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Purification of snRNPs U1, U2, U4, U5 and U6 with 2,2,7-trimethylguanosine-specific antibody and definition of their constituent proteins reacting with anti-Sm and anti-(U1)RNP antisera

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Small nuclear ribonucleoprotein particles (snRNPs) of the U-snRNP class from Ehrlich ascites tumor cells were purified in a one-step procedure by affinity chromatography with antibodies specific for 2,2,7-trimethylguanosine ($m_3^{2,2,7}G$), which is part of the 5′-terminal cap structure of snRNAs U1–U5. Antibody-bound snRNPs are desorbed from the affinity column by elution with excess nucleoside $m_3^{2,2,7}G$; this guarantees maintenance of their native structure. The snRNPs U1, U2, U4, U5 and U6 can be recovered quantitatively from nuclear extracts by this procedure. Co-isolation of U6 snRNP must be due to interactions between this and other snRNPs, as anti-$m_3^{2,2,7}G$ antibodies do not react with deproteinized U6 snRNA. We have so far defined nine proteins of approximate mol. wts. 10 000, 12 000, 13 000, 16 000, 21 000, 28 000, 32 000, 34 000 and 75 000. Purified snRNPs react with anti-(U1)RNP and with anti-Sm antisera from patients with mixed connective tissue disease and from MRL/l mice. As determined by the protein blotting technique, six of the snRNP polypeptides, characterized by apparent mol. wts. 13 000, 16 000, 21 000, 28 000, 34 000 and 75 000, bear antigenic determinants for one or the other of the above autoantibody classes. This suggests strongly that the U-snRNPs produced by the procedure described here are indeed representative of the snRNPs in the cell. With highly purified snRNPs available, investigation of possible enzymic functions of the particles may now be undertaken.

Key words: small nuclear ribonucleoprotein/anti-$m_3^{2,2,7}G$ IgG/affinity chromatography/lupus erythematosus/protein blotting

Introduction

The U class of small nuclear RNAs (U-snRNAs) encompasses a group of metabolically stable snRNAs which are highly abundant in eucaryotic cells. Six major species may be distinguished, of which one is nucleolus-specific (U3), while the others are found in the nucleoplasm. Except for U6, the snRNAs are marked by a 5′-terminal cap structure which contains the unusual nucleoside 2,2,7-trimethylguanosine ($m_3^{2,2,7}G$) at its 5′ end (for a recent review, see Busch *et al.*, 1982). The snRNAs are not present in the nucleoplasm as naked RNA molecules but probably exist as discrete ribonucleoprotein particles (U-snRNPs) (Raj *et al.*, 1975; Lerner and Steitz, 1979).

The discovery that snRNPs react with anti-(U1)RNP and anti-Sm antisera from patients with lupus erythematosus or mixed connective tissue disease (MCTD) has revealed a connection between U-snRNPs and autoimmune diseases. While anti-(U1)RNP precipitates only snRNP species U1, anti-Sm autoantibodies react with U1, U2, U4, U5 and U6. The antigenic determinants reacting with both classes of antibodies are located on the protein part of the snRNPs (Lerner and Steitz, 1979). These antigenic determinants of the snRNP proteins and the structures of the various snRNAs are highly conserved between species (Lerner and Steitz, 1979; Busch *et al.*, 1982).

While the exact function of the various snRNPs is still unknown, several lines of evidence suggest a role for at least some of them during processing of heterogeneous nuclear RNA (hnRNA). The first hint of this possibility came from reports demonstrating that several U-snRNAs co-sedimented with hnRNP particles (Sekeris and Niessing, 1975; Gallinaro and Jacob, 1979). Some of the snRNAs (U1 and U2) could even be cross-linked *in situ* to hnRNA by a psoralen derivative (Calvet and Pederson, 1981; Calvet *et al.*, 1982), indicating base pair interaction between the respective RNAs. Finally, much attention has been focussed on a possible role of snRNPs in splicing of pre-mRNA. It has been proposed that by base pairing with the consensus 5′ and 3′ ends of an intron, U1 snRNA/RNP might ensure the proper alignment for splicing of the exon boundaries of the respective pre-mRNAs (Lerner *et al.*, 1980; Rogers and Wall, 1980).

One of the prerequisites for a further analysis of structure-function relationships of snRNPs is the development of a procedure for the isolation of native particles on a preparative scale. Recently we showed that the $m_3^{2,2,7}G$-containing cap structures of snRNAs are accessible for $m_3^{2,2,7}G$-specific antibodies in intact snRNPs (Bringmann *et al.*, 1983). Here we report on the purification of snRNPs from Ehrlich ascites tumor cells by affinity chromatography with anti-$m_3^{2,2,7}G$ IgGs. The major advantage of this one-step procedure is the possibility of eluting the antibody-bound snRNPs from the affinity column with excess nucleoside $m_3^{2,2,7}G$, which guarantees maintenance of the native structure of the snRNPs. The purified particles have been characterized with regard to their protein and RNA composition. Furthermore, the proteins from isolated snRNPs which carry antigenic determinants reacting with anti-(U1)RNP and with anti-Sm autoantibodies have been determined by the protein blot technique.

