protuberances can only be seen very rarely (Fig. 6 III and Table I). Images of the Δ[C,A,70K]-U1 snRNPs, which lack all specific U1 snRNP proteins, containing only the set of common proteins (Fig. 1 *C*), show virtually nothing but the structure of the globular body; protuberances are hardly visible in these images (Fig. 6 IV, Table I). These experiments demonstrate that the removal of the A protein results in the loss of one protuberance and removal of the 70K protein results in the loss of the other protuberance.

Next we compared representative images selected for a high degree of structural similarity. This was done for each preparation in turn. Typical forms of complete U1 snRNPs (17) were taken as reference and typical images of the protein-deficient particles were selected for forms that appeared similar (except, of course, in respect of the lack of protuberances in Δ[C,A]-U1 and Δ[C,A,70K]-U1 snRNPs). As an example, images similar to the roughly symmetrical U1 snRNP-form are shown in Fig. 6 *B*. Images of the Δ[C]-U1 snRNP particles could be found showing all the typical features of the reference forms. Both the body and the two protuberances appear similar to the corresponding features in the U1 snRNP reference form. Within the resolution of the electron micrographs (~2 nm) no systematic differences between the complete particle and the particle deficient in C protein could be observed. Comparison of typical images of the Δ[C,A]-U1 snRNP with the reference forms showed no structural change beyond the lack of the one protuberance. Comparison of the core RNP structure of Δ[C,A,70K]-U1 snRNPs with that of the other two U1 snRNP species reveals a high structural similarity between the snRNP bodies of the complete and the depleted particles.

We conclude from these experiments that one protuberance of the U1 snRNP does indeed contain the A protein and the other the 70K protein. The binding of each of these proteins to the snRNP seems to be sufficient to generate the structural appearance of the respective protuberance. The location of the C protein could not be determined by visual comparison of negatively stained images of complete U1 snRNPs with those lacking this protein. The failure to detect morphological differences could be due to dissociation of the C protein during sample preparation for EM. To investigate this possibility we compared Δ[C]-U1 snRNPs with cross-linked U1 snRNPs which not only have the 70K and the A proteins efficiently covalently linked (see above), but also the C protein (Fig. 1 *B*). However, no morphological difference could be detected (data not shown), confirming that the removal of the C protein does not result in a detectable structural change in the U1 snRNP particle.

Table I. Number of Protuberances Visible on Images of U1 snRNP Particles Lacking Specific Proteins

Number of protuberances visible*	U1 snRNP particles				
	Complete	ΔC	Δ(C,A)	Δ(C,A,70K)	Cross-linked
2	35	31	4	2	44
1	28	29	31	7	16
0	7	6	15	48	8
X	30	34	50	43	27

* Structures protruding more than 2 nm from the main body of the particle were counted as protuberances. Images with smooth, approximately round outlines are counted as showing no protuberances. If the outline appeared irregular or could not be traced unambiguously, the image was denoted X.

We also compared the morphology of cross-linked and non-cross-linked complete U1 snRNPs. Although qualitatively there were no detectable differences, particles possessing two protuberances were observed with greater frequency with the cross-linked U1 snRNPs (Table I). This indicates that during sample preparation for EM proteins can dissociate from the U1 snRNP. As shown previously by antibody labeling experiments, the relatively weak association of the A protein in particular might be responsible for the less frequent appearance of the two protuberances in the absence of covalent cross-linking.

Positions of the A and 70K Protuberances Relative to the Site of the m_3G Cap

In previous work, we located the m_3G 5′-end of the U1 RNA on the main body of the U1 snRNP, by labeling the m_3G base with m_3G-specific antibodies (17). This site was found to be clearly separated from the base of the two protuberances. To investigate the positions of the protuberances with respect to the cap site, we conducted experiments that allowed simultaneously both the location of the cap site and the assignment of the protuberances. To label the cap site, we used the m_3G-specific monoclonal antibody H20 employed previously (17).

