A novel 40S multi-snRNP complex isolated from rat liver nuclei

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ABSTRACT

Two structurally distinct RNP complexes (MI and MII), each with a sedimentation value of approx. 40S, were isolated from rat liver nuclear extracts by sucrose gradient centrifugation and subsequent native gel electrophoresis of the 40S hnRNP-containing fractions. MII RNP contained the bulk of hnRNA and hnRNP proteins (i.e. the 32–45KD core proteins and polypeptides of 60–80 and 110–130KD). MI RNP was characterized by the exclusive presence of U-snRNAs (U1, U2, U4, U5 and U6), their well known snRNP polypeptides and a number of Sm-associated proteins in the range of 50–210KD. Immunoselection experiments employing a monoclonal antibody with an established specificity for the U2-snRNP-specific B″ polypeptide proved that the RNA and protein components characteristic of MI were part of a single multi-snRNP unit. The prominent 200/210KD protein doublet of MI was identified immunochemically as the rat homologue of the yeast PRP8 protein, a known U5-associated splicing component. Based on the major biochemical and immunochemical features of MI and MII RNP complexes, we conclude that MII represents the monomeric 40S hnRNP structure, whereas MI defines a novel multi-snRNP entity.

INTRODUCTION

Heterogeneous nuclear ribonucleoprotein particles (hnRNPs) are considered as the sites of conversion of pre-mRNA to mRNA. HnRNA released from nuclei by limited RNA digestion and sonic disruption is mostly recovered in hnRNP particles sedimenting at 40–50S, known as 40S hnRNPs or monoparticles (reviewed in ref. 1). The bulk of 40S hnRNP protein is represented by the 32–45KD core polypeptides: a group of three doublets known as A1/A2, B1/B2 and C1/C2 (2). In addition, a number of other polypeptides have been recognized as genuine components of 40S hnRNPs by UV cross-linking experiments (3) and by specific immunoprecipitation reactions (4, 5). These studies have verified previous evidence on the great protein complexity of hnRNP particles in animal cells (6, 7, 8).

The establishment of *in vitro* systems for RNA splicing has greatly advanced our understanding of the complex mechanisms involved in this process and has unequivocally proved the participation of U1, U2, U4, U5 and U6 small nuclear ribonucleoprotein complexes (U-snRNPs), as well as of hnRNP protein components in RNA splicing (reviewed in ref. 9). Analysis of *in vitro* splicing extracts by density gradient sedimentation combined with native gel electrophoresis has helped in identifying several RNP complexes involved in RNA splicing (10–12), including the *in vitro* assembled active splicing complex termed spliceosome (see review in ref. 9). The purification and visualization of the spliceosome has been achieved recently from HeLa (13) and yeast (14) cells.

In parallel to the *in vitro* studies, efforts have been initiated to identify endogenous hnRNP and snRNP complexes and ascribe specific activities to their components. Working along these lines we have reported on the application of native gel electrophoresis to the analysis of the 40S hnRNP-containing sucrose gradient fractions from rat liver nuclear extracts. These studies had led to a preliminary account on the identification of two discrete RNP complexes within the 40S fractions, which we have referred to as MI and MII RNPs (15). In this communication we describe the extensive biochemical analysis of MI and MII complexes. Most important is the immunological identification of a multi-snRNP complex contained in the 40S sucrose gradient fractions, which by its main biochemical characteristics corresponds to MI. This novel, endogenous multi-snRNP assembly consists of all the major U-snRNAs (U1, U2, U4, U5 and U6) and their snRNP proteins, as well as of additional polypeptide species of 50–210KD. We regard this study as an initial step towards ascribing biological function to this novel multi-snRNP entity.

MATERIALS AND METHODS

Preparation of 40S hnRNP-containing sucrose gradient fractions

40S hnRNPs were obtained from rat liver nuclei as described before (16). The main steps were extraction of nuclei in 10 mM Tris-HCl pH 8.0, 140 mM NaCl, 1 mM $MgCl_2$, followed by sonic disruption and fractionation of the nuclear extracts on 15–30% sucrose gradients with a 50% sucrose cushion at 55,000×g, for 17 h, at 4°C. 40–50S material was peletted at 70,000×g, for 18 h, at 4°C.

RNP gel electrophoresis and elution of MI and MII complexes

Pelleted 40–50S material was resuspended in 10 mM Tris-HCl pH 7.6, 30% glycerol and run on a 0.5% agarose gel in buffer A (6.4 mM Tris-HCl pH 8.0, 3.2 mM sodium acetate and 0.32

mM EDTA) at 40 V for 18 h at 4°C, as previously described (15). The RNP subpopulations (MI and MII) were localized by Coomassie brilliant blue R250 staining of strips cut from the sides of the gel. Corresponding areas on the gel were excised and extracted by freeze-thawing. After removal of the agarose by a brief spin, eluates were concentrated by vacuum dialysis against buffer A at 4°C. This elution protocol yielded over 50% of the material initially applied onto the agarose gel.

Protein analysis

Proteins were obtained either by TCA precipitation or following addition of two volumes of abs. ethanol at −20°C. They were then separated by the SDS-PAGE system of Laemmli (17). Unless otherwise stated, gradient (8−15%) gels are shown. Two-dimensional (NEPHGE/SDS-PAGE) electrophoresis was according to O'Farell (18).

For direct protein analysis (RNP/SDS-PAGE) of the electrophoretically resolved RNP complexes, the agarose strip was applied on an SDS-polyacrylamide gel and impregnated, prior to electrophoresis, in 2×-SDS sample buffer containing 1% agarose for 10min.