Results

Preparation of snRNP-containing nuclear extracts

Nuclei from Ehrlich ascites tumor cells were isolated using a low ionic strength buffer containing non-ionic detergent (Zieve and Penman, 1981). Nucleoplasmic ribonucleoprotein particles were obtained by salt extraction of purified cell nuclei according to the procedure of Weinberg and Penman (1968) but without the DNase hydrolysis step. Following separation of the chromatin by centrifugation at 23 000 *g*, the resulting supernatant was subjected to a second high speed centrifugation step so as to remove most of the high mol. wt. RNAs/RNPs. The identification of the small RNA species in both supernatants is summarized in the electropherogram of Figure 1. In the high speed supernatant, the snRNAs U1a, U1b, U2, U4, U5 and U6 predominate; in addition, some

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Fig. 1. RNA content of fractions obtained during preparation of nuclear extracts from Ehrlich ascites tumor cells. Salt extraction of nuclei from ^{32}P-labelled Ehrlich ascites cells and polyacrylamide gel electrophoresis of phenolized RNAs were performed as described in Materials and methods. Preparation of total RNA from sonicated cells was performed according to Lerner and Steitz (1979). The autoradiograph shows the RNAs extracted from: **lane 1**, cell sonicate equivalent to 5 x 10^5 cells; **lane 2**, supernatant 1 of nuclear salt extract equivalent to 5 x 10^6 cells; **lane 3**, supernatant 2 of nuclear salt extract equivalent to 1 x 10^7 cells.

tRNAs and the low mol. wt. rRNAs are present (Figure 1, lane 3). The snRNA content of this supernatant generally amounts to ~70% of total nucleoplasmic snRNAs, in good agreement with the results reported by Weinberg and Penman (1968). However, variation in recovery was sometimes observed for snRNA species U5 and U6. The high speed supernatant was taken as starting material for isolation of snRNPs.

Isolation of snRNPs from nuclear extracts by affinity chromatography with anti-$m_3^{2,2,7}G$ IgGs

Purified anti-$m_3^{2,2,7}G$ IgG was bound to Protein A-Sepharose and immobilized using the cross-linking agent dimethyl suberimidate (Gersten and Marchalonis, 1978). The ability of the covalently bound anti-$m_3^{2,2,7}G$ IgG to bind antigen was determined with ^{14}C-labelled 2,2,7-trimethylguanosine. 100 mg IgG cross-linked to 5 ml of Protein A-Sepharose bound 15 nmol of $[^{14}C]m_3^{2,2,7}G$ which means that ~1.1% of the IgG was specific for $m_3^{2,2,7}G$. The antibody-bound $[^{14}C]m_3^{2,2,7}G$ could be eluted quantitatively from the affinity column with excess unlabelled $m_3^{2,2,7}G$.

The high efficiency of the anti-$m_3^{2,2,7}G$ affinity column in binding snRNPs was demonstrated by RNA gel electrophoresis. As shown in Figure 2A, the snRNPs may be removed quantitatively from nuclear extracts of ^{32}P-labelled Ehrlich ascites tumor cells (Figure 2A; snRNP species U5 and U6 were under-represented in the nuclear extract used for this experiment). Most importantly, ~90% of the antibody-bound snRNPs could be recovered from the column by elution with excess nucleoside $m_3^{2,2,7}G$. The snRNPs thus obtained are essentially uncontaminated by other low mol. wt. RNAs (Figure 2A, lane 3).

An interesting change with regard to the relative efficiency in adsorption to the affinity column of the various snRNPs is observed if the snRNP-binding capacity of the column is exceeded. Under these conditions, snRNP species U1 is still completely removed from the nuclear extract while the other snRNPs are only partially retained (Figure 2B, lanes 2 and 3). This shows that U1 snRNP is much more strongly bound than the other snRNPs. The binding capacity for snRNPs of anti-$m_3^{2,2,7}G$ affinity gel is about one third of its capacity for the free nucleoside $m_3^{2,2,7}G$. Two possibilities may explain this observation: (i) only a fraction of anti-$m_3^{2,2,7}G$ antibodies which bind the isolated nucleoside $m_3^{2,2,7}G$ retain high apparent affinity for this moiety as part of the intact snRNA cap (Lührmann *et al.*, 1982); (ii) some antibodies on the affinity column may not bind the bulky snRNPs owing to steric hindrance by the Sepharose matrix.

