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Purification of the individual snRNPs U1, U2, U5 and U4/U6 from HeLa cells and characterization of their protein constituents

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A procedure is described for the purification of the individual major small nuclear ribonucleoproteins (snRNPs) U1, U2, U5 and U4/U6 from HeLa cells. The salient feature of the method is the combined usage of antibodies against 2,2,7-trimethylguanosine (m_3G) and 6-methyladenosine (m^6A) for differential immune affinity chromatography of the snRNPs. While anti-m_3G affinity columns allow the separation of snRNPs U1, U2 and U5 from U4/U6 RNPs, anti-m^6A antibodies selectively react with snRNPs U2 and U4/U6. Our technique further incorporates immune affinity chromatography of snRNPs with antibodies against snRNP proteins in addition to ion exchange chromatography. The procedure avoids the usage of denaturing agents, so as to maintain the native structure of the particles. This is mainly provided for by the possibility of eluting the anti-m_3G and anti-m^6A bound snRNPs with excess of the respective nucleosides. We have so far identified 12 polypeptides as constituents of the major snRNPs U1 to U6. Seven proteins of approximate mol. wts 29 kd (B′), 28 kd (B), 16 kd (D), 15.5 kd (D′), 12 kd (E), 11 kd (F) and 9 kd (G) were present in each of the individual snRNPs U1, U2, U5 and U4/U6. In addition to the common proteins, U1 RNPs contain three unique polypeptides of mol. wts 70 kd, 34 kd (A) and 22 kd (C). U2 RNPs are characterized by the presence of a 33-kd and a 28.5-kd protein, denoted A′ and B″. We could not detect any unique polypeptide confined to the purified snRNPs U5 or U4/U6. During our fractionation studies we noticed a U2 RNA – protein complex resistant to dissociation in SDS – polyacrylamide gels, which contained at least the proteins B′ and/or B, as demonstrated by immunoblotting. The procedure yields sufficient amounts of native purified snRNPs to study their roles in the processing of nuclear pre-mRNAs by *in vitro* complementation assays, as well as to investigate their role as an antigen and immunogen in the anti-Sm/RNP autoantibody production of patients with systemic lupus erythematosus.

Key words: Purification of snRNPs/nucleoside-specific antibodies/protein composition/splicing/lupus erythematosus

Introduction

Eucaryotic cells contain a group of metabolically stable RNAs known as U snRNAs (for recent reviews see Reddy and Busch, 1983; Steitz *et al.*, 1983; Brunel *et al.*, 1985). In the higher eucaryotes at least eight distinct snRNA species have been described so far, ranging in size between 56 and 215 nucleotides, of which the nucleoplasmic snRNAs U1, U2, U4, U5 and U6 are the major representatives. Except for U6 RNA, the snRNAs are marked by a 5′-terminal cap which contains the unusual nucleoside 2,2,7-trimethylguanosine (m_3G) at its 5′ end. The snRNAs U2, U4 and U6 are further notable in that they each possess a single 6-methyladenosine residue (m^6A) (for a compilation of snRNA sequences see Reddy, 1985).

The snRNAs are associated with a set of proteins to form ribonucleoprotein particles (snRNPs). While the snRNAs U1, U2 and U5 are organized within separate RNP particles, the U4 and U6 RNAs reside in one and the same ribonucleoprotein complex (Lerner and Steitz, 1979; Bringmann *et al.*, 1984; Hashimoto and Steitz, 1984; Rinke *et al.*, 1985). Some of the snRNP proteins bear the antigenic determinants reacting with the so-called anti-(U1)RNP and anti-Sm autoantibodies which are often developed by patients with systemic lupus erythematosus (SLE) (Lerner and Steitz, 1979; see Tan, 1982, for a review). Anti(U1)RNP antibodies react selectively with U1 RNPs only, while anti-Sm antibodies precipitate all nucleoplasmic snRNPs U1 to U6.

In the cell the snRNPs appear to play an essential role in the processing of nuclear pre-mRNAs. While it was first proposed that U1 RNP should contribute to splicing (Lerner *et al.*, 1980; Rogers and Wall, 1980), experimental evidence has been accumulated during recent years that all major nucleoplasmic snRNPs U1 to U6 participate in principle in the splicing of nuclear pre-mRNAs (Padgett *et al.*, 1983; Krämer *et al.*, 1984; Black *et al.*, 1985; Krainer and Maniatis, 1985; Chabot *et al.*, 1985; Black and Steitz, 1986). The exact function of the individual snRNP species at single steps of the splicing process is far from being understood, however. The minor snRNA U7 has been demonstrated to be an essential component in the generation of the 3′ termini of histone mRNA (Galli *et al.*, 1983; Strub and Birnstiel, 1986; reviewed by Birnstiel *et al.*, 1985).

Various attempts have been made to purify the snRNPs. So far U1 RNPs have been obtained in reasonably pure form by different procedures, but less progress has been made in the fractionation of the other snRNPs (reviewed by Brunel *et al.*, 1985).

We describe here the purification of the major individual snRNPs U1, U2, U5 and U4/U6 from HeLa cells and the analysis of their protein composition. Our procedure combines the usage of antibodies specific for the nucleosides m_3G and m^6A, as well as antibodies against selected snRNP proteins for differential immune affinity chromatography of the snRNPs in addition to ion exchange chromatography.

Results and discussion

Fractionation of snRNPs by affinity chromatography with antibodies specific for m^6A

Based on our previous observation that in immune precipitation assays the m^6A residues of the snRNAs U2 and U4/U6 are accessible for rabbit anti-m^6A IgG (Bringmann and Lührmann, manuscript submitted), we here investigated whether it was possible to isolate the respective snRNPs preparatively on anti-m^6A affinity columns. A high-salt nuclear extract from HeLa cells was used as a source of intact snRNPs. The high efficiency of the anti-m^6A affinity column in binding snRNPs was demonstrated