Nucleic acid hybridization studies

RNP material was blotted from the agarose gel onto a nitrocellulose filter by allowing one liter buffer A to diffuse through the blot. The filter was then baked at 80°C for two hours and used in hybridization studies as follows: Two nick-translated probes, namely a close to full-lenght mouse albumin cDNA clone (pMA1) obtained from U. Schibler, Geneva and a human U1-snRNA gene construct (pSP64/U1+, ref. 19) provided by R. Luehrmann, Marburg, were applied to identify albumin and U1-snRNA sequences, respectively. Also, a synthetic oligonucleotide complementary to nucleotide sequence +47 to +100 of the rat U5-snRNA (synthesized at the Institute of Molecular Biology and Biotechnology, Crete) was gel purified and 5′-end labeled with (γ-^{32}P) ATP (Amersham). Hybridizations using the nick-translated probes were performed at 42°C for 16 h in 50% deionized formamide, 5× SSC, 1× Denhardt's solution, 100 μg/ml denatured salmon sperm DNA, 20 mM sodium phosphate buffer pH 6.5. The final wash was performed at 60°C in 0.1× SSC containing 0.5% SDS. Hybridization with the U5-oligo probe was at 37°C for 16 h in 35% deionized formamide, 5× SET, 5× Denhardt's, 250 μg/ml denatured salmon sperm DNA, 0.05% sodium pyrophosphate, 0.1% SDS. Final wash was at 55°C in 3× SSC, 0.1% SDS.

RNA analysis

RNAs were extracted from the agarose gel and from pelleted sucrose gradient fractions as described before (16). They were, then, separated on 10% polyacrylamide-7M urea gel (20) and visualized by ethidium bromide (EtBr) or silver staining (21).

Antibodies

The 4G3 and 11A1 monoclonals (22) were the gift of W. van Venrooij, Nijmegen. The H20 anti-trimethylguanosine (23) and the anti-Sm-type Y12 (24) monoclonal antibodies were provided by R. Luehrmann, Marburg and J. Steitz, New Haven, respectively. The human anti-Sm and anti-(U1)RNP were standard autoimmune sera obtained from the Centers for Disease Control, Atlanta, Georgia, USA. The rabbit anti-8.2 and anti-8.3 sera for the yeast PRP8 protein (25) were the gift of J. Beggs, Edinburgh. Human non-immune sera were from healthy individuals and a control monoclonal antibody recognizing the rat glucocorticoid receptor was provided by N. Tsavdaroglou, Athens.

Immunoprecipitation

The immunoprecipitation reactions employing human sera were performed at 4°C essentially as described in (26), in 400 μl NET-2 buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.5, 0.05% NP-40) in the presence of 1 u/μl RNasin (Promega) and 3 mM DTT. When employing monoclonal antibodies, rabbit anti-mouse IgG was first bound to protein A-Sepharose (Pharmacia). Thereafter, immune reactions proceeded as with human sera by applying 100 μl of 10-fold concentrated hybridoma supernates. Immune pellets were washed five times, 10 min. each, in NET-2 containing 0.1% NP-40. For protein analysis immune pellets were dissolved directly in the SDS-PAGE sample buffer. RNAs were analysed following phenol extraction of the immune pellets.

In the experiments where the stability of the multi-snRNP complex was investigated, pelleted 40S hnRNP fractions were resuspended in 5 mM Hepes pH 7.6, 2 mM $MgCl_2$, 25 mM KCl, 0.1 mM EDTA, 3 mM DTT, 3.8% glycerol and 1 u/μl RNasin (essentially splicing buffer as in ref. 12, without ATP and creatine phosphate). Identical 100 μl aliquots were then incubated under the experimental conditions given in the legend of Figure 9. Thereafter, three volumes of NET-2 were added and immunoprecipitation using monoclonal 4G3 antibodies followed as usual.

Immunoblotting

Transfer, block and incubation conditions for immunoblotting were as described in ref. (28). Prior to immunostaining, the proteins on the nitrocellulose filter were stained by Ponceau S. For immunoblotting the human anti-Sm sera were used at 1:100 dilution and the rabbit anti-8.2 and −8.3 sera at 1:500.

RESULTS

MI and MII are two distinct RNP entities co-existing in the 40−50S sucrose gradient fractions

The material used in all studies presented here was recovered from the 40 to 50S fractions of a sucrose gradient (region 4 in Figure 1A). As pointed out previously (15), these fractions, known to contain the '40S hnRNPs', when submitted to electrophoresis on a 0.5% agarose gel under nondenaturing conditions (RNP gel) reproducibly yielded two well defined subpopulations. We referred to these as MI and MII for the slower and the faster moving complex, respectively (Figure 1Ba). The electrophoretic separation of these complexes is better evident in the densitometric scanning of the Coomassie blue-stained agarose strip (Figure 1Bb). The relative proportion of MI to MII in the 40S fractions, although subject to experimental variation, was within the range of 1:2 to 1:4.

The protein composition of MI and MII was determined by directly applying the agarose strip onto an SDS-polyacrylamide gel. As shown in Figure 2A, this two-dimensional gel electrophoresis (RNP/SDS-PAGE) revealed polypeptide species either exclusively localized or enriched in one of the two complexes. The polypeptides found in MII were ordered by size into three main groups. The first group included the core proteins (32−45KD polypeptides) of the 40S hnRNPs. The second and third group contained proteins of 60−80 and 110−130KD, respectively. As regards MI, roughly three groups of proteins