It is important to note that under either condition snRNP species U6 is isolated together with the other snRNPs, even though no $m_3^{2,2,7}G$-containing cap structure is present at the 5′ terminus of U6 snRNA (Harada *et al.*, 1980; Epstein *et al.*, 1980) and anti-$m_3^{2,2,7}G$ IgGs do not react with deproteinized U6 snRNA (Bringmann *et al.*, 1983). Thus, the snRNP preparation obtained by this procedure includes all snRNP species U1a, U1b, U2, U4, U5 and U6. Immune precipitates obtained after reaction of purified snRNPs with anti-Sm antisera contained all the snRNAs U1a, U1b, U2, U4, U5 and U6 (Figure 2B, lane 4). Since anti-Sm autoantibodies recognize antigenic determinants solely on the protein moiety of snRNPs (Lerner and Steitz, 1979), this demonstrates that genuine snRNPs are obtained by our procedure.

Protein composition of purified snRNPs

snRNPs were isolated by affinity chromatography with anti-$m_3^{2,2,7}G$ antibodies on a scale sufficiently large to allow the characterization of unlabelled snRNP proteins. Conditions were chosen for quantitative recovery of the various snRNPs from nuclear extracts (see above). Operation of the affinity column on a semi-preparative scale did not reduce the purity of isolated snRNPs, as verified by RNA gel electrophoresis (not shown).

Polypeptides from purified snRNPs were displayed by SDS-polyacrylamide gel electrophoresis and successive Coomassie blue and silver staining (Figure 3). Nine proteins with approximate mol. wts. of 10 000, 12 000, 13 000,

