Electron Microscopy of U4/U6 snRNP Reveals a Y-shaped U4 and U6 RNA Containing Domain Protruding from the U4 Core RNP

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Abstract. We describe the electron microscopic investigation of purified U4/U6 snRNPs from human and murine cells. The U4/U6 snRNP exhibits two morphological features, a main body ~8 nm in diameter and a peripheral filamentous domain, 7–10 nm long. Two lines of evidence suggest that the peripheral domain may consist of RNA and to contain U6 RNA as well as the 5' portion of U4 RNA. (*a*) Separation of the U4/U6 snRNA interaction regions from the core domains by site-directed cleavage of the U4 snRNA with RNase H gave filament-free, globular core snRNP structures. (*b*) By immuno and DNA-hybridization EM, both the 5' end of U4 and the 3' end of U6 snRNA were located at the distal region of the filamentous domain, furthest from the core. These results, together with our observation that the filamentous U4/U6 domain is often Y shaped, correlate strikingly with the consensus secondary structure proposed by Brow and Guthrie (1988. *Nature (Lond.)*. 334:213–218), where U4 and U6 snRNA are base paired in such a way that two U4/U6 helices together with a stem/loop of U4 snRNA make up a Y-shaped U4/U6 interaction domain.

EUKARYOTIC cells contain a number of small nuclear ribonucleoproteins (snRNPs)[1] of the Sm class, of which the principal members are snRNPs U1, U2, U4/U6, and U5. The snRNPs consist of either one (U1, U2, U5) or two (U4/U6) snRNA molecules and a set of polypeptides, which fall into two general classes: the common and the particle-specific ones. The common proteins B', B, D, E, F, and G are bound to the so-called Sm-binding site or domain A, a structural motif that is shared by the snRNAs U1, U2, U4, and U5 (6, 26, 29). In addition to the core proteins, at least U1, U2, and the 20S U5 snRNP contain specific proteins (1, 27). Proteins specific to U4/U6 snRNP have as yet not been identified in metazoan cells. By EM it has recently been shown that the snRNP core structures of the snRNPs U1, U2, and U5 are morphologically very similar and can be described as a round body ~8 nm in diameter. In addition to the core domain, snRNPs U1, U2, and U5 display peripheral structures, which arise from the snRNP-specific proteins, that give each of these snRNP species its characteristic shape (21, 22).

The major snRNPs U1, U2, U4/U6, and U5 are essential *trans*-acting factors in the pre-mRNA splicing process both in human and in yeast. They assemble with a pre-mRNA and a number of other proteins along an ordered pathway to form an active spliceosome (17, 19, 28, 33, 35). At an early stage of spliceosome assembly, U1 and U2 snRNPs interact directly with the 5' splice site and the branch point, respectively, and this step is followed by the integration of the snRNPs U4/U6 and U5 into the spliceosome. While interac-

tion of U5 snRNP with the 3' splice site has been suggested (13), there is, as yet, no indication for a direct interaction between U4/U6 snRNP and the pre-mRNA. In nuclear extracts, the snRNP U4/U6 and the 20S U5 snRNP form a 25S multi-snRNP complex which most likely represents an early spliceosome intermediate (2, 14, 23).

The exact role played by U4/U6 snRNP during splicing remains enigmatic. The U4/U6 snRNP displays several unique properties as compared with the other major Sm snRNPs. The U6 snRNA is the most highly conserved spliceosomal snRNA (11) and is the only one that lacks the trimethylguanosine (m3G) cap, instead, it contains a γ-methyl phosphate group at its 5' end (34). Since U6 snRNA lacks the domain A, the association of U6 snRNA with U4 snRNP in the U4/U6 particle appears to be largely determined by base pairing between U4 and U6 snRNAs (10, 20, 32). On the basis of phylogenetic comparison of U4 and U6 snRNA sequences from a variety of organisms, a conserved secondary structure model has been proposed. This consists of two intermolecular helices ~7 and 15 bp long, which are separated by an intramolecular stem of U4 snRNA producing a Y-shaped structure (11, 19, 40). During splicing, the U4/U6 snRNP appears to undergo a major conformational change prior to or concomitant with 5' splice site cleavage, measurable as a weakening of the U4/U6 interaction (4, 14, 24, 31). These data, together with the observation of the insertion of an intron into a highly conserved region of the S. *pombe* U6 snRNA (36), led to the hypothesis that U6 functions as a catalytic element in the splicing reaction (12, 18). In this view, the primary role of U4 snRNP would be to sequester a catalytically active domain of U6 until all spliceosom-

1. *Abbreviations used in this paper:* EATC, Ehrlich ascites tumor cell; snRNP, small nuclear ribonucleoprotein.

al components have properly assembled into a functional spliceosome.

To gain insight into the functional significance of the interaction between U4 and U6 snRNAs during splicing, knowledge of the three-dimensional structure of the U4/U6 snRNP is essential. As a step in this direction, we have investigated purified U4/U6 snRNPs at the ultrastructural level using EM. The U4/U6 snRNP exhibits two morphological features, a main body ~8 nm in diameter that resembles closely the core snRNP structure of snRNPs U1, U2, and U5 (21, 22) and a peripheral filamentous domain that often exhibits a Y-shaped appearance. This Y-shaped filamentous domain contains distal from the core structure the 5' end of the U4 snRNA and the 3' end of the U6 snRNA. This strongly supports a proposed conserved U4/U6 secondary structure model (11, 19, 40) that involves a Y-shaped filamentous U4/U6 interaction domain.