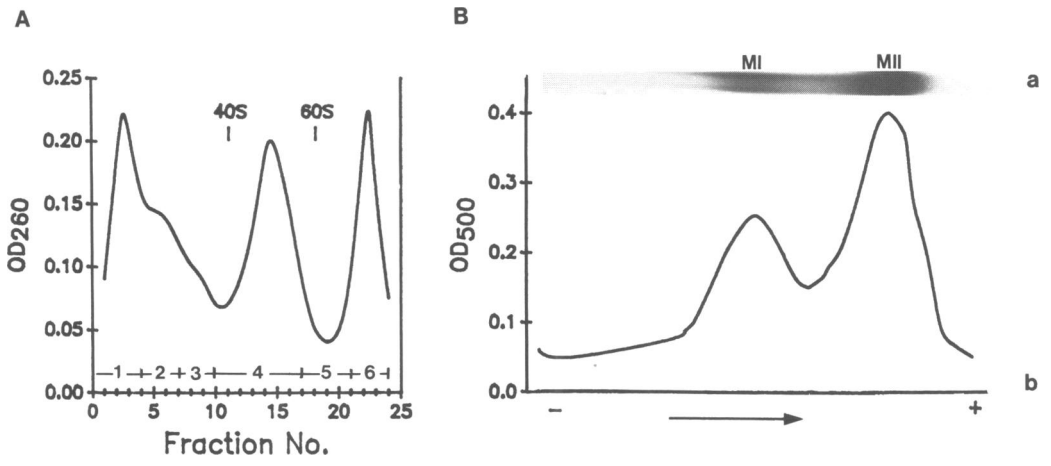

Figure 1. Identification of MI and MII complexes in endogenous 40S hnRNP-containing sucrose gradient fractions. Panel A. Absorbance profile of rat liver nuclear extracts fractionated on a 15–30% sucrose gradient. Region 4 corresponds to pooled fractions from which 40–50S material was taken for subsequent analysis. The position of migration of rat liver 40 and 60S ribosomal subunits run on a parallel gradient is also indicated. Panel B. Native agarose gel electrophoresis (RNP gel) of 40S sucrose gradient fractions. a. Coomassie blue-stained agarose strip. MI and MII refer to the two well defined centers on the RNP gel. b. Densitometric scanning of the agarose strip shown in a.

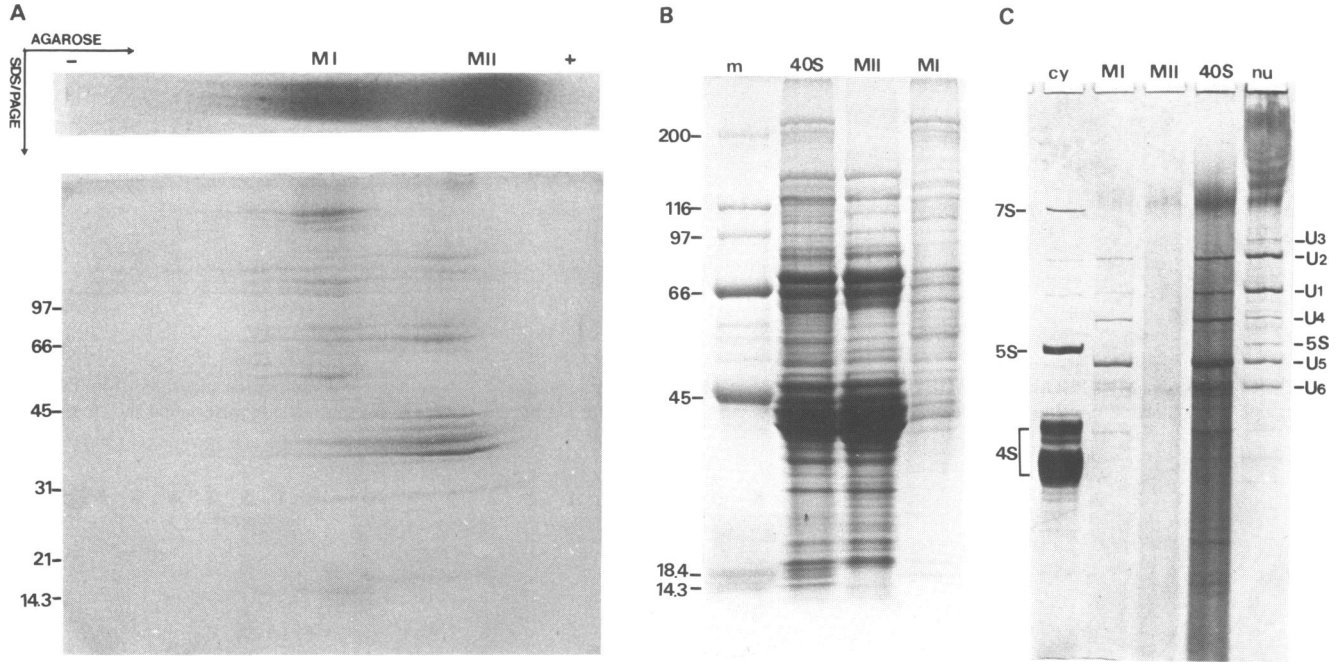

Figure 2. Protein and RNA composition of MI and MII complexes. Panel A. Two-dimensional (RNP/SDS-PAGE) gel electrophoresis of MI and MII protein components. Panel B. SDS-PAGE of agarose-eluted MI and MII and of 40S hnRNP-containing sucrose gradient fractions. Gels shown in both panels were Coomassie blue-stained. Panel C. RNA analysis of the same samples presented in panel B. Silver-stained RNA gel. Marker RNA was rat liver 4–5S cytoplasmic RNA (cy) and total nuclear snRNAs (nu).

in the range of 50–80, 105–126 and 200–210KD were recognized as the major protein species of this complex. The presence in MI of some of the very abundant (i.e. core) MII proteins was most likely due to streaking of MII into the slower migrating MI complex.

Following elution of MI and MII complexes from the agarose gel, their protein composition was compared to the initial 40S material on an SDS gel (Figure 2B). Since MII carried the major protein components of 40S monoparticles, its overall pattern closely resembled that of the initial 40S material. In contrast to MII, MI was enriched in minor polypeptides which, with the notable exception of the 200–210KD proteins, were originally not apparent in the 40S hnRNP-containing sucrose gradient fractions.

The RNA components of the agarose-eluted MI and MII complexes were similarly analysed in parallel with that of total 40S hnRNP material. As seen in Figure 2, panel C, all snRNA species recognized in the 40S fractions were found in MI. These included the major U-snRNAs (U1, U2, U4, U5 and U6), of which U5 was at the highest relative proportion. MII was devoid

of these RNA species but showed, instead, a smear which, as better seen below in Figures 4B and 5b, represented the degraded hnRNA molecules.