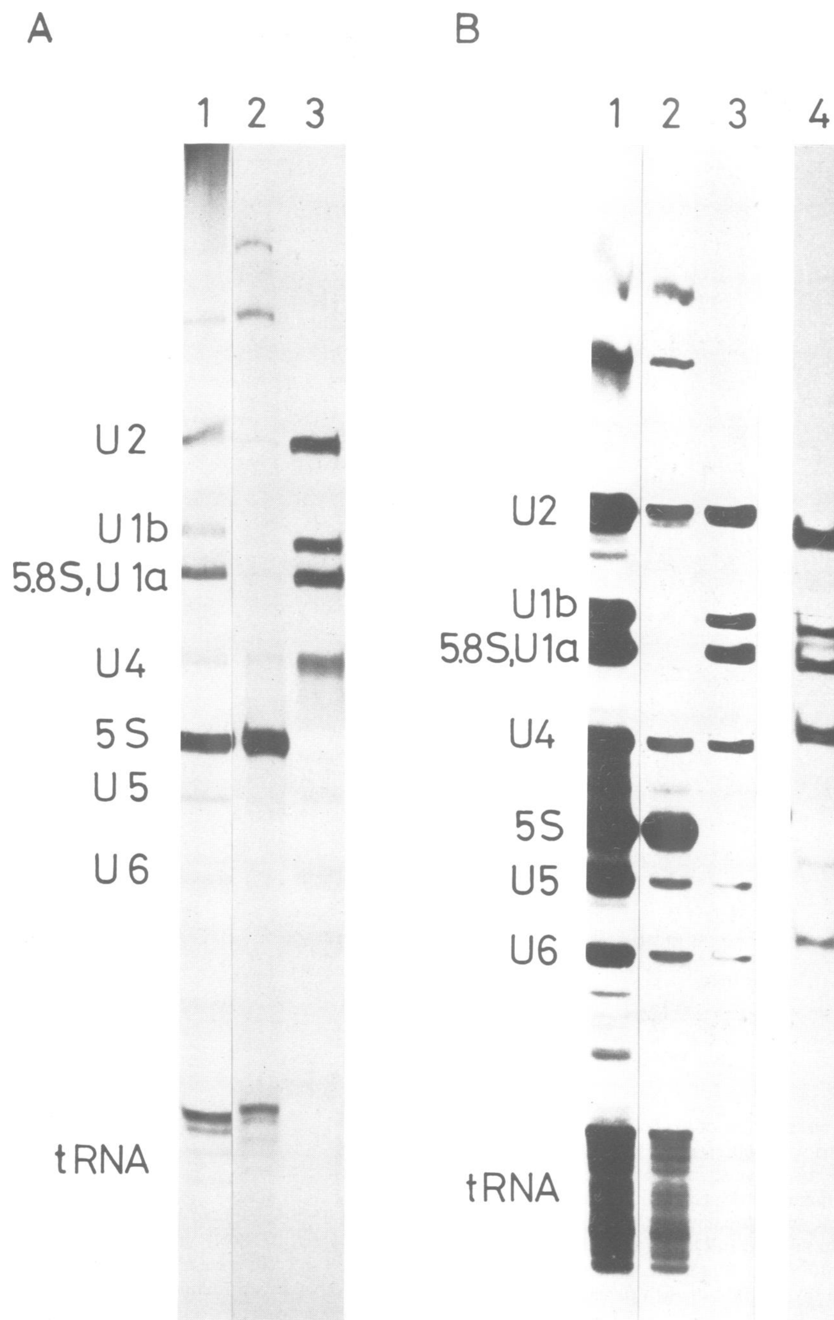

Fig. 2. Polyacrylamide gel electrophoresis of RNAs from fractions obtained during affinity chromatography of snRNPs with anti-$m_3^{2,2,7}G$ antibodies. Affinity chromatography of snRNPs from nuclear salt extracts (supernatant 2) equivalent to 5 x 10^7 **(panel A)** or 1 x 10^8 **(panel B)** ^{32}P-labelled Ehrlich ascites tumor cells was performed as outlined in Materials and methods, using a 1 ml column of anti-$m_3^{2,2,7}G$ Protein A-Sepharose. The autoradiographs in both panels show the RNAs extracted from: **lane 1**, nuclear salt extract used as starting material; **lane 2**, material not retained by the anti-$m_3^{2,2,7}G$ column; **lane 3**, $m_3^{2,2,7}G$ eluate (snRNPs). The various lanes exhibit RNAs extracted from samples, equivalent to 1.5 x 10^7 cells except for **lane 1** in **panel A**, which exhibits RNA from 5 x 10^6 cells. **Lane 4** in **panel B** exhibits the RNAs extracted from immune precipitates obtained after reaction of purified snRNPs ($m_3^{2,2,7}G$ eluate from affinity column) equivalent to 1.5 x 10^7 cells, with anti-Sm serum using *Staphylococcus aureus* cells (Kessler, 1975). The anti-Sm antiserum was the same as used for the protein blotting shown in Figure 4, lane 8).

16 000, 21 000, 28 000, 32 000, 34 000 and 75 000 were reproducibly found in our snRNP preparations. Individual proteins differ considerably in relative abundance, the polypeptides of approximate mol. wts. 16 000, 28 000 and 34 000 being the most prominent. The low staining intensity of the 21 000 and 32 000 proteins in the SDS gels indicate clearly that they are under-represented (Figure 3). It is interesting to note that, depending on the snRNP preparation, the 21 000 protein is seen either as a broad band or – less often – as a faint double band. The reason for this is unknown.

Identification of the snRNP proteins reacting with anti-(U1)RNP and anti-Sm autoantibodies

The antigenic activity of the proteins from purified snRNPs was investigated by the protein blotting technique (Towbin *et al.*, 1979) using autoimmune sera either from patients with MCTD or from MRL/l mice. Such mice spontaneously develop an autoimmune disease very similar to

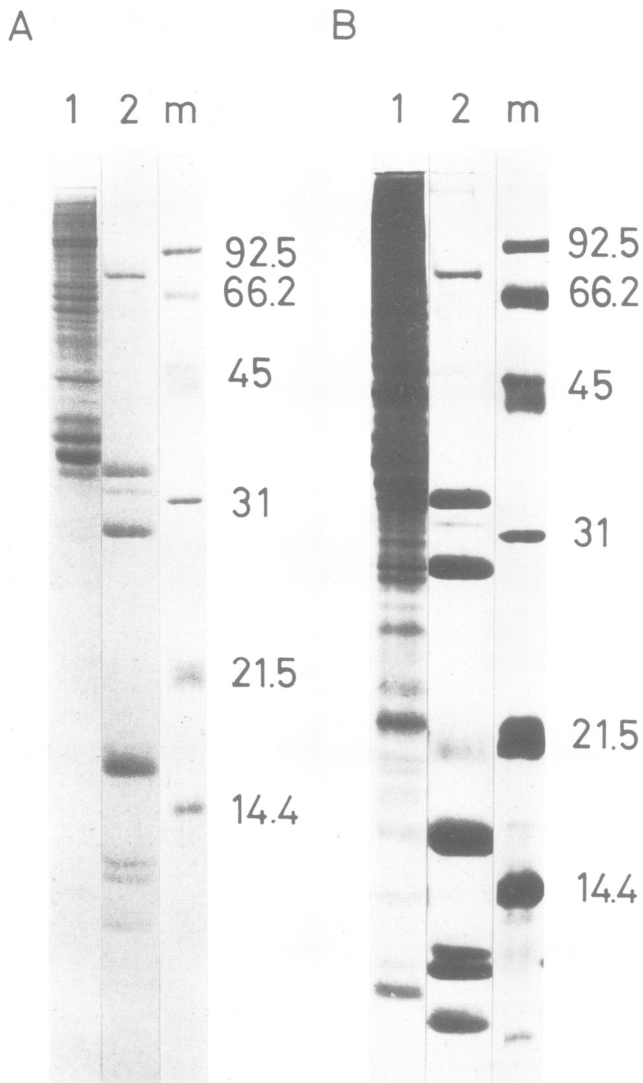

Fig. 3. SDS-polyacrylamide gel electrophoresis of proteins from nuclear extract and purified snRNPs. Proteins were separated by SDS-polyacrylamide gel electrophoresis and revealed by successive staining with Coomassie brilliant blue **(panel A)** and silver **(panel B)** (Materials and methods). **Lane 1** shows proteins from nuclear extracts equivalent to 1 x 10^6 cells (20 μg); **lane 2** shows proteins from purified snRNPs ($m_3^{2,2,7}G$ eluate of the affinity column) equivalent to 8 x 10^7 cells (10 μg); **lane 3** shows marker proteins whose mol. wts. are given on the right of each panel.