Materials and Methods

Preparation of U4/U6 snRNPs

U4/U6 snRNPs were isolated from HeLa and Ehrlich ascites tumour cells (EATCs) as described in detail elsewhere (37). In summary, nuclear extracts prepared from HeLa cells according to Dignam et al. (15) or from EATC according to Zieve and Penman (39) were partially depleted of the snRNPs U1 and U2 by passage over an anti-m3G immuno-affinity column. A second passage over a same column followed by competitive elution of the bound snRNPs with m⁷G (5) led to fractions highly enriched in U4/U6 snRNPs. Further fractionation by ion-exchange chromatography on Mono Q (Pharmacia Fine Chemicals, Piscataway, NJ) using an ascending KCl gradient (50 mM to 1 M) in elution buffer (20 mM Tris-HCl at pH 7.5, 1.5 mM MgCl₂, 0.5 mM PMSF, 0.5 mM DTT) yielded essentially pure U4/U6 snRNPs. The identity of the snRNA and protein constituents was determined by electrophoresis, as described by Bringmann et al. (9). The final concentration of the U4/U6 snRNPs from HeLa was 23 μg/ml, and that obtained from EATC was 272 μg/ml.

Immunocomplex Formation

HeLa U4/U6 snRNPs (6 μg) were allowed to react with 2.5 μg of monoclonal anti-m3G IgG H20 (5) by incubation for 60 min at 0°C in 50 μl of buffer A, which consisted of 20 mM Tris-HCl (pH 7.9), 50 mM KCl, and 15 mM MgCl₂. Samples for EM were prepared directly from the incubation mixture.

Gradient Centrifugation

Samples were diluted to 100 μl with buffer A and loaded onto a 5–20% glycerol gradient (1.3 ml) in buffer B (20 mM Hepes · KOH at pH 7.9, 150 mM KCl, 3 mM MgCl₂). Centrifugation was performed for 5 h at 55,000 rpm in a TLS-55 rotor (Beckman Instruments, Inc., Palo Alto, CA). The gradient was fractionated into 60-μl portions starting from the bottom.

Complex Formation between Streptavidin and the 3' End of the U6 snRNA

EATC U4/U6 snRNPs (10 μg) were incubated together with 7 μg 5'-biotinylated DNA "U6-oligonucleotide" in buffer A for 60 min at 30°C. The oligonucleotide (BioG-BioG-BioG-CGTTCACGAATTTGCGTGTC, where BioG is biotinylated guanosine) is complementary to nucleotides 75–95 at the 3' end of U6 snRNA. The oligonucleotide was a kind gift from Dr. A. Bindereif (Berlin) and was synthesized according to the protocol of Clontech Laboratories Inc. (Palo Alto, CA) using the Amino Modifier II and Biotin-X-NHS ester to incorporate three biotin-modified guanosine residues at the 5' end of the oligonucleotide. After the addition of 160 μg streptavidin (Pierce Chemical Co., Rockford, IL) and another incubation for 30 min at 30°C, the mixture was layered onto a 5–20% glycerol gradient in buffer B containing 300 instead of 150 mM KCl. The gradient was centrifuged and fractionated as described above. An oligonucleotide containing one Biotin-

11-dUMP at the 3' end (kindly provided by Drs. U. Albrecht and D. Schümperli, Bern) was also initially used to label the U6 snRNA. With this oligonucleotide a lower rate of streptavidin complex formation was observed and not used further.

Digestion of U4 snRNA with RNase H

EATC U4/U6 snRNPs (10 μg) were incubated together with 0.4 A₂₆₀ U DNA "U4-oligonucleotide" and 2 U of RNase H (Boehringer Mannheim Biochemicals, Indianapolis, IN) in a volume of 50 μl in buffer C (20 mM Tris-HCl at pH 7.5, 5 mM MgCl₂, 200 mM KCl, 1 mM DTE) for 60 min at 30°C. The oligonucleotide (GGGTATTGGGAAAAGTTTTCAATTAG) is complementary to nucleotides 58–84 of the U4 snRNA. After dilution with buffer A, the mixture was centrifuged in a glycerol gradient as described above.

EM

Negative staining with 2.5% uranyl formate was carried out by the double carbon film method as described by Kastner and Lührmann (21). The preparations were examined under a Zeiss EM 109 electron microscope with an acceleration voltage of 80 kV, and electron micrographs were taken at magnifications between 85,000 and 140,000.