To further characterize MI and MII complexes we proceeded to identify their proteins on the two dimensional (NEPHGE/SDS-PAGE) gel system of O'Farell (18). As seen in Figure 3, the bulk of the MII proteins corresponded to the easily identifiable A2, B1 and B2 core polypeptides. The 60−80KD proteins were resolved into distinct species, which were less basic than the major core polypeptides. The 110−130KD MII proteins could not be identified on these gels, most likely because they were too acidic to enter the NEPHGE gel. MI, on the other hand, contained unique polypeptides (included in circles in the schematic representation shown in Figure 3), as well as species common to MII. The latter, as suggested above, are considered contaminants of MII. Among the unique polypeptides of MI, those belonging to the group of 105−126KD (at least six different protein spots) were acidic proteins with pI less than 5.0. Moreover, a minimum of 15 polypeptides belonging to the group of 50−80KD proteins were reproducibly identified as unique species of MI and were widely distributed along the pH gradient. As was the case for the 110−130KD proteins of MII, the 200−210KD MI polypeptides could hardly be resolved on these gels. It is clear from the above that MI has a complex protein composition, distinct to that of MII.

The RNA components of MI and MII were identified by nucleic acid hybridization experiments using DNA probes specific for either albumin RNA sequences (an abundant RNA species in rat liver extracts) or U5- and U1-snRNAs. Material taken from the pooled fractions of the sucrose gradient shown in Figure 1A (regions 1−6) was first submitted to electrophoresis on an RNP gel and then transferred to nitrocellulose. Prior to hybridization

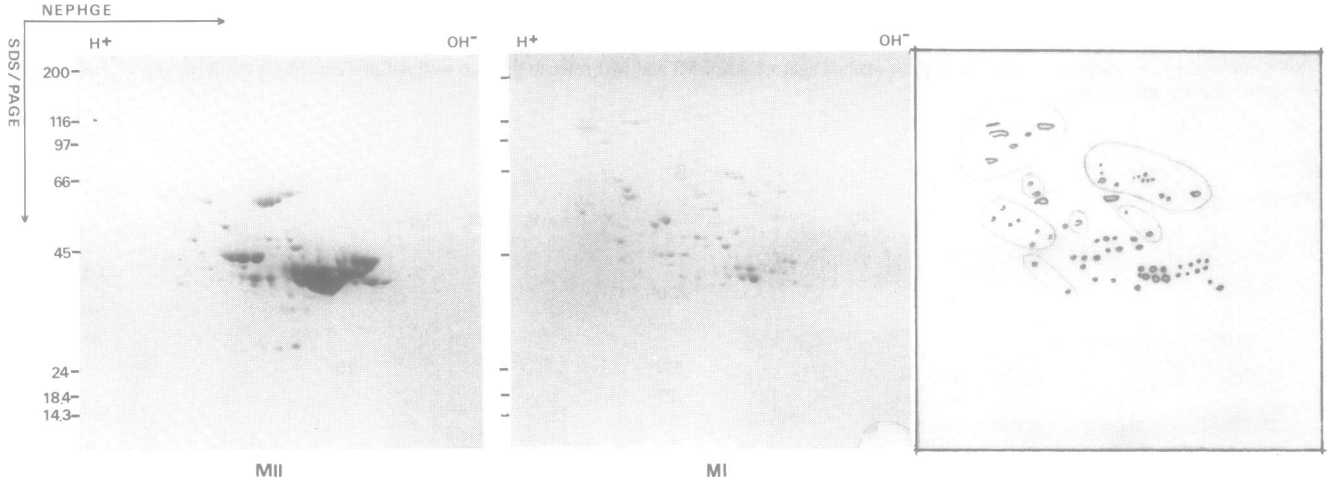

Figure 3. Two-dimensional (NEPHGE/SDS-PAGE) gel electrophoresis of MI and MII polypeptides shown at the relative proportion recovered from the agarose gel. Coomassie blue-stained SDS-polyacrylamide gels. The protein spots exclusive to MI are indicated in circles in the schematic representation shown at the right.

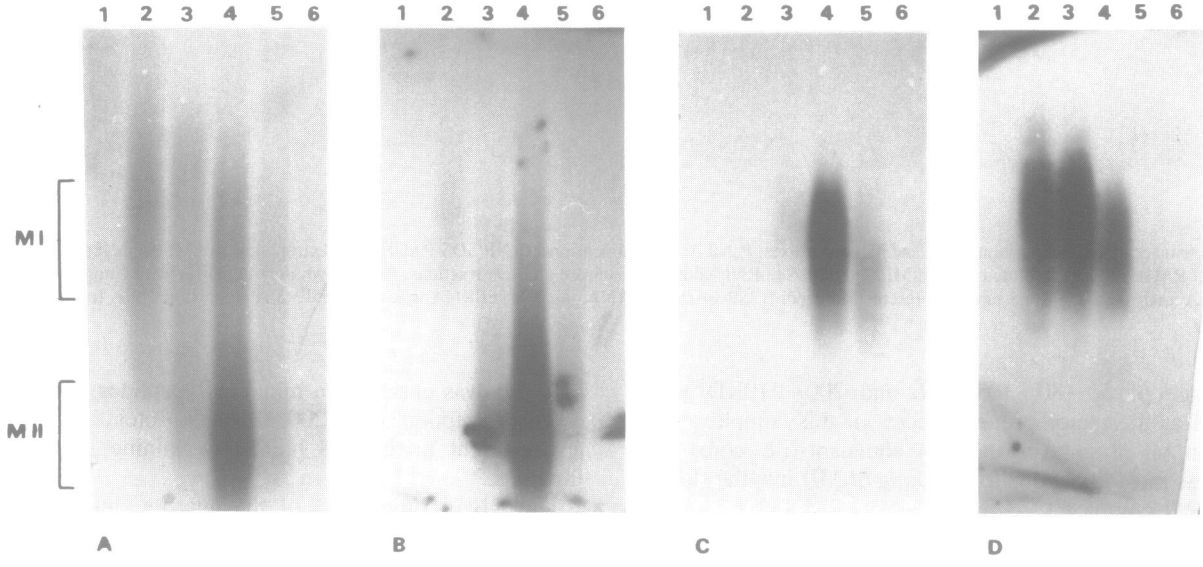

Figure 4. Specificity of MI and MII RNA components. Material corresponding to the pooled sucrose gradient fractions (regions 1 to 6 shown in Figure 1A) was electrophoresed on an agarose gel and then transferred to nitrocellulose. Identical blots were first stained with Ponceau S (A) and subsequently hybridized using either an albumin cDNA (B) or DNA probes complementary to U5 (C) and U1 (D) snRNA sequences as described in Materials and Methods.