First, we investigated the distance of the cap site from the 70K protuberance. Assignment of the 70K protuberance was made possible by using Δ[C,A]-U1 snRNPs. These particles lack the A protein and therefore have only one protuberance, the one associated with the 70K protein. The m_3G-specific mAb H20 was incubated with Δ[C,A]-U1 snRNPs, and fractions containing the immunocomplex were obtained by gradient centrifugation. Electron micrographs were taken from negatively stained samples. Fig. 7 *A* shows a general view and Fig. 7 *B* a gallery of selected typical complexes. In all cases, the anti-m_3G antibody binds to the main body of the

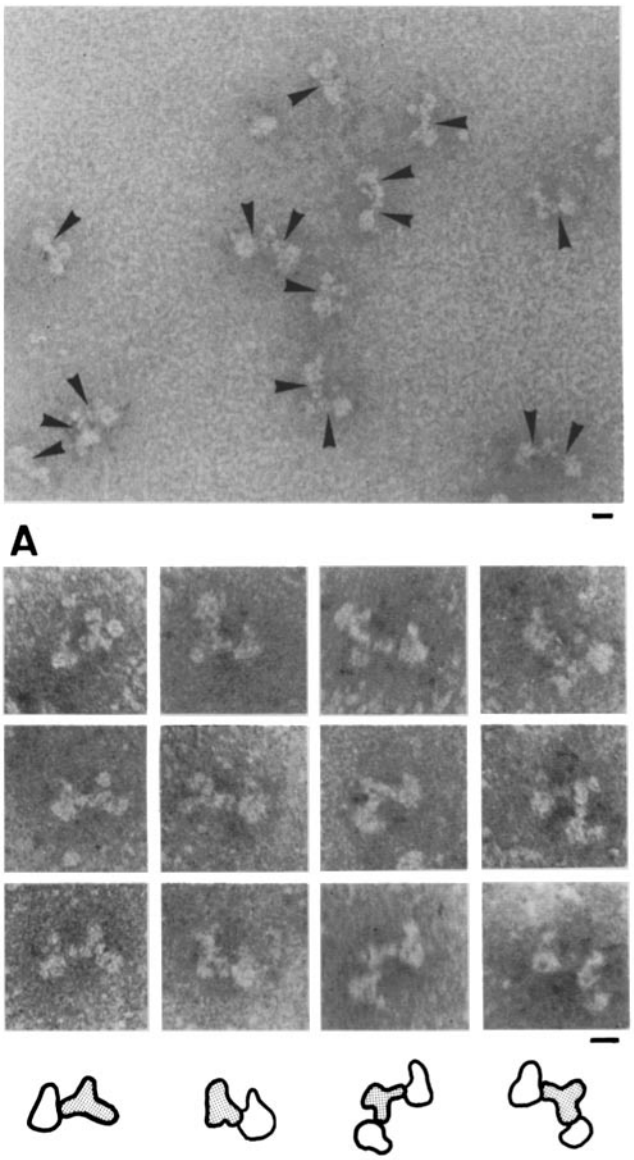

Figure 7. The binding of antibody against m3G to U1 snRNPs lacking both proteins C and A. (*A*) General view of a gradient fraction containing immunocomplexes formed by incubation of Δ[C,A]-U1 snRNPs with the m3G-specific mAb H20. Arrowheads point to the antibody-binding sites. (*B*) Gallery of selected [Δ[C,A]-U1-anti-m3G IgG] and [Δ[C,A]-U1-anti-m3G IgG Δ[C,A]-U1] complexes. The complex at the bottom of each column is illustrated by an interpretative sketch. The stippled area marks the antibody. Bar, 10 nm.

U1 snRNP at a site relatively close to the 70K protuberance. Antibody-binding sites directly opposite the base of the protuberance were not observed. This suggests that the 70K protuberance is located close to the m_3G cap.