Results

U4/U6 snRNPs Contain a Globular and a Filamentous Structural Domain

Fig. 1 shows the snRNA and protein compositions of the purified human (HeLa) and murine (Ehrlich ascites tumour cell, EATC) U4/U6 snRNPs. Besides minor contaminations by U1 or U2 snRNA, the preparations contain only U4 and U6 snRNAs and the common proteins B' (HeLa only), B, D, E, F, and G. No U4/U6-specific proteins were detected in
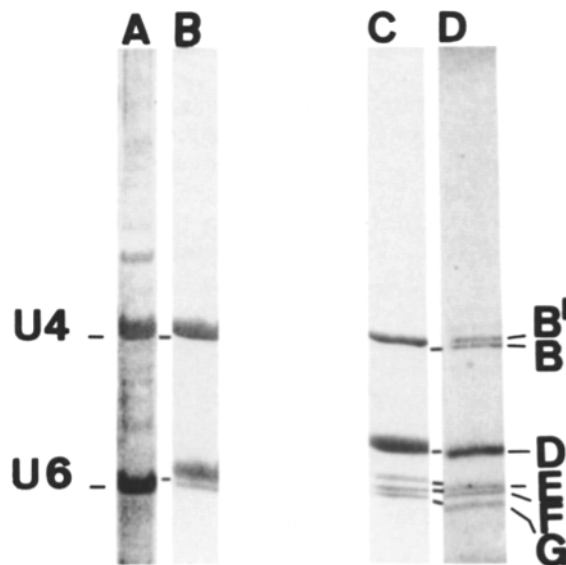

Figure 1. Polypeptide and snRNA composition of U4/U6 snRNPs from EATC and HeLa cells. (*A* and *B*) The snRNA was separated by electrophoresis on a urea-polyacrylamide gel and made visible by silver staining. (*C* and *D*) The proteins were extracted with PCA before electrophoresis on a 4-20% SDS-polyacrylamide gel and stained with Coomassie blue. (*A* and *C*) snRNA and proteins, respectively, of U4/U6 snRNPs from EATC. (*B* and *D*) snRNA and proteins, respectively, of U4/U6 snRNPs from HeLa cells. The separation of the U6 snRNA into a double band in *B*, representing conformational variants, is due to a much longer running time than used for the electrophoresis in *A*.
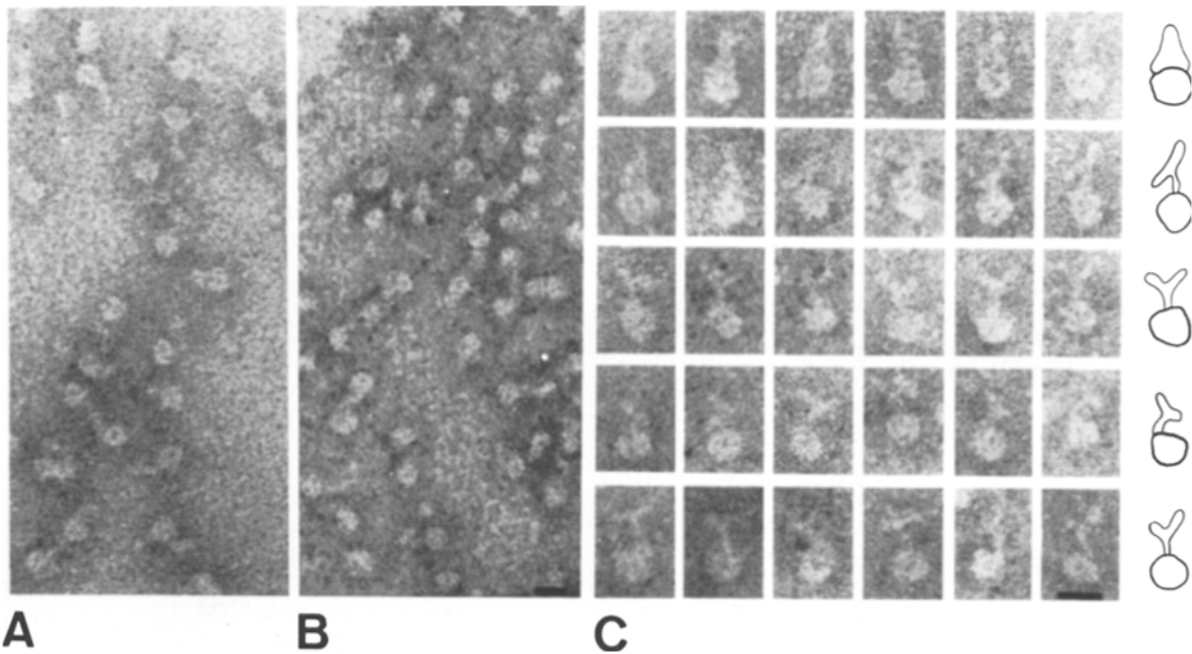
Figure 2. U4/U6 snRNPs negatively stained with uranyl formate. (*A* and *B*) General views of U4/U6 snRNPs purified from HeLa cells (*A*) and EATC (*B*). (*C*) Gallery of representative images of U4/U6 snRNPs from EATC. The views have been rotated so that the filamentous domain is above the core in each case. The filamentous domains of the particles in each row show similar structural details: some filaments appear more diffuse (*first row*), while in others a Y-shaped structure can be recognized (*second to fifth row*). One arm of this structure is always connected to the core domain and the second arm points more or less directly away from it, while, with respect to the second arm, the third arm points downwards (*second row*), upwards (*third row*), to the right (*fourth row*), or to the left (*fifth row*). In areas of optimal contrast the Y-shaped structure is visible in about one-third of the images and rows three and four show examples of such predominantly observed images. Bars, 10 nm.

these preparations (8). The U4/U6 snRNPs used in this study are functionally active insofar that they are capable of restoring splicing activity when added alongside purified U1, U2, and U5 snRNPs to HeLa cell nuclear extracts that have been predepleted of their endogenous snRNPs by micrococcal nuclease digestion (37; M. Bach, data not shown).