with the DNA probes, Ponceau S staining of the proteins on the filter indicated efficient transfer of material (Figure 4A). As shown in panels B and C, respectively, albumin RNA sequences and U5-snRNA were almost exclusively localized in the 40–50S fractions (region 4) of the sucrose gradient. Albumin RNA was confined to MII, whereas U5-snRNA was exclusively found in MI. In accordance to our previous observation on the distribution of U-snRNA species in sucrose gradient fractionated rat liver nuclear extracts (16), U1-snRNA, in contrast to U5, was mainly recovered from the light 10 to 30S fractions of the gradient (regions 2 and 3) and only a subset of it was found in the 40 to 50S fractions. The 40 to 50S-associated U1-snRNA was localized in the MI region of the agarose gel, as was U5. However, as evident from Figure 4D, the agarose gel alone cannot serve to discriminate the free or partially dissociated forms of U-snRNAs from those bound to the heavier 40–50S structures. Nonetheless, the presence of U1-snRNA in MI could not be attributed to contaminating material from the lighter fractions since this possibility is largely eliminated from the immunoselection experiments shown below.

Based on the data presented above, it was very likely that MI and MII represented discrete RNP entities co-existing within the 40S hnRNP-containing sucrose gradient fractions. This notion was supported by CsCl density determination of formaldehyde-fixed MI and MII complexes eluted from agarose gels, in relation to the density of total 40S material. MI and MII were found to have densities of 1.38 and 1.44 g/ml, respectively (data not shown). The density value 1.40 g/ml of the 40S hnRNP-containing fractions was taken as an indication that 40S represented a mixture of MI and MII structural units. If this is the case, both MI and MII should then have a sedimentation coefficient of approx. 40S. This was confirmed by sedimenting the agarose-eluted MI and MII on sucrose gradients identical to those shown in Figure 1A. Fractionated MI and MII were identified by subsequent protein and RNA analysis (Figure 5a and 5b, respectively) of the pooled sucrose gradient fractions (regions 1–6). This analysis revealed that material recovered from the 40–50S fractions (region 4) had the biochemical characteristics expected for either MI or MII. In the case of MI, this was more evident from the protein gel and mainly from the characteristic presence of the 200–210KD proteins. The U-snRNA species, with the notable exception of U5, are rather difficult to detect, as they are more sensitive to degradation during experimental manipulations. As to the MII protein species, it is interesting to note the apparent tight association of the larger than 66KD polypeptides, evident by their exclusive localization in the 40 to 50S fractions of the gradient (region 4) together with the degraded hnRNA species. We conclude from all data presented thus far that MI and MII are two distinct RNP entities, each with a sedimentation coefficient of approx. 40S.

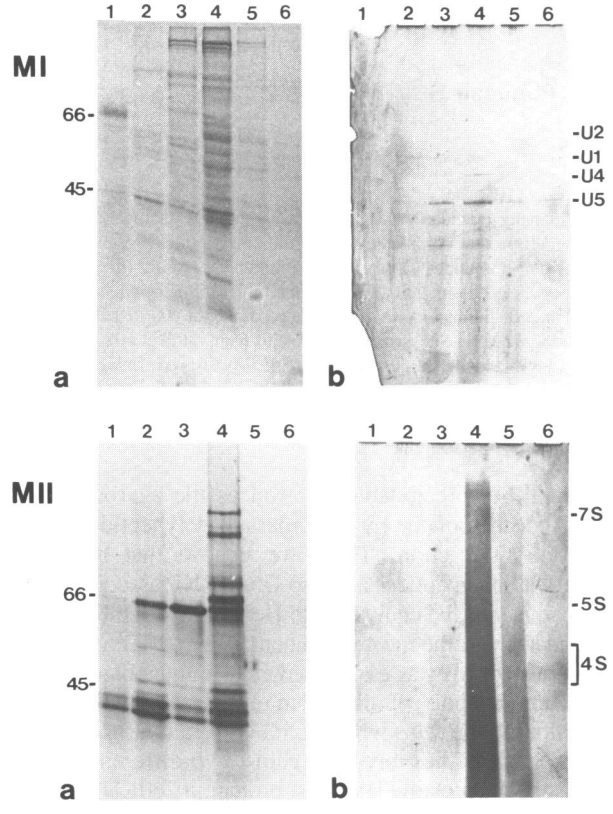

Figure 5. Estimation of sedimentation coefficient of MI and MII RNP complexes. Agarose-eluted MI and MII were fractionated on sucrose gradients similar to the one shown in Figure 1A and the corresponding 1 to 6 regions of the gradients were subsequently analysed with respect to protein and RNA composition. a. Coomassie blue-stained protein gels. b. Silver-stained RNA gels.

The major 50 to 210KD polypeptides of MI are Sm-associated protein components

Based on its main biochemical features, MI appeared to correspond to a novel 40S multi-snRNP assembly. We, therefore, exploited alternative experimental approaches to identify such an RNP entity and characterize its protein and RNA composition further. To this extent, we performed specific immunoselection experiments on 40S nuclear fractions using antibodies against well characterized snRNP components.