In the second experiment, we attempted to investigate the distance of the cap site from the A protuberance. Unfortunately, U1 snRNPs lacking the 70K protein but containing the A protein, so that only the A protuberance is present, have not yet been observed. We therefore pursued a more complex strategy involving antibody double labeling, in order to discriminate between the two protuberances. Complete U1 snRNPs were labeled with two antibodies simultaneously: the anti-m3G antibody to mark the cap site and the anti-A antibody to label the protuberance containing A protein. U1 snRNPs cross-linked with DSP were incubated with both antibodies and, after gradient centrifugation, fractions containing immunocomplexes were examined under the electron microscope. Fig. 8 *A* shows an overview of particles from the fraction in which the greatest number of doubly labeled U1 snRNPs was found. Alongside [U1-IgG] complexes, free IgG molecules and U1 snRNPs, five [IgG-U1-IgG-U1] complexes can be seen.

Several electron micrographs were searched for such immunocomplexes containing at least two antibodies. In most complexes, the typical U1 snRNP and IgG structures can hardly be recognized. Nevertheless, 11 doubly labeled U1 snRNP complexes could be recognized where the antibody-binding sites were found at opposite sites of the U1 snRNP. Fig. 8 *B* shows some examples at higher magnification. Large distances between the m_3G-cap site and the A protuberance are seen, showing that these two sites are well separated in the U1 snRNP particle.

Similar experiments involving double labeling with the anti-m_3G-cap antibody and the anti-70K antibody were also performed. However, here we have so far been unable to detect immunocomplexes for which the U1 snRNP and the two bound antibodies could all be seen clearly. This inability could be the consequence of a relatively close location of the two antibody binding sites, because the closer the two bound antibodies are, the more difficult is an unambiguous recognition of these antibodies.

Discussion

In this work, we have used electron microscopic techniques to investigate the distribution of the proteins in the U1 snRNP particle. Two strategies were used to locate the proteins: (*a*) successive removal of U1-specific proteins and (*b*) labeling with monoclonal IgG antibodies.

Complete U1 snRNPs consist of two types of domain: a globular main body and two protuberances that originate from adjacent sites on the main body. The removal of all three specific proteins, 70K, A, and C, from the U1 snRNP leaves behind a globular structure ~8 nm in diameter. As shown by gel electrophoresis, all the common proteins are contained in this core RNP. The core is very similar in shape to the main body of the complete U1 snRNP particle (see Fig. 6 *B*). It is therefore highly probable that all the common proteins are located in the body. This is in agreement with the results of previous studies of snRNPs U2, U5, and U4/U6; in each of these, a globular structure very similar in size, shape, and fine structure to the main body of U1 snRNP was observed and was found to contain all the common proteins (18, 19). These results suggest that the two characteristic protuberances of U1 snRNP may represent the U1-specific proteins, an idea confirmed in this work both by successive removal of the U1-specific proteins and by labeling with antibodies. While loss of protein C alone did not change to a significant extent the appearance of the U1 RNP as compared with the complete particle, the loss of protein A in addition to protein C resulted in the disappearance of one of the typical protuberances of U1 snRNP. This implies that one protuberance of the U1 RNP may consist principally