For electron microscopic investigations, the purified U4/U6 snRNP particles were negatively contrasted with uranyl formate as described previously (21). Fig. 2 *A* shows a general field of HeLa U4/U6 snRNPs. The snRNP particles are characterized by two morphological features. The main feature is a round body, or core, ~8 nm in diameter. On close inspection of individual views, further structural details in the core can be recognized. These are revealed by regions of high stain density within the globular domain. Sometimes, forms are seen with a line of stain that roughly bisects the round body. In regard to size, appearance, and fine structure, this main body closely resembles the core snRNP particles of the snRNPs U1, U2 and U5, that is, the snRNPs U1, U2, and U5 lacking their complement of snRNP-specific proteins (22). It is therefore likely that the round body of the U4/U6 snRNP particle also represents the core snRNP domain of this snRNP, consisting of domain A of U4 snRNA and the common proteins B' to G.

The second feature that can be distinguished is a filamentous structure that protrudes from the core domain. This filamentous protrusion regularly appears relatively dark and, for this reason, is often hard to distinguish from the stain surrounding it. The contours of the U4/U6 snRNP particle and its internal fine structure appear similar irrespective

of whether the U4/U6 are taken from HeLa (Fig. 2 *A*) or from EATC (Fig. 2 *B*). The visibility of the filamentous protrusion varies greatly, on account of the poor contrast between them and the background. On a single grid, the protrusions in some areas can be nearly invisible, while in other areas of the same grid they appear almost as clearly as the globular core-snRNP domains. In some instances, "dumb bell-shaped" particles are also seen, in which two globular bodies ~8 nm in diameter appear to be connected by a filamentous structure 8–17 nm in length. While these structures could be interpreted by assuming that two U4/U6 monomers can dimerize by an association involving two distal Y arms, they are not considered further here.

Upon closer examination, the more clearly visible filaments reveal fine structures that differ from one view to another but which follow certain reproducible patterns. Fig. 2 *C* contains examples of typical U4/U6 snRNPs at high magnification. The first row shows U4/U6 snRNP particles as they appear most frequently; the filamentous structure is very diffuse, and at its base it appears up to 5 nm wide. The U4/U6 snRNP particles in the second row show very similar filaments, but on closer inspection these are seen to possess a well-defined fine structure. The 5-nm base consists of two thin, elongated structures that join some 3–5 nm from the globular core domain. Each line has a diameter of ~2 nm. The line running towards bottom left does not appear to be connected with the core domain. The branching point of the lines frequently shows a characteristic Y-like structure. In the third, fourth, and fifth rows of Fig. 2 *C*, U4/U6 snRNP particles with more easily discernible Y-shaped domains are
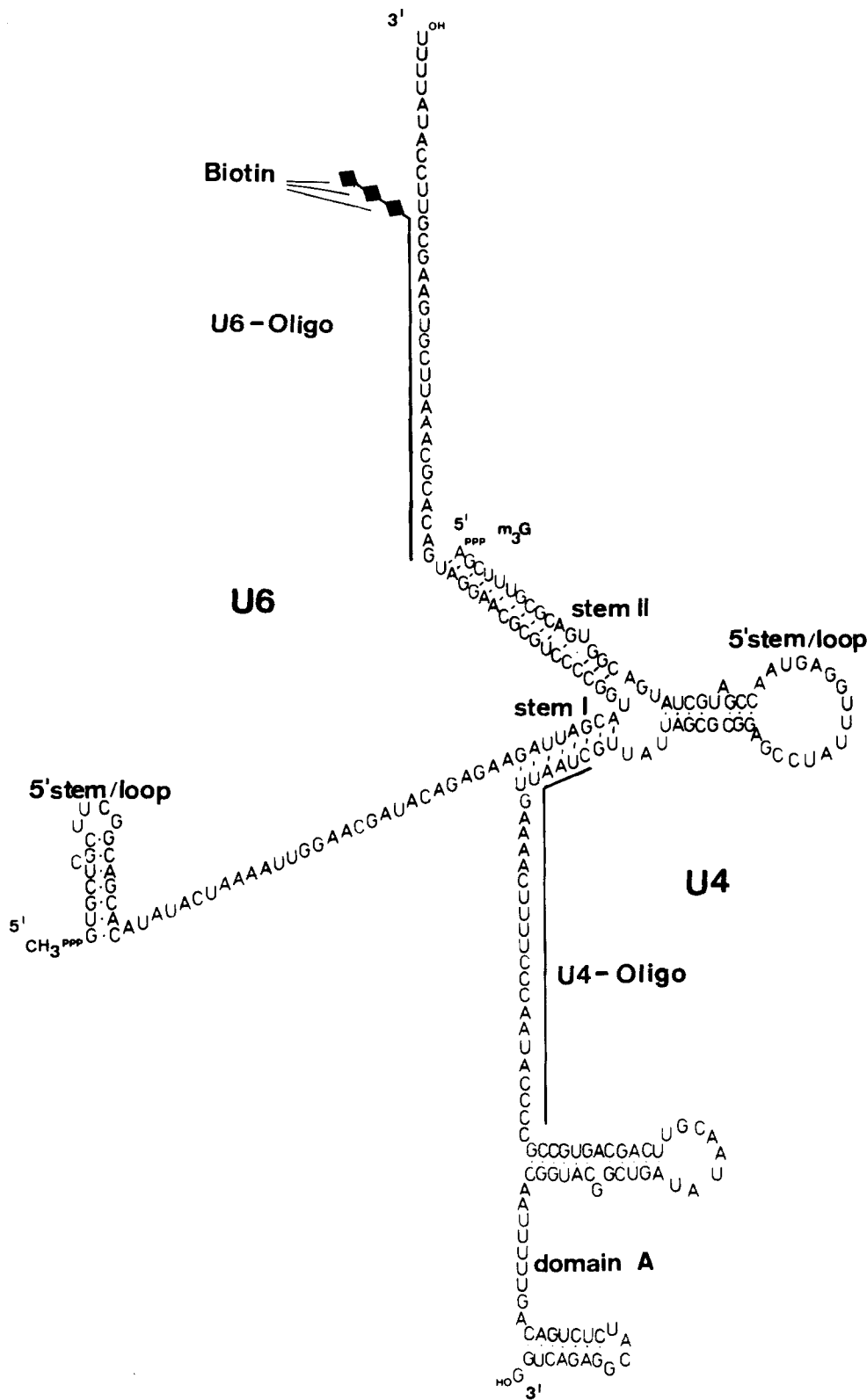
Figure 3. Model of the secondary structure of U4/U6 snRNA. The sequences of U4 and U6 snRNA are shown in the consensus secondary structure proposed by Guthrie and Patterson (19). In addition, the regions complementary to the DNA-oligonucleotides used in this work are shown by black lines. The black lozenges on the U6 DNA oligonucleotide represent biotinylated guanosine residues. The single-stranded PuAUUUUUGPu sequence, together with the two flanking hairpin loops, represents the domain A (6).