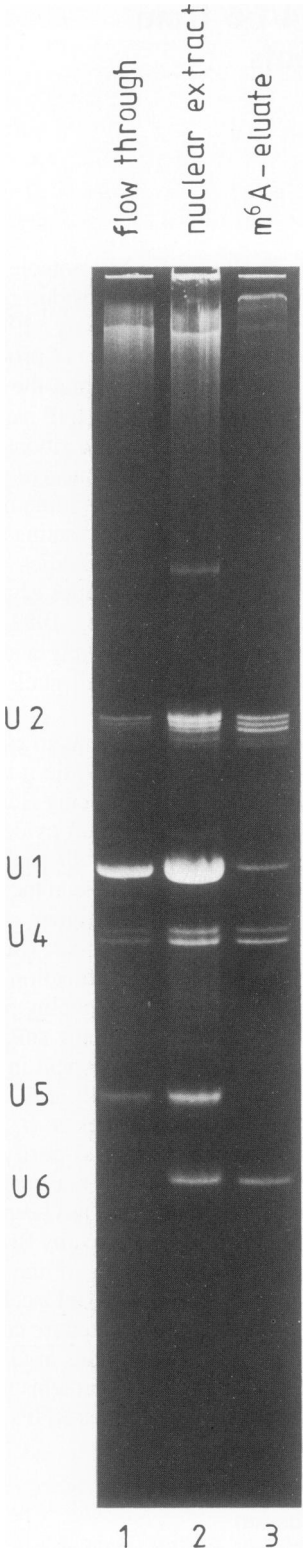

Fig. 1. Polyacrylamide gel electrophoresis of RNAs from fractions obtained during affinity chromatography of snRNPs with anti-m^{6}A IgG. Affinity chromatography of snRNPs from nuclear high-salt extracts was performed essentially as described for the separation of U2 and U5 RNPs on anti-m^{6}A IgG columns which is outlined in detail in Materials and methods. RNAs from respective RNP fractions corresponding to equivalent cell numbers (2×10^7 HeLa cells) were extracted with phenol and analyzed by electrophoresis on 10% polyacrylamide gels containing 7 M urea and TBE. The photograph shows the ethidium bromide-stained RNA species extracted from: **lane 2**, nuclear high-salt extract used as starting material; **lane 1**, flow-through fraction; **lane 3**, m^{6}A eluate.

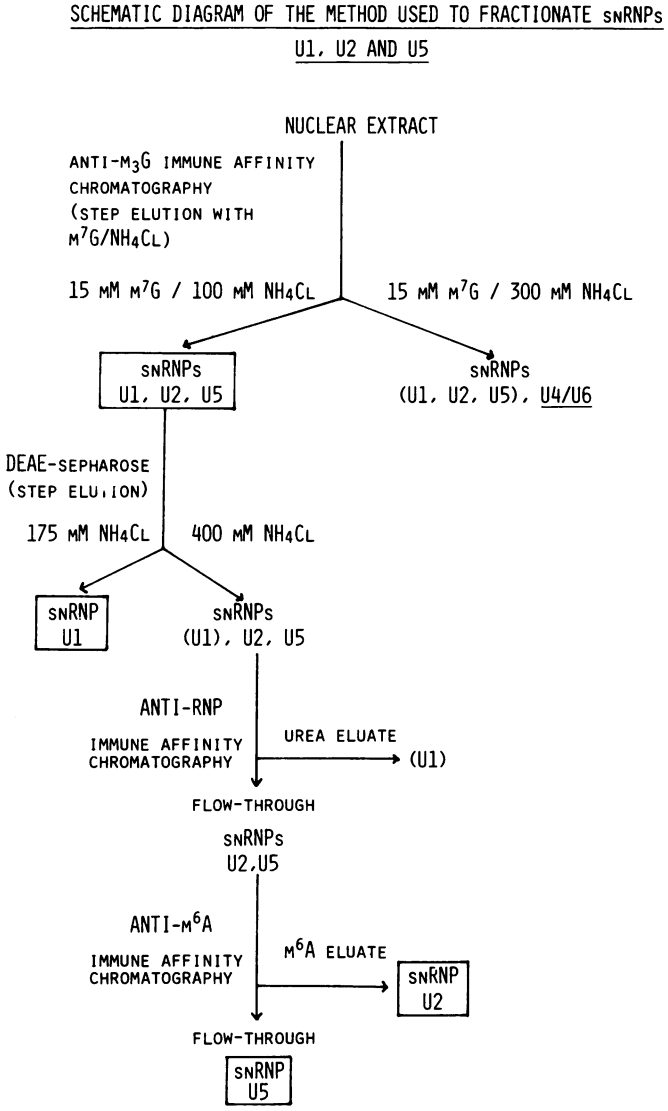

Fig. 2. Schematic representation of the fractionation protocol used for the isolation of snRNPs U1, U2 and U5. Fractions containing the individual snRNPs are boxed.

by RNA gel electrophoresis. As shown in Figure 1 (lane 1) snRNPs U2 and U4/U6 may be removed almost quantitatively from the nuclear extract. Most importantly, the snRNPs can be recovered almost quantitatively from the column by elution with excess nucleoside m^{6}A (Figure 1, lane 3). The m^{6}A eluate was essentially free of U5 RNPs. However, despite the fact that U1 RNA, like U5 RNA, does not contain m^{6}A, a fraction of U1 RNPs (~10% of the U1 RNPs originally contained in the nuclear extract) was reproducibly co-eluted from the anti-m^{6}A affinity column, which is most probably due to intermolecular interaction of U1 with U2 RNP (P.Bringmann and R.Lührmann, submitted).

The above results demonstrate that the anti-m^{6}A affinity column can be used for the rapid preparation of snRNP fractions containing predominantly the U2 and U4/U6 RNP particles as well as for the further fractionation of the two snRNP species from a mixture of total U snRNPs.