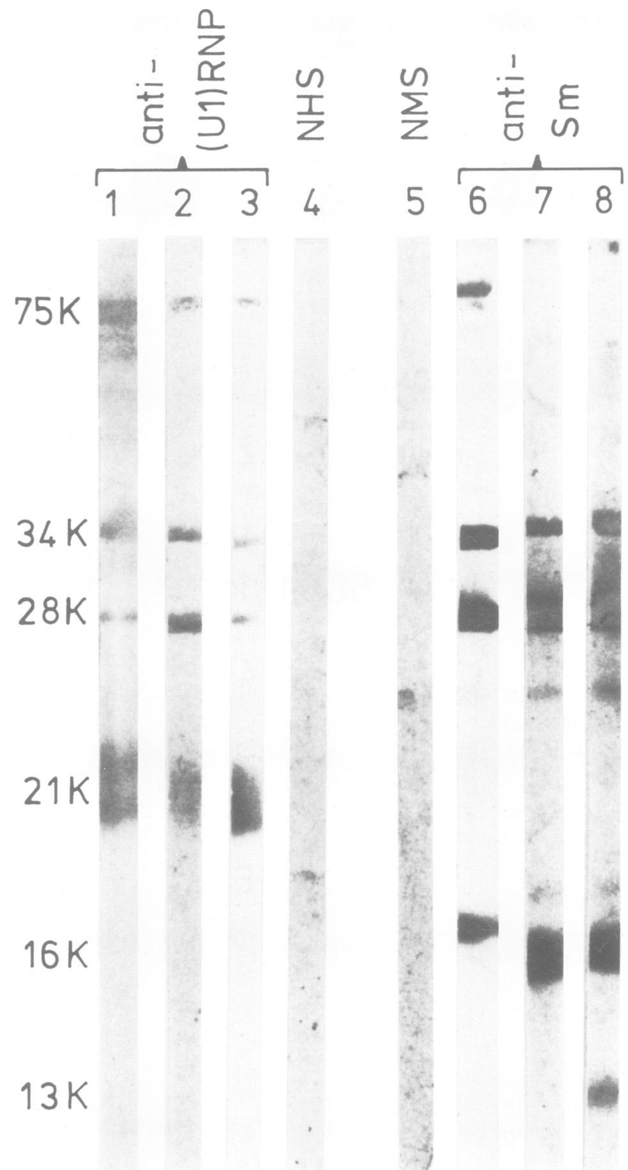

Fig. 4. Protein blots of immobilized proteins from purified snRNPs with various anti-(U1)RNP and anti-Sm antisera. Proteins from affinity column-purified snRNPs were electrophoresed on SDS-polyacrylamide gels and transferred to nitrocellulose filters which were probed by a double antibody procedure (Materials and methods). Sera used as a source of the first antibody were as follows: **lanes 1–3**, various anti-(U1)RNP sera from patients with MCTD; **lane 4**, normal human serum (NHS); **lane 5**, normal mouse serum from a BALB/c mouse (NMS); **lanes 6–8**, various anti-Sm sera from MRL/l mice.

human lupus erythematosus with circulating anti-Sm autoantibodies (Andrews *et al.*, 1978; Eisenberg *et al.*, 1978). Anti-(U1)RNP sera from MCTD patients and anti-Sm sera from MRL/l mice were selected by the criterion of precipitating either U1 snRNP only or total snRNPs U1, U2 and U4–U6 from extracts of ^{32}P-labelled HeLa cells (Lerner and Steitz, 1979).

Proteins from purified snRNPs were separated on SDS-polyacrylamide gels and transferred electrophoretically to nitrocellulose. The nitrocellulose filters were probed using the various autoimmune and non-immune sera as sources of the first antibody. Reactivity of antibodies with individual snRNP proteins was determined using peroxidase-labelled anti-Ig (Karcher *et al.*, 1981) as shown in Figure 4 (the blots shown are assembled from various experiments, so that the R_F values appear to differ slightly).

All three anti-(U1)RNP autoantisera react with the same set of four snRNP proteins characterized by approximate mol. wts. 75 000, 34 000, 28 000 and 21 000 (Figure 4, lanes 1–3). The 21 000 protein sometimes appears as a double band (Figure 4, lane 2), a feature which was already observed when SDS gels were stained directly for protein (see above).