shown. The arms of the Y-like domains are between 3 and 6 nm in length, giving the Y structure an overall length of some 10 nm.

The Filamentous Protrusion Contains the 5' End of the U4 snRNA and the 3' End of the U6 snRNA

If the U4/U6 secondary structure proposal which includes

a Y-shaped U4/U6 interaction domain (11) is considered, the Y-shaped appearance of the filamentous structure in some of the U4/U6 electron micrographs (Fig. 2 C) suggests that this structural feature might consist of RNA and represent the domain in which the interaction between U4 and U6 takes place.

To test this working hypothesis, we first determined the

location of the 5'-terminal m3G cap of U4 snRNA at the surface of the U4/U6 snRNP. For this purpose, we made immunocomplexes between U4/U6 snRNPs and the m3G-specific mAb H20 (5); these were then investigated under the electron microscope without further purification. Fig. 4 A shows a typical general view, in which free U4/U6 snRNPs and snRNP particles with an attached IgG molecule (marked by arrowheads) can be seen. More than 200 U4/U6 H20 immunocomplexes with recognizable filamentous structures were examined. The IgG antibodies can be recognized by their typical Y shape, and they are appreciably larger than the filamentous U4/U6 domains. Fig. 4 B shows a selection of typical immunocomplexes. The antibody-binding site was always located in the filamentous domain, and in >90% of the complexes it was clearly separated from the core domain. Distances of up to 8 nm were measured between the core and the antibody-binding site. The observed position of the antibody-binding site allows the conclusion that the m3G cap, and thus the 5' end of the U4 snRNA, lie in the filamentous domain and are clearly separated from the core.

Next, we attempted to determine whether U6 snRNA was also contained in the filamentous protrusion. U6-specific antibodies were not available, so we adopted a different approach (30) to this question: streptavidin was used to locate a biotinylated nucleotide complementary to the 3' end of the U6 snRNA. The 3' end of the U6 snRNA was chosen because (a) in earlier studies, it had been shown that the 3' end of U6 snRNA is single stranded in the U4/U6 snRNP particle (3, 4, 16, 38); and (b) according to Guthrie's consensus secondary structure model for U4/U6 snRNA, the 3' end of the U6 snRNA is situated far from the core snRNP domain (11, 19). Streptavidin is well suited for this electron microscopic analysis, because with its diameter of ~5 nm it is large enough to be clearly recognizable in the electron microscope, while its shape makes it clearly distinguishable from the large U4/U6 core domain. In addition, streptavidin does not bind to unlabeled U4/U6 snRNPs (data not shown).