We first established by immunoblotting (data not shown) that the 28 and 16KD Sm antigens could be recognized, as expected, amongst the MI proteins and that they were absent from MII. Thus, the anti-Sm antigens could be considered immunological marker of MI in the 40S sucrose gradient fractions. It was then examined whether the MI snRNA and protein components could be co-immunoprecipitated from 40S material using anti-Sm sera. Parallel reactions were performed on unfractionated nuclear extracts as well. The RNA and protein content of the immunoprecipitate was analyzed electrophoretically and the presence of the Sm antigens was monitored by probing the immunoprecipitated proteins with anti-Sm. Because the rat liver proteins could not be sufficiently radiolabelled *in vivo*, unlabelled extracts were used in these reactions and the immunoprecipitated proteins were visualized by Ponceau S staining upon their transfer onto nitrocellulose filter. Figure 6 shows a compilation of data obtained from such immunoprecipitation experiments. As seen by reference to the non- immune serum, the anti-Sm antibodies co-precipitated a large number of polypeptides (panel Aa) together with the Sm-antigens (panel Ab) and the U-snRNAs (panel C). Indeed, the major species of the 50–210KD proteins initially identified in MI were detected amongst the anti-Sm immunoselected polypeptides, in addition to the Sm-antigens and the polypeptides corresponding by relative mol. mass to the well known U-snRNP proteins. An almost identical set of proteins was seen in the immunoprecipitate of 40S fractions and of unfractionated nuclear extracts, the only notable difference being the larger amounts of polypeptides with mol. wt. less than 60K in nuclear extracts. As pointed out before (16), this could be accounted for by the extra pool of free 10–12S U-snRNPs,

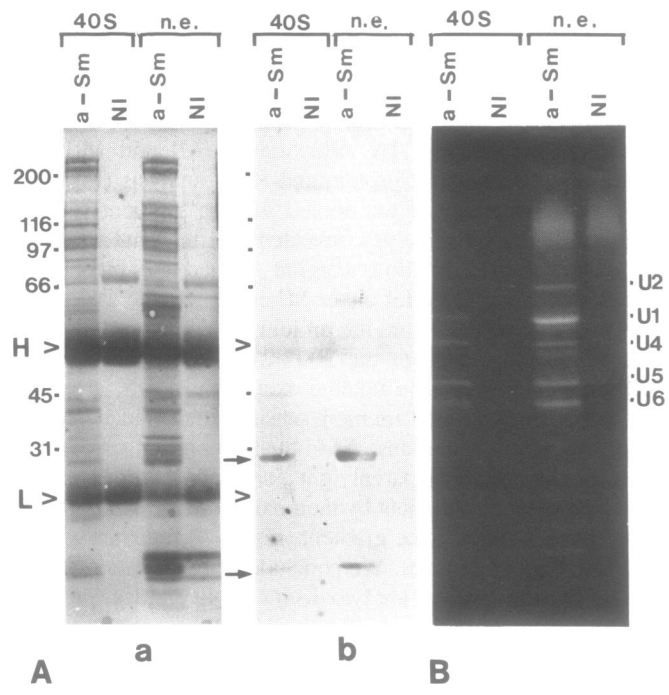

Figure 6. Sm-associated polypeptides and RNA components in 40−50S sucrose gradient fractions and in unfractionated nuclear extracts. 40−50S material (40S) or unfractionated nuclear extracts (n.e.) were precipitated using anti-Sm or non-immune (NI) sera. Protein species present in the immune pellets were analysed by SDS-PAGE and transferred to nitrocellulose. Panel A. a. Ponceau S-stained nitrocellulose filter. H and L refer to the position of migration of heavy and light IgG chains, respectively. b. Immunoblot with anti-Sm sera of the same filter shown in a. Panel B. RNA gel (EtBr-stained) of the snRNAs present in the immune pellets.

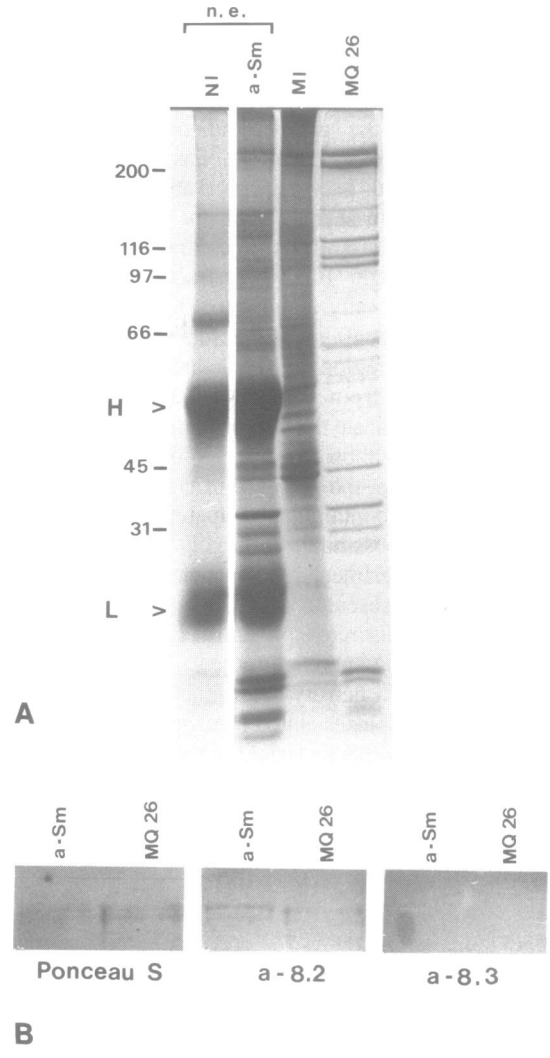

Figure 7. Characterization of MI polypeptides. Panel A. Comparison by SDS-PAGE of the MI protein components (MI), the polypeptides present in the anti-Sm (a-Sm) or non-immune (NI) precipitate of a nuclear extract (n.e.), as well as of the polypeptides contained in a HeLa Mono Q, fraction 26, aliquot (MQ26), ref. in (29). Panel B. Immunological identification of the rat 200/210KD Sm-associated polypeptides. The anti-Sm precipitable proteins from rat liver 40S sucrose gradient fractions (a-Sm) and the HeLa Mono Q sample (MQ26) were analysed by SDS-PAGE, transferred to nitrocellulose and stained by Ponceau S. Duplicate filters were immunoblotted using the rabbit anti-8.2 and −8.3 sera with specificity for the yeast PRP8 protein (30).

mainly of the U1-snRNP type (see also Figure 4, Panel D), present in nuclear extracts. It is also interesting to note the almost complete absence of the abundant MII polypeptides, notably of the core proteins, from the anti-Sm immunoprecipitates. We conclude from this latter finding that MI and MII RNPs are not tightly associated to each other either in the 40S hnRNPs or in the unfractionated nuclear extracts.