Isolation of individual snRNPs U1, U2 and U5

A schematic representation of the fractionation procedure used for the isolation of the snRNPs U1, U2 and U5 is shown in Figure

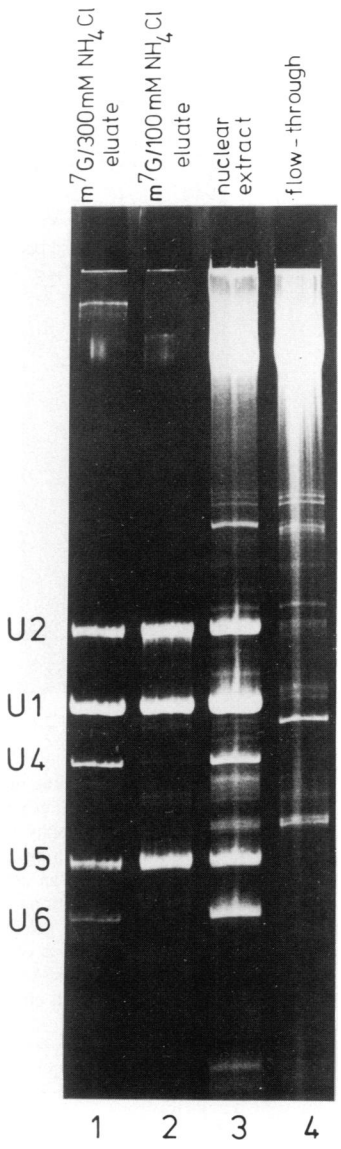

Fig. 3. Fractionation of total nucleoplasmic snRNPs U1 – U6 by differential salt elution of anti-m_3G immune affinity columns. Affinity chromatography of snRNPs from nuclear high-salt extracts was carried out as described in Materials and methods, using a 25 ml column of anti-m_3G IgG Protein A – Sepharose. The photograph exhibits extracted RNA-species from: **lane 1**, 0.3 M NH_4Cl/m^7G eluate; **lane 2**, 0.1 M NH_4Cl/m^7G eluate; **lane 3**, nuclear high-salt extract; **lane 4**, flow-through fraction. Each fraction analyzed corresponds to 4×10^7 HeLa cells.

2. In the first step snRNPs were isolated by immune affinity chromatography with anti-m_3G antibodies. All snRNPs U1 to U6 may be quantitatively removed from a high-salt HeLa nuclear extract by the anti-m_3G column (Figure 3, lanes 3 and 4). In agreement with our previous observation (Bringmann *et al.*, 1984), it was possible to fractionate the snRNPs on the anti-m_3G affinity column by sequential elution of the particles with excess nucleosides 7-methylguanosine (m^7G) under differential salt concentrations. [Since the anti-m_3G antibody cross-reacts with non-phosphorylated m^7G (Reuter *et al.*, 1984) this nucleoside may be used instead of m_3G for the desorption of antibody-bound snRNPs.] When the affinity column was eluted with m^7G in the presence of 100 mM NH_4Cl (low-salt m^7G eluate), a fraction of snRNPs was obtained which contained only the snRNP species U1, U2 and U5 (Figure 3, lane 2). This fraction represents ~50 – 60% of the total antibody-bound snRNPs. The U4/U6 RNP particle was only desorbed from the column alongside a further fraction of snRNPs U1, U2 and U5 when the NH_4Cl concentration was raised to ~0.3 M in the m^7G elution buffer (high-salt m^7G eluate) (Figure 3, lane 1).

Further fractionation of snRNPs U1, U2 and U5 contained in the low-salt m^7G eluate proceeds via ion exchange chromatography on DEAE – Sepharose. Following adsorption of the snRNPs to the DEAE – Sepharose at 0.05 M NH_4Cl the column is first washed with a buffer containing 0.075 M NH_4Cl, which separates the snRNPs from the nucleoside m^7G and some minor contaminating proteins. Stepwise elution of the DEAE column with a buffer containing 0.175 M NH_4Cl (DE175 fraction) yields a fraction of essentially pure U1 RNPs. Increasing the elution buffer concentration to about 0.4 M NH_4Cl (DE400 fraction) results in a fraction which comprises predominantly snRNPs U2 and U5 in addition to U1 RNPs. The RNA components of the respective fractions are shown in Figure 4. The reason why U1 RNPs are eluted from the DEAE – Sepharose column at both salt concentrations is not known and has not been further investigated.

In order to remove selectively the fraction of U1 RNPs contained in the DE400 eluate, this eluate was passed over an affinity column which contained immobilized anti-(U1)RNP autoantibodies. The U1 RNPs were quantitatively retained on the affinity column and a flow-through fraction was obtained which contained only the snRNPs U2 and U5 (Figure 5, lane 1).

In a final step the flow-through of the anti-(U1)RNP column containing U2 and U5 RNP was passed over an anti-m^6A immune affinity column. The m^6A-containing U2 RNP was retained on the column, while pure U5 snRNPs were recovered in the flow-through fraction of the anti-m^6A column (Figure 5, lane 2). The antibody-bound U2 RNPs could be quantitatively desorbed from the column by elution with an excess of nucleoside m^6A. This m^6A eluate contained pure U2 RNPs as demonstrated by RNA gel electrophoresis (Figure 5, lane 3).

The yields of the snRNPs in the various purification steps are given in Table I(A). Recoveries of the snRNPs were nearly quantitative when they were directly isolated from nuclear extracts by anti-m_3G affinity chromatography. Loss of snRNPs was always observed when the snRNPs purified in this way were subjected to further fractionation, which is most probably due to the tendency of the pure particles to stick to the Sepharose matrix either by hydrophobic or electrostatic interactions.

Protein composition of snRNPs U1, U2 and U5

The proteins of the fractions containing pure snRNPs U1, U2 and U5, respectively, were analyzed on SDS – polyacrylamide gels by direct Coomassie staining (Figure 6). The three snRNPs share six major polypeptides denoted B′ (29 kd), B (28 kd), D (16 kd), E (12 kd), F (11 kd) and G (9 kd). An additional protein of apparent mol. wt 15.5 kd, which we term D′, is also found to be present in each of the three snRNPs. It should be emphasized that, due to the lower abundance and the similar mol. wt of D′ as compared with D, the two proteins often resolve badly on SDS – polyacrylamide gels, in particular when the gels are overloaded. This may explain why an snRNP protein of similar mol. wt has not been noted previously.