Purified U4/U6 snRNPs were incubated together with the U6 DNA oligonucleotide. This oligonucleotide is complementary to nucleotides 78–95 of U6 snRNA and carries three biotinylated G nucleotides at its 5' end (Fig. 3). It hybridizes exclusively with the 3' end of U6 snRNA, and shows

no interaction with any other part of this molecule or with U4 snRNA. After the addition of an excess of streptavidin and brief incubation, the preparation was fractionated on a glycerol gradient. The snRNPs in the various fractions of the gradient were then examined under the electron microscope. Fig. 5 A shows a general field of a fraction containing U4/U6–streptavidin complexes, labeled with arrows. Beside these complexes, the fraction contains mainly oligomeric streptavidin aggregates (labeled with arrowheads), and occasional free U4/U6 snRNPs. The binding site of the streptavidin molecules on the U4/U6 snRNPs is clearly identifiable in the filamentous domain, well separated from the core. This fact also aids the easy identification of these complexes, of which >100 were examined under the electron microscope.

Fig. 5 B shows a gallery of streptavidin-labeled U4/U6 snRNPs. The complexes are oriented such that the U4/U6 core domains (the larger globular structure) are at the bottom and the streptavidin (the smaller, round structure) is at the top. The streptavidin binds to the filamentous domains, some 10 nm from the cores. In some of the complexes, the filamentous domain can be recognized as a Y-shaped fine structure (Fig. 5 B, bottom row). In these cases, the streptavidin-binding site lies on one of the arms of the Y structure that point away from the core domains. The 3' end of the U6 snRNA thus resides on one of the distal arms of the Y structure of the U4/U6 snRNPs, clearly removed from the core domain.

As a third experimental demonstration that the filamentous domain consist of U4 and U6 RNA, we treated the U4/U6 snRNPs with RNase H and a DNA oligonucleotide complementary to the nucleotides 58 to 84 of the U4 snRNA (Fig. 3) so as to destroy specifically the U4 snRNA between domain A and the U6 snRNA interaction domain (see Fig. 3). The conditions were chosen such that about one-quarter of the U4 snRNA remained intact as determined by RNA gel electrophoresis (not shown). The residual intact U4/U6 snRNPs served as an internal check on the visibility of the filamentous domains in a given field. After treatment with RNase H, the products were fractionated by glycerol density gradient centrifugation, and the snRNA and protein content of the fractions was analyzed. While the intact U4/U6
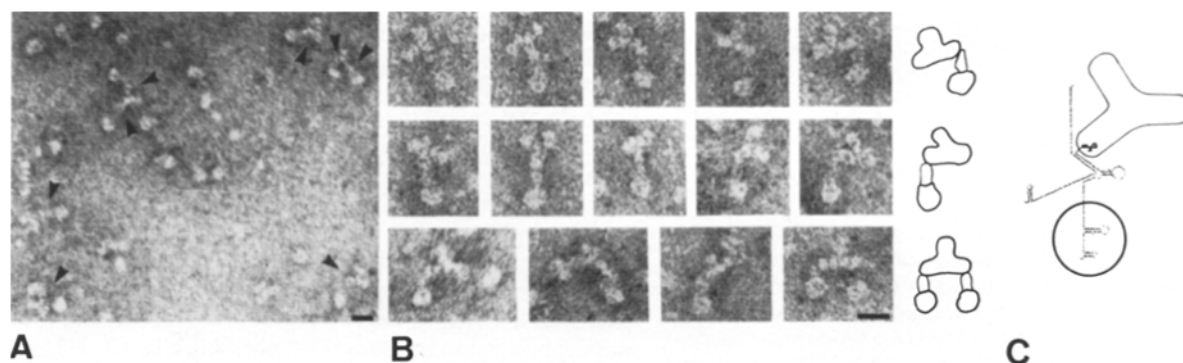

Figure 4. The binding of anti-m3G to U4/U6 snRNPs. (A) General view showing complexes visible after incubation of HeLa U4/U6 snRNPs with monoclonal m3G-specific IgG antibodies. Antibody-binding sites on the U4/U6 snRNPs are indicated by arrowheads. (B) Gallery of selected [U4/U6.IgG] (two upper rows) and [U4/U6.IgG.U4/U6] complexes (last row) obtained with the anti-m3G antibody. The U4/U6 snRNPs in the complexes are oriented with the filamentous domains pointing upwards. The complex at the far right of each row is illustrated by an interpretative sketch. (C) Schematic drawing of anti-m3G IgG binding to the U4/U6 snRNP. The U4/U6 snRNA is shown by its secondary structure model and the core domain is indicated by the large circle. Bars, 10 nm.
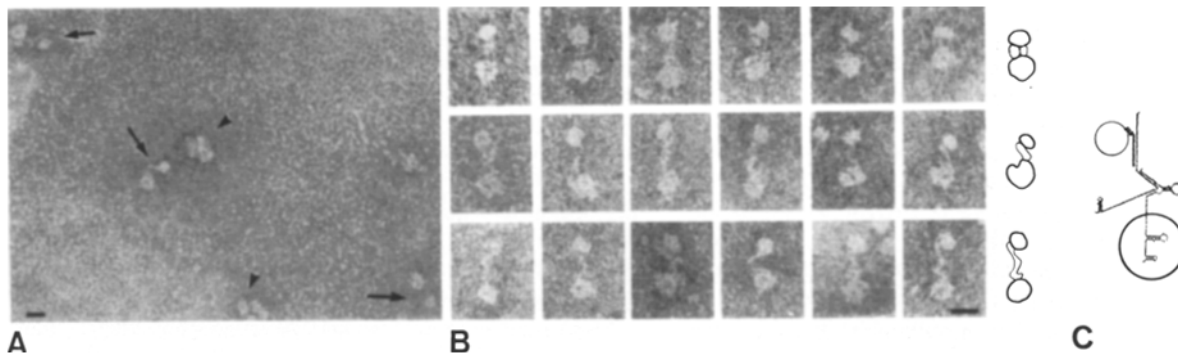
Figure 5. The binding of an oligonucleotide complementary to the 3' end of U6 snRNA and its detection by streptavidin when bound to U4/U6 snRNPs. EATC U4/U6 snRNPs were first hybridized with the biotinylated DNA oligonucleotide complementary to the 3' end of the U6 snRNA and then incubated with streptavidin. Complexes were separated from the excess of unbound streptavidin by gradient centrifugation. (*A*) General view of a fraction enriched in streptavidin-U4/U6 snRNP complexes. Arrows point to U4/U6 snRNP-bound streptavidin. Arrowheads mark streptavidin aggregates. (*B*) Gallery of selected U4/U6-streptavidin complexes. The U4/U6 snRNPs in the complexes are oriented with the filamentous domain pointing upwards. The snRNP core is the lower and the streptavidin is the upper globular structure. The complex at the far right of each row is illustrated by an interpretative drawing. (*C*) Model of streptavidin binding to the U4/U6 snRNP via the biotinylated DNA oligonucleotide complementary to the 3' end of the U6 snRNA. The U4/U6 snRNP is shown as in Fig. 4 *C*. The streptavidin is indicated by a small circle (*top left*) and is connected to one of the biotin molecules (black lozenges) of the DNA oligonucleotide. Bars, 10 nm.