As shown above, the pattern of the anti-Sm precipitable polypeptides from 40S RNPs, as well as from unfractionated nuclear extracts, resembled very closely the protein composition of MI. That this is the case is shown in Figure 7, panel A, in which polypeptides immunoprecipitated from nuclear extracts by anti-Sm and the proteins of agarose-purified MI are directly compared by SDS-PAGE. As evident, almost all of the Sm-precipitable polypeptides, with emphasis on the proteins in the range of 50−210KD, were major protein species included in MI. Despite the fact that a comparison on an one to one basis between the polypeptides present in MI and the anti-Sm precipitable species is not easy, the overall resemblance between them is, nonetheless, quite obvious.

Since U5 was the major U-snRNA species represented in MI (see Figure 2C), we also compared the anti-Sm-precipitable proteins of nuclear extracts, as well as the agarose-eluted MI protein species, to the polypeptides present in a sample of HeLa cell extracts (Mono Q, fraction 26) that is highly enriched in a recently identified 20S U5-snRNP complex (29). This complex contains a set of U5-associated polypeptides of ~200 (mostly a doublet), 116, 102, 100, 52 and 40KD, which are recognized as the major protein species of the Mono Q fraction shown here in Figure 7A (lane MQ26). This comparison revealed that many of the anti-Sm precipitable, as well as the agarose-eluted MI proteins, corresponded by mol. mass to polypeptides present in the Mono Q fraction. Thus, we believe that many of the 50−210KD polypeptides are also U5-snRNP associated and that MI encompasses the equivalent to HeLa 20S U5-snRNP particle.

The identity of the most prominent 200/210KD Sm-associated polypeptides of MI was established after immunoblotting the anti-Sm precipitable polypeptides from rat liver 40S material and the HeLa Mono Q proteins with two rabbit sera (anti-8.2 and −8.3) containing antibodies directed against two different portions of the yeast PRP8 protein. The yeast protein, a 260KD polypeptide, has been identified as a splicing factor stably associated with U5-snRNP (25, 30). As recently established (31), antibodies in the anti-8.2 serum cross-react with the HeLa 200KD protein doublet associated to 20S U5-snRNP, while the anti-8.3 does not. As shown here (Figure 7B), the MI 200/210KD protein doublet was similarly recognized by the anti-8.2, but not the anti-8.3

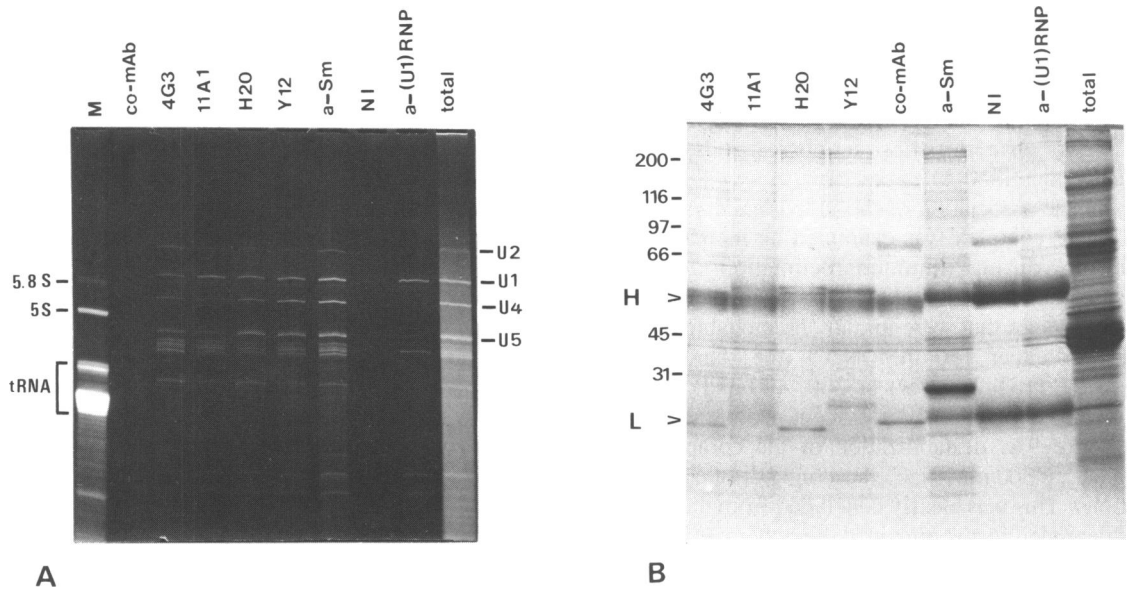

Figure 8. Identification of the multi-snRNP complex in 40–50S sucrose gradient fractions. Identical aliquots of 40S hnRNP-containing fractions were immunoprecipitated using the antibody specificities described in Materials and Methods. NI is human non-immune sera and co-mAb a control monoclonal antibody. Total refers to an aliquot of 40S hnRNP fraction representing 1/10th of that used in each immune reaction. Panel A. RNA species present in the immune pellets resolved on an RNA gel (EtBr-stained). Panel B. Protein species of the same immune pellets (Coomassie blue-stained protein gel).

antibodies. Therefore, the rat 200/210KD protein doublet is immunologically related to the yeast PRP8 splicing factor and the HeLa U5-associated ~200KD protein.