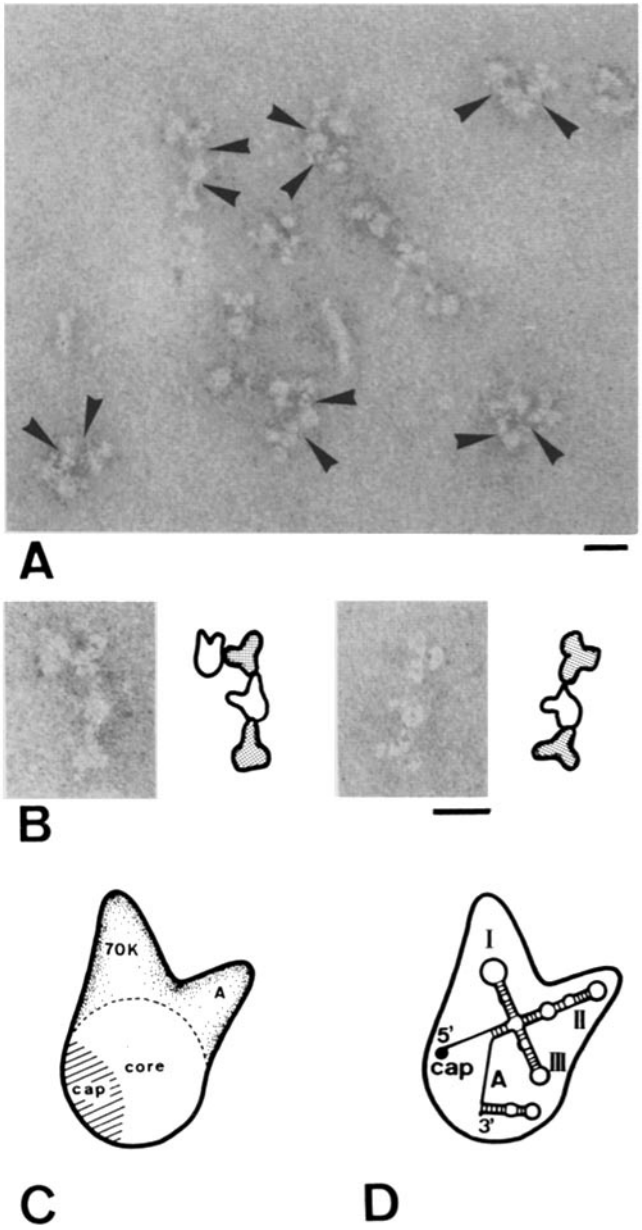

Figure 8. Simultaneous binding of the antibodies against A protein and the m3G cap to DSP-cross-linked U1 snRNPs. (*A*) General view of a gradient fraction containing double antibody-labeled U1 snRNPs. The arrowhead points to the antibody-binding sites at the doubly labeled U1 snRNPs. (*B*) Selected complexes showing double antibody-labeled U1 snRNPs. An interpretative sketch is shown at the right of each complex. The stippled area marks the antibody. Bar, 20 nm. (*C*) Locations of proteins A and 70K and the m3G cap at the U1 snRNP. In the interpretative drawing, the round core domain is indicated (*dotted line*), and the locations of the specific proteins A and 70K (*stippled area*) and the m3G cap site at the 5′ end of the U1 snRNPs (*hatched area*) are shown. (*D*) Tentative location of the U1 RNA at the U1 snRNP. The 5′-terminal cap and stem/loops I and II are located according to the localization of the m3G cap, the 70K, and the A protein, respectively. The locations of stem/loop III and the domain A are arbitrary.

of the A protein and the other of the 70K protein. The distribution of these two proteins in the two protuberances was confirmed by our finding that mAbs against the 70K protein or the A protein each bound only to one of the protuberances. No immunocomplexes were seen in which one kind of mAb had reacted with both protuberances. The molecular masses of the two proteins deduced from their sequences are 52 kD for the 70K and 32 kD for the A protein (29, 39, 40, 44). If a specific volume of 0.73 $cm^3\ g^{-1}$ and a spherical form are assumed, the calculated diameters are 4.9 nm (70K) and 4.2 nm (A). These correspond reasonably well with the observed shapes of the two protuberances, ~3–4 nm in width and 4–7 nm in length, the estimate varying somewhat with the orientation of the U1 RNP particle on the grid.

Because of the symmetric appearance of the two protuberances in most of the electron micrographs of U1 RNPs, it is not possible to see directly which protuberance is associated with 70K and which with A. We therefore attempted to define the positions of the two protuberances with respect to a third topological site on the surface of U1 snRNP, namely, the 5′-terminal m_3G cap structure of the snRNA. For this purpose, we conducted two series of experiments based upon our earlier observation that the m_3G cap structure is located on the main body of the U1 snRNP close to one of the protuberances (17). First, we labeled U1 snRNP particles with an anti-m_3G antibody and, at the same time, with a mAb specific for the A protein. Secondly, we labeled U1 snRNP that lacked the A and C proteins (i.e., particles containing only the 70K protuberance) with antibodies against the m_3G cap. The results of both studies suggest that the m_3G cap is closer to the 70K protuberance than to the A protuberance. Fig. 8 *C* shows a two-dimensional model of the U1 snRNP summarizing these data.