Three proteins of mol. wts 70 kd, 34 kd and 22 kd are uni-

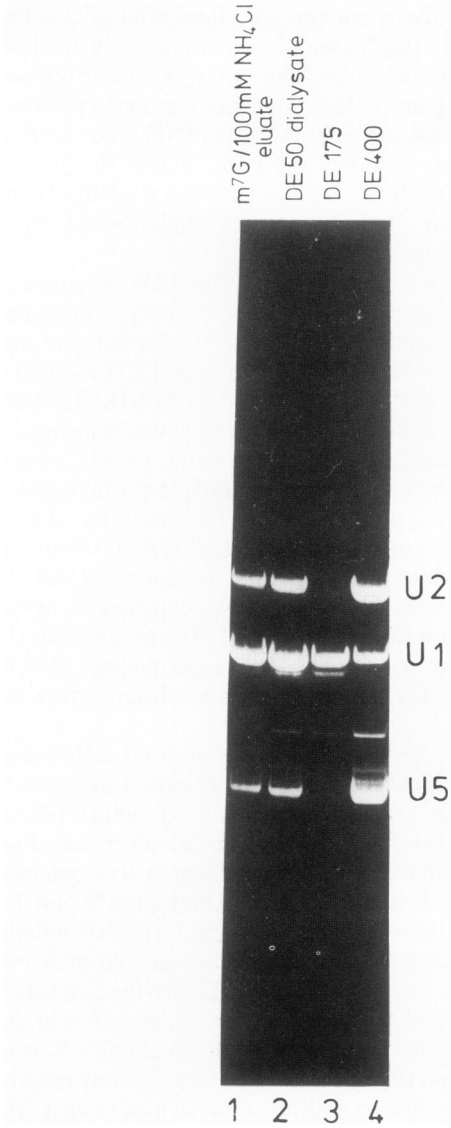

Fig. 4. Separation of snRNP U1 from U2 and U5 RNPs by DEAE chromatography. The snRNPs U1, U2 and U5 contained in the 0.1 M salt/m^{7}G eluate of the anti-m$_3$G affinity column were dialyzed and fractionated on DEAE–Sepharose as outlined in Materials and methods. The photograph shows the gel fractionated RNAs from: **lane 1**, 0.1 M NH_4Cl/m^{7}G eluate; **lane 2**, DE50 dialysate; **lane 3**, DE175 fraction; **lane 4**, DE400 fraction. RNAs were visualized by staining with ethidium bromide.

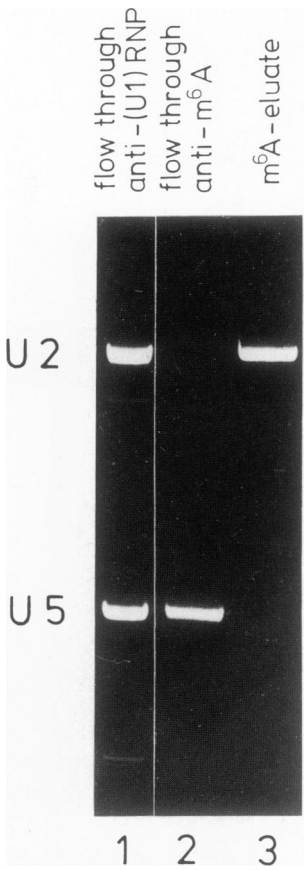

Fig. 5. Separation of snRNPs U2 and U5 by immune affinity chromatography with anti-m^{6}A antibodies. The DE400-fraction was depleted from residual U1 RNPs by immune affinity chromatography using anti-(U1)RNP autoantibodies. For a further fractionation the resulting flow-through fraction containing the snRNPs U2 and U5 was applied to an anti-m^{6}A affinity column and affinity chromatography was carried out as described in Materials and methods. The photograph shows the gel fractionated RNA-species extracted from: **lane 1**, flow-through fraction of the anti-(U1)RNP column; **lane 2**, flow-through fraction of the anti-m^{6}A column; **lane 3**, m^{6}A-eluate. RNAs were visualized by staining with ethidium bromide.

que for the U1 snRNP particle only, the latter two denoted A and C, respectively (Figure 6, lane 2). The amount of C protein found in distinct U1 RNP preparations varies somewhat, which is probably due to the salt sensitivity of its binding to U1 RNPs.

The U2 snRNP contains two unique polypeptides, the A′ protein (33 kd) and a polypeptide of mol. wt 28.5 kd, denoted B″, which in most cases resolves badly from the B protein (Figure 6, lane 3). An example where B″ clearly separates from B is shown in lane 2 of Figure 7A. Interestingly, the U5 RNP particle obtained by our procedure does not appear to contain any unique polypeptide, its protein composition being confined to the seven polypeptides that are also shared by the snRNPs U1 and U2 (Figure 6, lane 4). (The faint band around 21.5 kd in lane 4 is not reproducibly seen in U5 RNP preparations and is therefore not considered to be a genuine snRNP protein.)

It should be noted that all proteins described above, including D′, are also found when total proteins of the starting snRNP frac-

Table I. Recoveries of the snRNPs during isolation

snRNP-containing fractions	Protein content (μg)
(A) Fractionation of snRNPs U1, U2 and U5[a]	
1. 0.1 M NH_4Cl/m^{7}G eluate of anti-m$_3$G column (U1, U2, U5)	1050
2. 0.3 M NH_4Cl/m^{7}G eluate of anti-m$_3$G column (U1–U6)	1920
3. DE175 (isolated U1 RNPs)	400
4. DE400 (U2, U5, (U1))	200
5. Flow-through anti-(U1)RNP column (U2, U5)	140
6. Flow-through anti-m^{6}A column (purified U5 RNPs)	45
7. m^{6}A-eluate (isolated U2 RNPs)	55
(B) Fractionation of snRNPs U2 and U4/U6[b]	
1. m^{6}A eluate of anti-m^{6}A column (U2, U4/U6, (U1))	80
2. Flow-through of mAb D5 column (U4/U6, (U2))	15

[a]Recoveries of snRNPs are expressed in μg snRNP protein. Nuclear extracts from 1.2 × 10^{10} HeLa cells were used as starting material for the initial fractionation of snRNPs on anti-m$_3$G affinity columns (steps 1 and 2). The snRNPs contained in the low-salt m^{7}G eluate (1050 μg) were used for further fractionation (steps 3–7).

[b]Nuclear extracts from 1.2 × 10^9 HeLa cells were used for the initial anti-m^{6}A affinity chromatography.