The various antisera containing anti-Sm antibodies reveal a more complex picture since they give different patterns of recognition of the snRNP proteins. Most importantly, only the proteins of approximate mol. wts. 13 000 and 16 000 react with these sera and not with the anti-(U1)RNP sera (Figure 4). While all three sera react with the 16 000 protein they are heterogeneous as regards their content of antibodies against the 13 000 protein (Figure 4). The other proteins reac-

ting with the anti-Sm sera are also recognized by the anti-(U1)RNP sera (the two minor bands in lanes 6 and 7 do not overlap with the position of any of the snRNP proteins and most probably are due to unspecific staining). All three anti-Sm sera react strongly with the polypeptides of approximate mol. wts. 28 000 and 34 000, while they differ in their reaction with the 75 000 protein (Figure 4).

Discussion

In this report, a procedure is described which has been developed to isolate the U-snRNPs of nucleoplasmic origin from eucaryotic cells. The snRNPs are selected from the nuclear extract by complex formation of the snRNAs 5′-terminal cap structure with Sepharose-bound $m_3^{2,2,7}G$-specific antibodies. The particles are released from the antibodies by competition with an excess of the homologous hapten $m_3^{2,2,7}G$. The rationale underlying this procedure is that any protein co-chromatographing with the snRNAs displaced by $m_3^{2,2,7}G$ must be regarded as a genuine compound of the snRNPs or at least as associated with the snRNPs inside the cell, a rationale which is the more important because an assay for functional activity of the snRNPs has not yet been developed.

The snRNPs obtained by our procedure may be regarded as representative of the native snRNP population in the cell for the following reasons. (i) The desorption of the snRNPs from the affinity column with $m_3^{2,2,7}G$ avoids the use of denaturing agents, generally required to dissociate immune complexes. This minimizes the risk of producing 'core' particles. Indeed, no degradation of the RNA in the snRNPs was observed throughout the isolation procedure (Figure 2). (ii) High yields of snRNPs may be obtained. (iii) Purified snRNPs react with anti-Sm autoantibodies (Figure 2).

Nine polypeptides of approximate mol. wts. 10 000, 12 000, 13 000, 16 000, 21 000, 28 000, 32 000, 34 000 and 75 000 have been found consistently in our snRNP preparations. The large number of antigenic polypeptides reacting both with anti-(U1)RNP and with anti-Sm antisera (Figure 4) further corroborates the contention that these proteins are constituents of the native snRNPs. Four of these, characterized by mol. wts. of 21 000, 28 000, 34 000 and 75 000 react consistently with all three anti-(U1)RNP sera (Figure 4, lanes 1 – 3). The pattern of reactivities found for the three anti-Sm sera is less uniform (Figure 4, lanes 6 – 8). Let us first examine the features which distinguish anti-Sm from anti-(U1)RNP sera. First of all the 16 000 and 13 000 proteins react exclusively with anti-Sm sera, which suggests that these proteins are common to all snRNP species. Note that the pattern of reactivity with individual snRNP proteins differs among the various anti-Sm sera (Figure 4), a feature which has also been reported by Billings *et al.* (1982). Secondly, the three anti-Sm sera do not react with the 21 000 protein which was strongly stained by all three anti-(U1)RNP sera. Therefore, the 21 000 protein may be regarded as unique to U1 snRNP particles.

Two possibilities may be raised to explain the finding that the proteins of apparent mol. wt. 28 000, 34 000 and 75 000 react with both anti-(U1)RNP and anti-Sm sera. (i) Anti-Sm sera may contain antibodies of (U1)RNP specificity as well. This possibility is hard to exclude, since anti-Sm sera are defined and were selected by the criterion of precipitating total snRNPs. (ii) A given protein band in the SDS gel may contain two polypeptides of the same mol. wt. but different antigenicity. At this stage of purification we cannot distinguish between these possibilities as regards the 28 000 and 34 000 proteins. However, indirect evidence suggests that reaction of the 75 000 protein with one anti-Sm serum (Figure 4, lane 6) is indeed likely to be due to contaminating anti-(U1)RNP antibodies: attempts to fractionate snRNPs further into individual snRNP species indicate that the 75-K protein is unique to U1 snRNPs (Lührmann *et al.*, unpublished results).

How do our data relate to other published results on snRNP constituents? Polypeptides with mol. wts. similar to those observed here have been reported (reviewed in MacGillivray *et al.*, 1982). Comparison with some of the results, however, is complicated for the following reasons. (i) Cell lines and tissues of various species have been used as a source of snRNPs or snRNP constituents by the various investigators. Therefore, deviation in mol. wts. in the range of several kilodaltons may be expected when comparing homologous proteins. (ii) Some investigators have tried to identify the antigens reacting with anti-(U1)RNP and anti-Sm autoantibodies without paying attention to the 'nativeness' of intact snRNP particles.