The observation of two distinct locations for proteins 70K and A in the two protuberances also agrees well with data from studies of binding between the two free proteins and the isolated stem/loops I and II from U1 snRNA: protein 70K binds to stem/loop I but not to II, and protein A binds to stem/loop II but not to I (22, 30, 34, 35, 42). However, the binding of protein 70K to complete U1 RNA is not always completely independent of the binding of protein A, because in *Xenopus* a direct or indirect mutual stimulation of binding of these two proteins has been observed (13, 14). In our model, the bases of the two protuberances lie close to one another, so that proteins 70K and A could be in contact, which would explain the observed cooperativity of their binding.

The location of proteins 70K and A in the protuberances reveals indirectly the positions of stem/loops I and II. If these sequences are involved in binding proteins 70K and A to the U1 snRNP, then each must lie at the base of its respective protuberance. In Fig. 8 *D*, a possible localization of the U1 RNA is shown in the U1 snRNP model. The localization of stem/loop I and II, as well as the 5′ terminus, can be determined by our electron microscopic data, while stem/loop III and the domain A, including the 3′-terminal stem/loop, can only be positioned arbitrarily. However, domain A should be located in the core body, since it is the binding site of the core proteins.

As discussed above, we were not able to locate protein C by selective removal of this protein from U1 snRNPs, because no morphological changes were seen when normal U1 snRNPs were compared with U1 snRNPs depleted of this protein. Furthermore, attempts to locate protein C directly by using currently available anti-C antibodies also failed, probably because of the low affinity of these antibodies towards this

protein. For these reasons, only a tentative location can be made, on the basis of circumstantial evidence from the requirements for the binding of protein C. The fact that U1 snRNP particles can be isolated that lack only protein A indicates that protein A probably plays only a minor role in binding protein C. This is supported by assembly experiments with mutated U1 RNA, where the incorporation of both 70K and C was affected by the same set of mutations (12) and by our finding that it is possible to incorporate protein C, translated in vitro, into Δ[C,A]-U1 particles (26). These results, along with the finding that protein C does not bind to naked U1 RNA, suggest that the 70K protein may be a part of the binding site of protein C. However, in human U1 snRNPs protein C can be cross-linked to the 70K and A proteins (4, 15), suggesting that protein C is in close proximity to the other two. These data are consistent with our two-dimensional model of U1 RNP if the binding site for the C protein is close to the bases of the two protuberances on the core RNP body. Such a location would also explain our failure to observe a structural change upon protein C removal. That is, depletion of a spherical protein with a diameter of 3.4 nm (the value calculated for protein C when it has a molecular mass of 17.4 kD [26]) from the bulky core body should be much more difficult to detect than deletion from an exposed structure like the protuberance.

We showed previously that protein C strengthened significantly the binding of a 5′ splice site RNA to the 5′ end of U1 RNA, while the presence or absence of protein A at the U1 RNP particle showed only marginal effects (16). These data are in good agreement with the U1 RNP model discussed above, where proteins 70K and C are positioned close to the 5′ end of U1 RNA and protein A is furthest away from this site. The exposed positions of protein A and part of protein 70K suggest that these two proteins could provide interaction sites with other components of the spliceosome.

The distribution of the common and the specific U1 snRNP proteins may also be of importance in connection with certain autoimmune diseases. As described in the Introduction, SLE and MCTD patients both produce antibodies against U1 snRNPs, but with the important difference that SLE patients have antibodies against the common proteins (anti-Sm), while MCTD patients have antibodies against the specific proteins of U1 snRNP (anti-RNP) (43). It is not known why the immune system responds in these two distinct ways to the U1 snRNP particle as an antigen. Since snRNP-specific autoantibodies can be induced experimentally by immunization with snRNPs (31), it is probable that the pathological immune response is also induced directly by U1 snRNPs. It follows that the cause of the different autoantibody specificities in SLE and MCTD patients may reside in the structure of the snRNPs. In support of this hypothesis, the work described in this paper has shown that the two sets of epitopes are located in different places on the U1 snRNP particle: SLE autoantibodies are directed against the round, compact main body and MCTD autoantibodies against the exposed proteins of the protuberances. The physical grouping of the epitopes could result in the one or the other set being presented to the immune system, and it may thus provide a physical basis for the specificity of the autoimmune response.

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