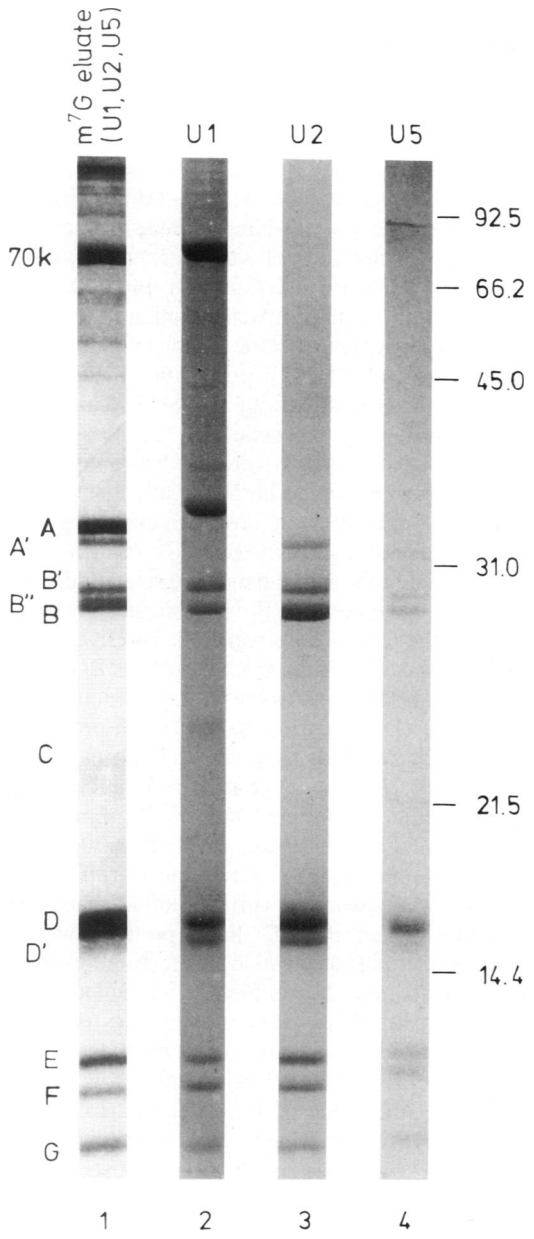

Fig. 6. Protein composition of isolated snRNPs U1, U2 and U5. Individual snRNPs U1, U2 and U5 were purified as schematically shown in Figure 2 and described in detail in Materials and methods. Following phenol extraction of the final snRNP fractions, proteins were precipitated from the organic phase and the interphase by addition of 5 volumes acetone and fractionated on SDS – polyacrylamide gels. The photograph shows the Coomassie-stained proteins obtained from: **lane 1**, 0.1 M NH_4Cl/m^7G eluate (snRNPs U1, U2 and U5); **lane 2**, DE175 fraction (isolated U1 RNPs); **lane 3**, m^6A eluate of the anti-m^6A column (isolated U2 RNPs); **lane 4**, flow-through of the anti-m^6A column (isolated U5 RNPs).

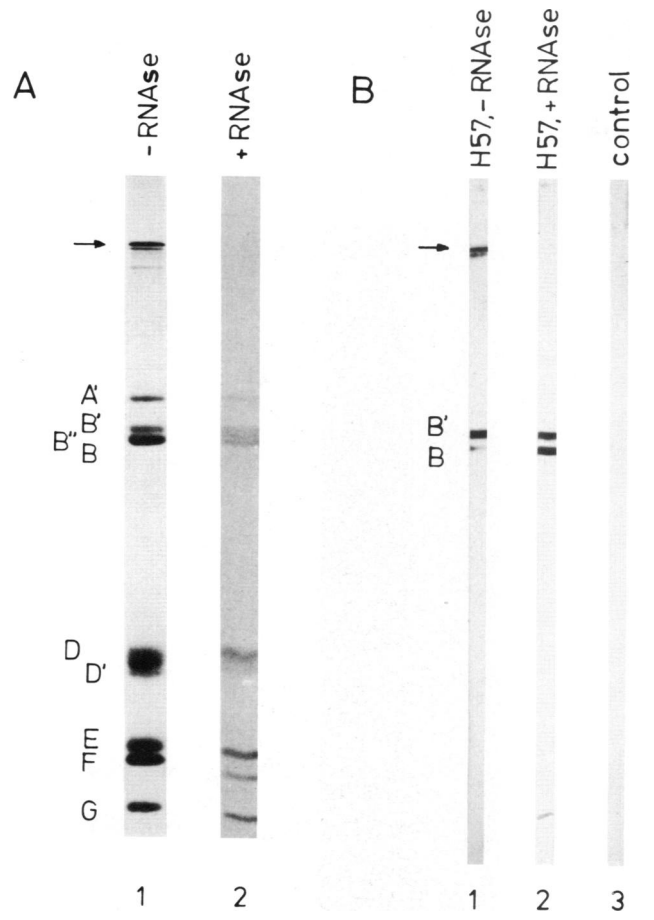

Fig. 7. Evidence for a SDS-resistant U2 RNA – protein complex. Isolated U2 RNPs were digested with RNAse A and T_1 (1 mU and 10 mU per 10 μg of U2 RNP protein respectively) for 30 min at 37°C. Proteins prepared from digested and undigested samples were separated by SDS – polyacrylamide gel electrophoresis and visualized directly by successive staining with Coomassie and silver **(panel A)**. In a separate experiment the SDS – polyacrylamide gel fractionated proteins from RNase treated and non-treated U2 RNPs were blotted onto nitrocellulose and probed with a monoclonal antibody specific for the proteins B/B′ (mAb H57) essentially as described by Reuter and Lührmann (1986) **(panel B)**. The photograph in panel A shows directly stained U2 RNP proteins before **(lane 1)** and after **(lane 2)** digestion of the RNA with nucleases. Panel B exhibits the immunoblot analysis of U2 RNP proteins with mAb H57 before (lane 1) and after RNA-digestion (lane 2). **Lane 3** shows a control with a monoclonal antibody non-related to snRNP proteins.

tion, i.e. the low-salt m^7G eluate containing a mixture of the snRNPs U1, U2 and U5, are examined by SDS – polyacrylamide gel electrophoresis (Figure 6, lane 1). This excludes the possibility that some of the polypeptides might have been generated by proteolytic degradation during the various fractionation steps necessary to purify the individual snRNP species.

Except for the protein D′ (see our discussion above) the U1 RNP protein composition determined here is in good agreement with previous reports on the polypeptides contained in biochemically fractionated U1 snRNPs (Hinterberger *et al.*, 1983; Kinlaw *et al.*, 1983; Billings and Hoch, 1984; Lelay-Taha *et al.*, 1986). The proteins A′ and/or B″ have previously been noted in snRNP fractions which were enriched in snRNP U2 (Hinterberger *et al.*, 1983; Kinlaw *et al.*, 1983; Billings and Hoch, 1984; Bringmann *et al.*, 1983). The notion that the two polypeptides are U2 specific is most strongly supported by the finding that autoantibodies from SLE patients as well as monoclonal antibodies against A′ or B″ selectively precipitate U2 snRNPs (Mimori *et al.*, 1984; Habets *et al.*, 1985; Reuter *et al.*, 1986).