Despite these uncertainties, several reports agree upon the existence of smaller polypeptides of mol. wts. between 9000 and 14 000 as structural components of snRNPs (for a summary see MacGillivray *et al.*, 1982). These most probably correspond to our 10 000, 12 000 and 13 000 proteins. As these polypeptides remain strongly associated with U-snRNAs, even during purification on CsCl gradients (Brunel *et al.*, 1981), they may be regarded as primary RNA-binding proteins. A 13 000 mol. wt. polypeptide from rabbit thymus reacted with anti-Sm antibodies, as shown by protein blotting (White and Hoch, 1981). The same authors reported that a lapine 70 000 mol. wt. protein which may correspond to our 75 000 protein, is reactive towards monoclonal anti-(U1)RNP antibodies (Billings *et al.*, 1982). Immune precipitation of cellular proteins from a human cell line with systemic lupus erythematosus autoantisera in the presence of SDS revealed that Sm determinants resided primarily in polypeptides of mol. wts. 16 000 and 25 000, whereas the RNP determinants resided in a 19 000 mol. wt. protein (Conner *et al.*, 1982). These polypeptides have to be considered as a minimum number since it cannot be ruled out that the SDS solubilization denatured other potentially immunoreactive proteins. The 19 000 mol. wt. protein could well correspond to our anti-RNP reactive snRNP protein of approximate mol. wt. 21 000. Good agreement also exists as to the Sm reactivity of the 16 000 polypeptide. It is difficult, however, to correlate their 25 000 mol. wt. polypeptide with any of our snRNP proteins.

The polypeptide content of our purified snRNPs resembles most closely that of the snRNPs immunoprecipitated with anti-(U1)RNP and anti-Sm antibodies from fresh Ehrlich ascites cell extracts, as investigated by Lerner and Steitz (1979). By comparison of mol. wts., their polypeptides A – G probably correspond to our proteins of approximate mol. wts. 34 000, 28 000, 21 000, 16 000, 13 000, 12 000 and 10 000. More recently, Hinterberger *et al.* (1983) succeeded in purifying snRNPs from mouse erythroleukemia cells by a series of gel filtration, ion-exchange and hydrophobic chromatography steps. They found that total proteins of purified snRNPs embrace the same set of their previously determined polypeptides A – G together with two additional ones of approximate mol. wts. 32 000 and 68 000. There is

thus good agreement between our results and theirs, in spite of the use of completely different preparation procedures, the only disagreement is over the mol. wt. of the longest polypeptide (68 000 or 75 000 daltons).

While our data are primarily related to structural aspects of snRNPs, our finding that U1 snRNP may be removed selectively from nuclear extracts by affinity chromatography (Figure 2B) is of functional significance for this particular snRNP species. As the cap structures of the various snRNAs are identical, the high affinity of U1 RNP for anti-$m_3^{2,2,7}$G antibody, i.e., the better fit of the U1 snRNA cap for the combining site of the antibody, must be due to a higher flexibility of the 5'-terminal region of U1 snRNA in the intact RNP particle. This suggests that the 5' terminus is not intimately involved in intramolecular secondary structure, which would be a prerequisite for U1 snRNP to facilitate splicing by base pairing with the 5' and 3' ends of an intron (Lerner *et al.*, 1980; Rogers and Wall, 1980). We believe that the purity, homogeneity and high yield of the procedure described in this paper may provide a basis for further investigation of this and similar questions at the level of isolated particles.

Materials and methods

Anti-$m_3^{2,2,7}$G antibodies and source of autoimmune sera

Antibodies of high specificity for 2,2,7-trimethylguanosine were obtained as previously described (Lührmann *et al.*, 1982). Sera from patients diagnosed as having systemic lupus erythematosus or MCTD which were used as a source of anti-(U1)RNP autoantibodies were obtained from the Medizinische Hochschule, Hannover through the kind help of Professor Peter. Sera from MRL/l mice (The Jackson Laboratory, Bar Harbor) were used as a source of anti-Sm autoantibodies.

Cell growth and labelling conditions

Ehrlich ascites cells were grown in suspension culture at 37°C in Eagle's minimum essential medium (Flow Laboratory) supplemented with 5% newborn calf serum, 50 μg/ml penicillin and 100 μg/ml streptomycin. Cultures were kept at a cell density of $6-8 \times 10^5$ cells/ml by daily feeding. For preparation of ^{32}P-labelled nuclear extract, cells were harvested at mid-log phase ($5-6 \times 10^5$/ml), resuspended in phosphate-free medium to a starting density of 3×10^5 cells/ml and labelled with [^{32}P]orthophosphate (Amersham/Buchler, Braunschweig) at 20 μCi/ml for 14 h. Growth in suspension culture of HeLa cells (S_3 strain) and conditions for labelling with [^{32}P]orthophosphate were as described for Ehrlich ascites cells, except that HeLa cells were kept at $4-5 \times 10^5$ cells/ml by daily feeding.