snRNPs sedimented at about 10S, the S value of the fragmented core U4 snRNPs, i.e., U4 RNPs that contained only the 3'-terminal half of U4 RNA together with the core proteins, was reduced by 2–3 U (7–8 S) (not shown). For comparison, intact U4/U6 snRNPs that had not been treated with RNase H were centrifuged and analyzed in the same way.

The fractions from the glycerol density gradients were investigated under the electron microscope. Fig. 6 *B* shows a general view of the 7-8S snRNPs, i.e., fragmented core U4 snRNPs. Round, globular structures can be seen. Closer examination of these reveals fine-structural elements such as bisecting lines and a central dot. Outside the core domain,
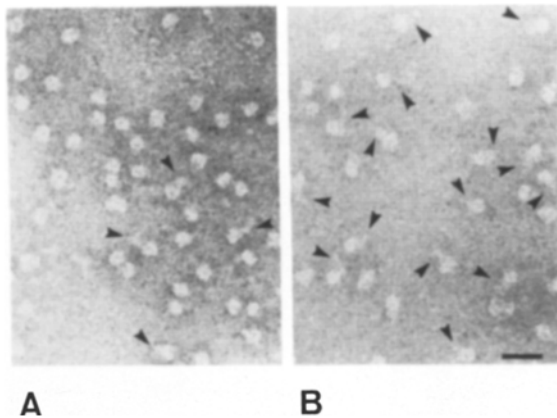
the vast majority of these particles show no further structure. However, in a few of them (indicated with arrowheads in Fig. 6 *A*), the filamentous domains and the Y-shaped structures can be seen. This confirms that these domains are indeed visible in principle in this experiment, so that they really are absent from most of the particles and not just invisible owing to poor contrast. For comparison, a general view of the 7-8S fraction from the glycerol gradient with native U4/U6 snRNPs shows that U4/U6 snRNP particles with the filamentous domain predominate (Fig. 6 *B*). The absence of the filamentous domain from RNase H-fragmented core U4 snRNPs provides independent confirmation of the hypothesis discussed above that the filamentous domain contains the 5' portion of U4 RNA and the U6 RNA molecule base paired to the U4 RNA.

Discussion

According to the results from EM presented here, the isolated U4/U6 snRNP lacking U4/U6-specific proteins consists of two different structural domains, one globular and one filamentous. The globular domain is more or less spherical and has a diameter of ~8 nm. Frequently, a central dot or bisecting line can be seen. The globular domain of the U4/U6 snRNPs resembles, in shape, size, and fine structure, the core particles of the snRNPs U1, U2, and U5 (22). Core particles are obtained by the removal of all particle-specific proteins until only the common proteins B–G are left. It is consistent with this that the globular structure is the core in U4/U6 snRNP as well.

The filamentous domain of the U4/U6 snRNP proceeds out of the core domain and is up to 10 nm long. It frequently possesses a Y-shaped fine structure, in which one arm is always connected to the core. The other snRNPs U1, U2, and U5 also show other domains in addition to the round core, for example, elongated protrusions (21) or more globular extensions (22). In earlier work, we were able to show by immunoelectron microscopy that these additional domains consist of snRNP-specific proteins (22; Kastner, B., U. Kornstädt,

Figure 6. Electron micrographs of gradient fractions obtained after centrifugation of native and U4 snRNA-cleaved U4/U6 snRNPs. Samples corresponding to the 7-8S regions of the glycerol gradient were negatively stained with uranyl formate. *A* shows the fractions of U4/U6 snRNPs cleaved by RNase H and the U4-oligonucleotide. *B* shows the fractions of native U4/U6 snRNPs. Different times were used for adsorption of the particles to the carbon films, in order to obtain specimens with roughly the same particle density. Therefore, the number of particles seen on each micrograph is not proportional to the particle concentration in the respective gradient fraction. Bar, 20 nm.