A discrepancy may be noted between the protein composition of the U5 RNPs described here and those isolated by Lelay-Taha *et al.* (1986). They identified a 25-kd protein which co-fractionated with U5 RNA during various purification steps, including CsCl gradient centrifugation, and which, therefore, may represent a candidate for a U5-specific protein. Furthermore, their U5 RNPs contained the proteins D, E, F and G while the polypeptides B/B′ appeared to be absent or only present in marginal amounts.

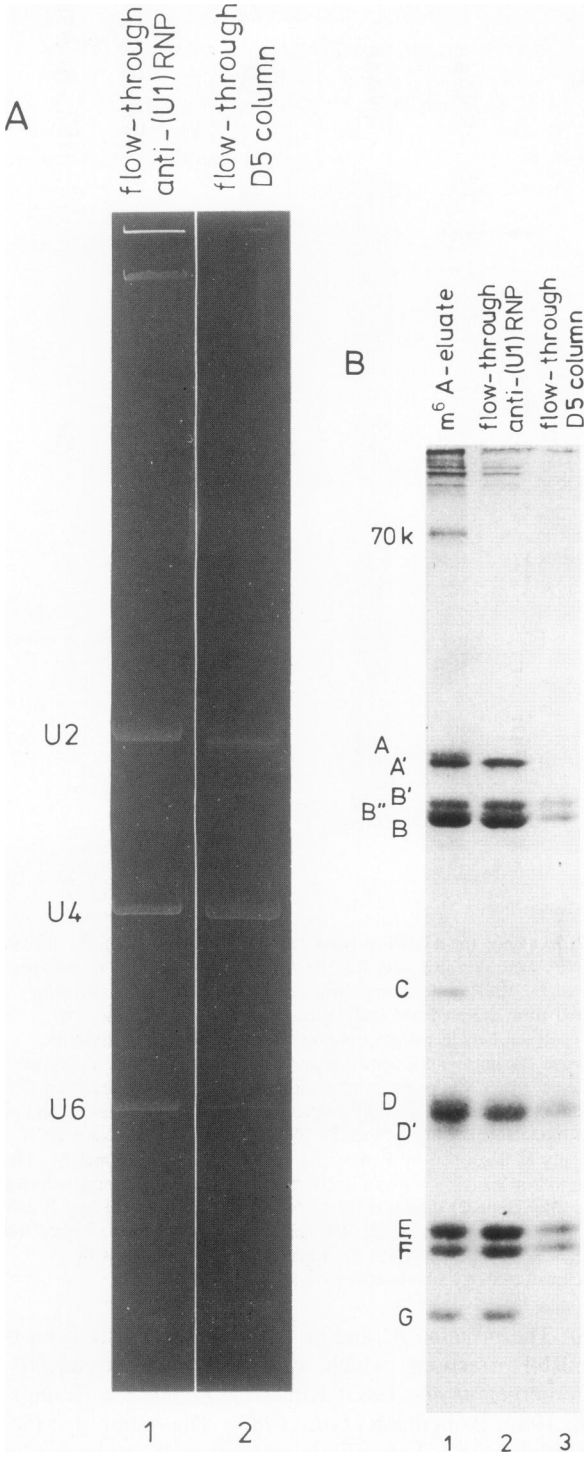

Fig. 8. Protein composition of isolated U4/U6 RNPs. snRNPs U2 and U4/U6 were purified from nuclear high-salt extracts of HeLa cells by immune affinity chromatography with m^{6}A-specific IgGs (see Figure 1). Following depletion of the m^{6}A eluate from residual U1 RNPs by immune affinity chromatography with anti-(U1)RNP autoantibodies U2 RNPs were separated from snRNPs U4/U6 by immune affinity chromatography using the (U1, U2)RNP-specific monoclonal antibody D5 (Reuter *et al.*, 1986). **Panel A** shows the gel fractionated RNA species extracted from: **lane 1**, flow-through fraction of the anti-(U1)RNP column; **lane 2**, flow-through fraction of the mAb D5 column (enriched U4/U6 RNPs). The photograph in **panel B** represents the SDS-gel fractionated proteins visualized by Coomassie-staining from: **lane 1,** m^{6}A eluate (snRNPs U2, (U1), U4/U6); **lane 2**, flow-through fraction of the anti-(U1)RNP column (snRNPs U2 and U4/U6); **lane 3**, flow-through fraction of the mAb D5 column (enriched snRNPs U4/U6).

A U2 RNA-protein complex resistant to SDS treatment

When the SDS – polyacrylamide gel fractionated proteins of the individual snRNPs were displayed by the more sensitive silver stain, additional bands became visible which were normally not visualized by the Coomassie stain. This was particularly true of U2 RNPs, which reproducibly exhibited a doublet or sometimes a triplet with an apparent mol. wt of ~60 kd (Figure 7, lane 1) in addition to the protein bands already observed by the Coomassie stain. When the gel was overloaded with U2 RNP proteins, these bands were also faintly visible in the Coomassie stain. While in the first place it was tempting to suggest that the 60-kd bands might represent some minor U2 specific proteins, they turned out to be U2 RNA – protein complexes which were resistant to treatment with phenol and SDS. This was demonstrated by the following experiments.

(i) When U2 RNP proteins were used as antigenic material for immunoblots with various anti-Sm patient sera, we noticed staining of the 60-kd bands with those sera exhibiting strong titers of antibodies against the proteins B and B′. Our notion that the proteins contained in the 60-kd bands are structurally related to, or identical with, the proteins B/B′ was convincingly corroborated by the fact that the 60-kd bands were also reactive with a monoclonal antibody specific for the snRNP proteins B/B′ (Figure 7B, lane 1). The mAb H57 was isolated from a mouse immunized with snRNPs U1 (Daser *et al.*, manuscript in preparation). (ii) Staining of the 60-kd bands by mAb H57 is sensitive to RNase treatment (Figure 7B, lane 2). Furthermore, RNase treatment does not yield additional protein bands on the immunoblots apart from proteins B and B′. This indicates that the 60-kd bands represent RNA – protein complexes which most probably contained the B and/or B′ polypeptides. (iii) The 60-kd bands were also RNase-sensitive when the U2 RNP proteins were directly displayed by SDS – polyacrylamide gel electrophoresis and silver stianing (Figure 7A, lane 2). (iv) Finally, our notion that the 60-kd bands represent U2 RNA – protein complexes is supported by our findings that these bands are only observed upon gel fractionation of proteins from fractions containig U2 RNPs and that the RNA isolated from the 60-kd bands migrates as U2 RNA in denaturing polyacrylamide gels upon treatment with proteinase K (not shown). The question of whether the observed RNP complexes represent direct interactions between U2 RNA and proteins B and/or B′, or whether additional snRNP proteins are also present has not been investigated any further and remains open at this point.