Cell fractionation

Ehrlich ascites cells were fractionated into cytoplasm and nuclei essentially as described by Zieve and Penman (1980). For preparation of nuclear extracts, purified nuclei were resuspended in a buffer containing 10 mM Tris-HCl, pH 7.4, 500 mM NaCl, 50 mM $MgCl_2$, 1 mM dithiothreitol and 1 mM phenylmethylsulfonylfluoride at a density of 4×10^7 nuclei/ml and vigorously agitated on a Vortex mixer for 2 min. Chromatin was separated from nucleoplasma by centrifugation at 12 000 r.p.m. for 20 min on a Sorvall RC-3 with an HB 4 rotor (Weinberg and Penman, 1968). The resulting supernatant (supernatant 1) was freed from higher mol. wt. RNAs and RNPs by centrifugation for 1 h at 40 000 r.p.m. in a Ti45 rotor. The high speed supernatant (supernatant 2) was taken as starting material for isolation of snRNPs.

Affinity chromatography

Anti-$m_3^{2,2,7}$G IgGs were bound covalently to Protein A-Sepharose Cl-4B according to the procedure of Gersten and Marchalonis (1978) using dimethyl suberimidate as a cross-linking agent. Approximately 20 mg IgG were cross-linked to 1 ml of Protein A-Sepharose.

For isolation of snRNPs from unlabelled Ehrlich ascites cells, the Protein A-Sepharose anti-$m_3^{2,2,7}$G column (0.8 cm x 10 cm, 5 ml bed volume) was washed with 50 ml of buffer A containing 10 mM Tris-HCl, pH 7.4, 500 mM NaCl and 50 mM $MgCl_2$. Typically, the nuclear extract (supernatant 2, see above) from 1×10^9 Ehrlich ascites cells was passed over the column at a flow rate of 0.5 ml/min followed by a 75 ml wash of buffer B (20 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 300 mM KCl). snRNPs were desorbed from the affinity column by elution with 5 ml buffer B containing 3 μmol nucleoside $m_3^{2,2,7}$G. The affinity column was regenerated by washing with 50 ml 6 M urea in buffer B and then with 50 ml of buffer A. The capacity of the affinity column to bind $m_3^{2,2,7}$G was determined with ^{14}C-labelled $m_3^{2,2,7}$G by following the same procedure.

RNA and protein analysis

RNAs prepared by phenol extraction were fractionated on 10% polyacrylamide gels containing 7 M urea, 100 mM Tris-borate, pH 8.3 and 2 mM EDTA. Samples for SDS-gel electrophoresis were prepared from snRNP containing fractions by precipitating the proteins with 10% TCA. The precipitates were pelleted by centrifugation, washed twice with acetone and dissolved in sample buffer (50 mM Tris-HC1, pH 6.8, 2% SDS, 10% glycerol and 5% 2-mercaptoethanol). SDS-gel electrophoresis was performed on 15% polyacrylamide gels (Laemmli, 1970). Proteins were stained with Coomassie brilliant blue R250 followed by a silver stain (Merril *et al.*, 1981).

Protein blot assays with anti-Sm and anti-(U1)RNP antisera

Immunologically reactive proteins were identified in the anti-$m_3^{2,2,7}$G-purified snRNPs by the protein blot procedure of Towbin *et al.* (1979). snRNP proteins were separted by SDS-polyacrylamide gel electrophoresis loading 20 μg protein/0.8 cm of sample well on 15% gels. One sample well was loaded with marker proteins (1 μg per protein). The separated proteins were transferred electrophoretically to nitrocellulose filter sheets at 60 V for 4 h in 12.5 mM Tris/96 mM glycine buffer containing 20% (v/v) methanol. Longitudinal strips of the filters were cut and the strip containing marker proteins was directly stained for protein with amido black (Towbin *et al.*, 1979). Additional protein binding sites on the other nitrocellulose filter strips were saturated by immersion for 2 h at room temperature in phosphate-buffered saline (PBS), pH 7.4, containing 5% bovine serum albumin (BSA), 5% ovalbumin (OVA) and 0.1% Tween 20. The filters were washed with PBS for 10 min and incubated with an appropriate dilution of the human or mouse test antibody in PBS, pH 7.4 containing 3% BSA and 0.1% Tween 20. The strips were washed in four changes of PBS, pH 7.4 every 15 min. They were then treated for 2 h at room temperature with either goat anti-human Ig peroxidase conjugate or goat anti-mouse Ig peroxidase conjugate (Cappel Diagnostics, Inc.) diluted 1/500 in PBS, pH 7.4, containing 3% BSA and 0.1% Tween 20. The nitrocellulose strips were then rinsed in three changes of PBS, pH 7.4, every 45 min. The peroxidase was localized by immersion of the nitrocellulose strips in a dimethyl formamide solution containing 3-amino-9-ethylcarbazole (Sigma) and H_2O_2 prepared as described by Karcher *et al.* (1981). The proteins became visible as reddish brown bands.

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