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M. Bach, and R. Lührmann, unpublished observations). It was therefore surprising at first that- U4/U6 snRNPs also show a further structure, the filamentous domain, even though these snRNPs do not contain specific proteins. We therefore pursued the possibility that these filamentous structures consisted solely of snRNA, as snRNA is the only component of U4/U6 snRNP that is specific for this particle only.

Three lines of evidence show clearly that the filamentous extension represents part of U4 and U6 snRNA. (a) Separation of the U4/U6 snRNA interaction regions from the core domains by cleavage of the U4 snRNA with RNase H gave filament-free, globular core snRNP structures. (b) Anti-m₃G antibodies bind to the end of the filamentous domains furthest from the core, showing that the 5' terminus of the U4 snRNA must lie here. (c) Hybridization with a biotin-labeled DNA-oligonucleotide complementary to the 3' end of the U6 snRNA, and subsequent electron-microscopic localization of streptavidin bound to the biotin, showed that the 3' end of the U6 snRNA is also located in the very distal region of the filamentous structure.

The findings (a) that the 3' end of U6 and the 5' end of U4 are in close proximity to each other at the tip of the filamentous domain and (b) that this domain is often Y-shaped are in striking agreement with the secondary structure proposed by Brow and Guthrie (11) and Zucker-Aprison et al. (40), where U4 and U6 snRNA are base paired in such a way that two U4/U6 helices together with a stem/loop of U4 snRNA make up a Y-shaped U4/U6 interaction domain. Moreover, if A-form RNA-helices are assumed, a width of 2 nm and a length of 2–4 nm is expected for the double helical stems (7). This is in reasonable agreement with the dimensions observed for the Y-structure, where the arms are 2 nm thick and 3–6 nm long.

Such a two-domain structure of U4/U6 would imply that the major part of the snRNAs in this particle is completely unprotected. This agrees with the results of published studies of nuclease digestion of U4/U6 RNPs in solution, demonstrating that U6 RNA and the 5' half of U4 RNA are vulnerable to attack by RNase (3, 16, 25, 26). It is further significant that, after digestion with RNase H and the oligonucleotide complementary to U4 snRNA, the filamentous domain can no longer be seen, that is, the U6 snRNA dissociates together with the 5'-terminal part of U4 snRNA. This means that the U6 snRNA in the isolated U4/U6 snRNP monomer is associated principally by base pairing, while the core proteins do not make any important contribution to the stability of this complex (38).

Recent evidence suggests that in nuclear extracts U4/U6 snRNP may interact with U5 snRNP and form a 25S [U4/U6·U5] tri-snRNP (2, 14, 23). Though it has not yet been proved it is likely that the tri-snRNP complex represents an early spliceosome intermediate that binds to pre-spliceosomes as such. While we have observed the Y-shaped filamentous structure in the purified U4/U6 snRNP, it may be asked whether this structure is functionally meaningful, i.e., whether it is preserved in the [U4/U6·U5] tri-snRNP complex. While at present we have no direct proof for this, there are strong arguments in favor of it. We have shown recently that U4/U6 snRNPs purified according to the same protocol as the particles used in this study are functionally active, i.e., they restore splicing activity when added alongside purified U1, U2, and U5 snRNPs to a HeLa cell nuclear

extract depleted of the endogenous snRNPs by prior treatment with micrococcal nuclease (37). This indicates further that the purified U4/U6 snRNPs may readily interact with U5 snRNP and additional proteins of the nuclear extract needed for stable [U4/U6·U5] tri-snRNP complex formation (Behrens, S., and R. Lührmann, manuscript submitted for publication). This would not be expected if the peripheral Y-shaped domain observed in the purified U4/U6 snRNP (this paper) were to represent an artifact arising from the purification method.

The structure of U4/U6 snRNPs as presented here is compatible with the proposal that U6 snRNA may play a part in the catalysis of splicing of pre-mRNA. In this model, U4 snRNP could be considered as an snRNP particle delivering U6 snRNA to the site of action inside the spliceosome (12). In U4/U6 snRNP, the presumed catalytic snRNA is found, bound by base pairing to the U4 snRNA, stretched out, and at a distance from the bulky core snRNP region. This positioning would make easier the approach of the U6 snRNA to the catalytic center of the spliceosome. The sequences exposed on isolated U4/U6 snRNPs would consequently be within the interior of the spliceosome and thereby protected. This is supported by oligonucleotide-binding experiments (4): most of the regions of U4 and U6 snRNAs that in free U4/U6 are accessible for the binding of oligonucleotides appear to be blocked in the spliceosome. As U4/U6 snRNP most probably becomes integrated into the spliceosome in the form of a 25S [U4/U6.U5] tri-snRNP complex (see above), it will be important to investigate whether the Y-shaped domain of U4/U6 snRNP is still exposed at the surface of the tri-snRNP complex or already shielded by the interaction with the protein-rich U5 snRNP.

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