When proteins from isolated U5 RNPs were displayed by SDS – polyacrylamide gel electrophoresis and staining with silver a band migrating at ~26 kd was sometimes observed. This band was also RNase sensitive and most probably represents SDS-resistant U5 RNA protein complexes. However, due to the low abundance of this complex we could not investigate the proteins contained in this band by immunoblotting.

Protein composition of snRNPs U4/U6 and conclusions

For the purification of individual U4/U6 RNP particles snRNPs were isolated from HeLa nuclear extracts by affinity chromatography with anti-m^{6}A IgG in the first step. The m^{6}A eluate of the affinity column yields a fraction which is essentially free of U5 RNPs and contains predominantly snRNPs U2 and U4/U6 in addition to minor amounts of U1 RNPs, as already demonstrated by RNA gel electrophoresis in Figure 1. The U1 RNPs were removed by passage of the m^{6}A eluate over an anti-(U1)RNP autoantibody affinity column, yielding a fraction con-

taining essentially only snRNPs U2 and U4/U6 (Figure 8A,lane 1).

In order to remove the U2 RNPs the flow-through fraction of the anti-(U1)RNP affinity column was passed over an immune affinity column which was prepared with a monoclonal antibody that cross-reacts with the U2-specific protein B″ and the U1-specific polypeptide A (mAb D5). This antibody selectively precipitates snRNPs U1 and U2 in immune precipitation assays (Reuter *et al.*, 1986). RNA analysis of the flow-through of the D5 column showed that most of the U2 RNPs had been removed by this step, the U4/U6 RNPs representing ~70–80% of this RNP fraction (Figure 8A, lane 2). The residual amounts of U2 RNPs could not be removed by a second passage of the first D5 flow-through over the D5 column, suggesting that this U2 RNP fraction lacked protein B″. The analysis of the proteins contained in this fraction supports this notion. Neither protein B″ nor A′ are found in the flow-through of the D5 column while they are both present in the snRNP fraction passed over the D5 column (Figure 8B). When compared with the U4 RNA, the intensity of the U6 RNA band decreases with additional purification steps as a result of the exceptional nuclease sensitivity of U6 RNA even as part of the U4/U6 RNP particle. Further attempts to obtain pure U4/U6 RNP particles from the D5 flow-through fraction by separation methods such as ion exchange and heparin Sepharose chromatography as well as by differential centrifugation were not successful. The recoveries of the U4/U6 RNP particles during isolation are given in Table I(B).

The protein composition of the final U4/U6 RNP containing fraction is shown in lane 3 of Figure 8B. The proteins B′, B, D, E, F and G are clearly visible in the SDS–polyacrylamide gel. A faint band migrating below protein D may also be recognized, indicating that D′ is also contained in the U4/U6 RNP particle in addition to the other common polypeptides. As was the case for U5 RNPs we failed to detect U4/U6-characteristic proteins in this fraction. Since such a putative protein was also not observed in the original m^{6}A eluate of the anti-m^{6}A affinity column (Figure 8B, lane 1), this excludes the possibility that it might have been degraded by proteolysis during the further fractionation steps. Thus the protein composition of U4/U6 RNPs appears to be confined to the common polypeptides.

The absence of unique proteins from snRNPs U5 and U4/U6 could indicate that the exertion of the two particles' specific functions in the processing of nuclear pre-mRNAs is governed primarily by their RNA components. We note, however, that our failure to detect U5- and U4/U6-characteristic proteins does not exclude their existence, in principle, in the cell. In contrast to the situation for the U1- and U2-specific proteins they could be so loosely bound to the snRNPs U5 and U4/U6 that they are quantitatively dissociated from the two particles during the extraction of the HeLa cell nuclei with 0.5 M salt buffer (see Materials and methods).

Taken together, we have identified 12 distinct polypeptides as constituents of the snRNPs U1, U2, U5 and U4/U6 from HeLa cells. The protein composition of the individual purified snRNP particles is summarized in Table II. In so far that we cannot formally exclude the presence of minor variants of some of the common snRNP proteins which would not resolve in the one-dimensional SDS–polyacrylamide gel, the 12 proteins given in Table II should be considered the minimum number of distinct polypeptides contained in the major nucleoplasmic snRNPs. A final answer to this question may come from the analysis of the respective snRNP protein genes.

Table II. Protein composition of individual snRNPs U1, U2, U5 and U4/U6

snRNP particle	Common proteins							Characteristic proteins
	B′	B	D	D′	E	F	G	
U1	+	+	+	+	+	+	+	70 kd, A, C
U2	+	+	+	+	+	+	+	A′, B″
U5	+	+	+	+	+	+	+	
U4/U6	+	+	+	(+)	+	+	+	

Materials and methods

Cell growth

HeLa S3 cells were maintained in suspension culture at 37°C in Eagle's minimum essential medium supplemented with 5% (v/v) newborn calf serum, 50 μg/ml penicillin and 0.1 mg/ml streptomycin. Cells were grown logarithmically by daily dilution and were harvested at a density of 5–6 × 10^5/ml.

Preparation of affinity columns

Rabbit anti-m$_3$G and anti-m^{6}A sera were obtained as described previously (Lührmann *et al.*, 1982; Bringmann and Lührmann, manuscript submitted). Human anti-(U1)RNP sera were obtained from patients diagnosed as suffering from SLE and were characterized for the selective reactivity with U1 snRNPs by immunoprecipitation and immunoblotting assays. Total IgG was prepared from anti-m$_3$G and anti-(U1)RNP sera by fractionation with $(NH_4)_2SO_4$ and subsequent chromatography on Sephadex G150 (Pharmacia). Anti-m^{6}A IgGs were affinity purified on m^{6}A-aminohexyl–Sepharose 4B columns, and m^{6}A-specific antibodies were eluted by competition with 25 mM m^{6}A. The m^{6}A eluate was concentrated by $(NH_4)_2SO_4$-precipitation and further separated into IgM, IgG and m^{6}A by gel filtration on Sephadex G150. IgGs were coupled to Protein A–Sepharose CL-4B (Pharmacia) according to Gersten and Marchalonis (1978). Approximately 20 mg IgG could be immobilized per ml gel using this method. The (U1,U2)RNP-specific monoclonal antibody D5 (Reuter *et al.*, 1986) was isolated from supernatants of the hybridoma culture using the monoclonal antibody purification system (MAPS^{TM}) purchased from Bio Rad Laboratories. For preparation of immune affinity columns purified D5 IgGs were immobilized to CNBr-activated Sepharose 4B (Pharmacia) according to the manufacturer's protocol.

Isolation of snRNPs U1, U2 and U5

All buffers were autoclaved before use and all operations were carried out on ice or at 4°C. All buffers used for the extraction of snRNPs, from HeLa cell nuclei and for the immune affinity chromatography of the snRNPs contained the following protease inhibitors: phenylmethylsulfonyl fluoride (PMSF) (0.2 mM) aproteinin (10 μg/ml) and leupeptin (5 μg/ml). The preparation of the nuclear extract was as described by Bringmann *et al.* (1983), but without the low-speed centrifugation step after extraction of isolated nuclei. One hundred ml of the final high salt nuclear extract were applied to an anti-m$_3$G affinity column (bed volume 25 ml). After overnight passage at a flow rate of 0.15 ml/min (using a P1-pump, Pharmacia) the affinity column was washed with two bed volumes TMN300 buffer (20 mM Tris–HCl pH8, 10 mM $MgCl_2$, 300 mM NH_4Cl, 1 mM DTT) followed by two volumes of TMN100 buffer (this buffer is the same as TMN300 except for the presence of 100 mM NH_4Cl). snRNPs U1, U2 and U5 were desorbed from the anti-m$_3$G column by elution with 25 ml TMN100 buffer containing 15 mM m^{7}G (flow rate 0.5 ml/min). Four-ml fractions were collected. Following a 25-ml TMN100 wash the affinity column was eluted a second time using 25 ml TMN300 buffer containing 15 mM m^{7}G. This high-salt m^{7}G-eluate contained the U4/U6-complex and a further fraction of snRNPs U1, U2 and U5. After a 30-ml wash of buffer TMN300 the column was regenerated by washing with 50 ml 6 M urea/TMN300 and 150 ml TMN300 containing 0.02% (w/v) NaN_3. The elution profile of snRNPs was monitored by protein determination according to Bearden (1978). snRNP-containing fractions of the 100 mM eluate were pooled, frozen in liquid nitrogen and stored at −80°C. Subsequently this material was dialyzed for 5 h against 80 volumes of DE50 buffer (20 mM Tris–HCl pH7, 15 mM $MgCl_2$, 50 mM NH_4Cl, 0.2 mM DTT) with a change of dialysis buffer after 2.5 h.

For a further fractionation the resulting U1-, U2- and U5-containing dialysate was applied to a DEAE–Sepharose (Pharmacia) column (1 ml bed volume/4 mg protein applied, flow rate 1 ml/min) which had been pre-equilibrated with DE50 buffer. Under these conditions all snRNPs bind to the DEAE matrix. We were unable to detect any snRNAs in the flow-through (not shown). However, some basic high mol. wt proteins still contaminating the m^{7}G-eluate could be separated here and in the following DE75 wash without loss of snRNPs. Following a wash with three bed volumes of DE75 buffer (salt concentrtions as in DE50 but containing 75 mM NH_4Cl), snRNP U1 was eluted with three volumes of DE175

buffer (as DE50 but containing 175 mM NH_4Cl); 0.5-ml fractions were collected. The peak fraction contained snRNP U1 with a protein concentration in the range 0.6–0.8 mg/ml. Subsequently snRNPs U2, U5 and a residual amount of U1 were eluted with DE400 buffer (as DE50 but with 400 mM NH_4Cl). Again 0.5 ml fractions were collected and the protein concentration was about 0.15 mg/ml. The pooled DE400 eluate was directly applied to an anti-(U1)RNP column (bed volume 1 ml) equilibrated in DE400 buffer (flow rate 0.1 ml/min). The flow-through obtained is essentially depleted from snRNP U1. The anti-(U1)RNP column was washed with 3 ml DE400 buffer and regenerated by a 5-ml wash of 6 M urea in DE400 followed by equilibration in DE400 containing 0.02% (w/v) NaN_3. Finally, the flow-through fraction was pooled with the first DE400 wash fraction and loaded on a 2-ml anti-m^6A affinity column which had been pre-equilibrated in TMN300 buffer. Following the sample passage (flow rate 0.1 ml/min) the column was washed with 6 ml TMN300 buffer. Two-ml fractions were collected. The flow-through and the first wash fraction contained pure snRNPs U5. Desorption of snRNP U2 was achieved by elution with 2 ml 10 mM m^6A in TMN300 buffer followed by 4 ml TMN300 only. The affinity column was regenerated with 6 M urea in TMN300 and subsequent equilibration in TMN300/0.02% (w/v) NaN_3. The U2- and U5-containing fractions exhibited protein concentration of about 10 μg/ml and could be concentrated by successive dialysis and DEAE chromatography (see above).

RNA and protein analysis

Samples were made 0.5% (w/v) in SDS and were extracted with 1 volume of PCA (phenol, chloroform, isoamylalcohol 50:50:1). Following phase separation by centrifugation RNAs were prepared from the aqueous phase by ethanol precipitation. Proteins were obtained from the organic phase by precipitation with 5 volumes of acetone.

RNAs were fractionated on 10% polyacrylamide gels containing 7 M urea, 10 mM Tris–borate pH 8.3 and 2 mM EDTA (TBE). Analysis of proteins was carried out by SDS– gel electrophoresis as described previously (Laemmli, 1970). RNA bands were visualized by staining with ethidium bromide (0.5 μg/ml) in TBE buffer or with silver (Merrill *et al.*, 1983). Protein bands were detected by successive staining with Coomassie G250 and silver.

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