MI RNP represents a novel multi-snRNP complex

The immunoprecipitation experiments using anti-Sm antibodies established that the 50–210KD polypeptides characteristic of MI were indeed snRNP-associated since they co-precipitated with the snRNAs and the Sm-antigens. These experiments could not, however, answer the question as to whether all components were constituents of a single 40S multi-snRNP complex or whether they existed as individual snRNP units that precipitated together because of the shared Sm-epitope. This latter interpretation seemed rather unlikely when considering the experiments presented in Figure 5, unless every individual snRNP found in MI sedimented as a 40S particle. Nonetheless, to directly address this question, we included in our studies an antibody probe specific for a polypeptide unique for only one of the U-snRNPs and examined whether it could as well co-precipitate all of the other snRNA and protein constituents of MI. As a unique snRNP probe we used the monoclonal 4G3 antibody reacting solely with the B″ polypeptide of the U2-snRNP (22) and repeated the immunoprecipitation experiments of 40–50S sucrose gradient fractions, in direct comparison to the anti-Sm sera. This study also included other monoclonal antibodies, such as the 11A1 recognizing the A and B″ polypeptides of U1- and U2-snRNP, respectively (22), the H20 recognizing the tri-methylguanosine cap of U-snRNAs (23) and the Y12 with an Sm-type specificity (24). In addition, a standard autoimmune anti-(U1)RNP serum was included in this comparison. Figure 8 shows the data obtained when identical aliquots of 40S material were immunoprecipitated using the different antibodies described above and the RNA and protein components of the immune pellets were resolved on RNA and protein gels (panels A and B, respectively). As clearly seen in this Figure, the anti-U2 specific monoclonal antibody (4G3) could immunoprecipitate all the RNA and protein components identified with the anti-Sm and Y12 antibodies, establishing that the individual U-snRNPs in the 40S sucrose gradient fractions are indeed associated together in the form of a single multi-snRNP assembly. The same result was reproduced with the anti-U1/U2 (11A1), as well as the anti-cap (H20) monoclonals, the latter showing that at least some of the U-snRNA cap structures are exposed in this multi-snRNP complex.

A different immunoprecipitation pattern was, however, obtained with the standard anti-(U1)RNP serum. Apart from U1, this serum failed to immunoprecipitate appreciable amounts of the other U-snRNAs or of the high mol. wt. Sm-associated proteins present in the 40S fractions (compare a-Sm and a-(U1)RNP lanes in panels A and B). The same inefficient immunoprecipitation was reproduced (data not shown) with two monoclonal antibodies, H304 (32) and H111 (R. Reuter, personal communication), that recognized the A and the 70K U1-specific polypeptides, respectively. This was an unexpected finding, considering the fact that U1-snRNA was readily seen amongst the U-snRNA species immunoprecipitated by 4G3 (Figure 8A) and that, as revealed by immunoblotting (data not shown), the U1-specific A and 70K polypeptides were included in the proteins immunoprecipitated by monoclonal 4G3 antibodies. The simplest way to reconcile these findings is to consider a loose association of the U1-snRNP within the multi-snRNP complex, as already alluded to from the data presented in Figure 4D. Furthermore, we have to assume that within the complex the U1 antigenic polypeptides are not accessible to the anti-(U1)RNP antibodies but they become available for interaction upon U1-snRNP dissociation. Another possibility could be that U1-snRNP cannot maintain its unstable binding to the complex when immunoprecipitation is via U1-specific antigens.

As evident from Figure 8, panel A, in addition to the intact forms of U-snRNA species, a number of bands smaller in size than U5-snRNA existed in the immune pellets. These bands have been lately verified by hybridization experiments using antisence U-snRNA probes (data not shown) to be fragments of the U-

snRNA species, mostly of U1- and U2-snRNA. These fragments remained, nonetheless, immunoprecipitable.

Taken together, the results presented in Figures 7 and 8 prove that a novel, endogenous multi-snRNP complex is present in the rat liver 40S hnRNP-containing sucrose gradient fractions, and that in its major characteristics it corresponds to the electrophoretically identified MI.

The stability of the association of the individual components of this multi-snRNP complex was examined by increasing the salt concentration during immunoprecipitation reactions employing monoclonal 4G3 antibodies. Thus, we looked for the RNA and protein species that were immunoprecipitable by 4G3 in the presence of either 150 mM NaCl (the standard salt concentration of the NET-2 buffer) or 300, 400 and 500 mM NaCl during immune reaction. As evident from the results presented in Figure 9A, destabilization of the complex was already taking place at 300 mM NaCl, becoming drastic at higher salt concentrations. This was clearly concluded from the finding that U2 and its degraded forms remained the only major snRNA species detected in the immune pellets (Figure 9Ab), while the other snRNAs, as well as the 50−210KD proteins (Figure 9Aa), were extensively diminished. These findings are in accord with previous data (22) showing that U2-snRNA is the only RNA species immunoprecipitated by 4G3 from HeLa nuclear extracts in IPP buffer which contains 500 mM NaCl. We conclude from the above that electrostatic interactions are important in holding together the components of the multi-snRNP complex.

We further tested the stability of the multi-snRNP entity represented in MI by employing conditions known to affect the assembly of other multi-component RNP structures (27, 33, 34), as well as the conformation of individual snRNPs (35). To this end, we employed a variety of treatments to the 40S hnRNP fractions and, thereafter, analysed which snRNA species remained immunoprecipitable by 4G3. Such treatments included, incubating at 30°C, varying the ATP concentration either by adding ATP or reducing the endogenous ATP pool by hexokinase/glucose treatment, adding heparin, increasing the Mg^{++} concentration from 2 to 15 mM, as well as digesting with micrococcal nuclease. As seen in Figure 9B, only heparin and micrococcal nuclease affected the integrity of the multi-snRNP complex, evident by the sole presence of U2 snRNA and its fragments in the immune pellets. The other treatments did not appear to have any appreciable effect, since all U-snRNAs were still immunoprecipitable by 4G3. These results are, then, indicative of a rather stable association of the components of the multi-snRNP complex.

DISCUSSION

We have presented in this communication evidence on the existence of two structurally distinct RNP entities (MI and MII) within the 40S hnRNP- containing sucrose gradient fractions of rat liver nuclei.

Initial identification of MI and MII RNP subpopulations was on a native agarose gel system (RNP gel) that we developed for nuclear RNPs. This system, as pointed out previously (15), does not dissociate protein-RNA complexes and, as shown in the present study, it can be considered an important fractionation step for both analytical and preparative purposes. Several RNP gel systems (either agarose/polyacrylamide or polyacrylamide alone) have been since applied to the analysis of splicing extracts (10−12) and have allowed detection of multiple electrophoretic forms of the *in vitro* assembled spliceosome and of endogenous snRNP complexes (33, 36, 37). A direct comparison of the resolution of the agarose gel employed by us and the other published gel systems has not been presently established.

Biochemical analysis of the agarose-resolved MI and MII clearly identified them as discrete RNP entities. From all data

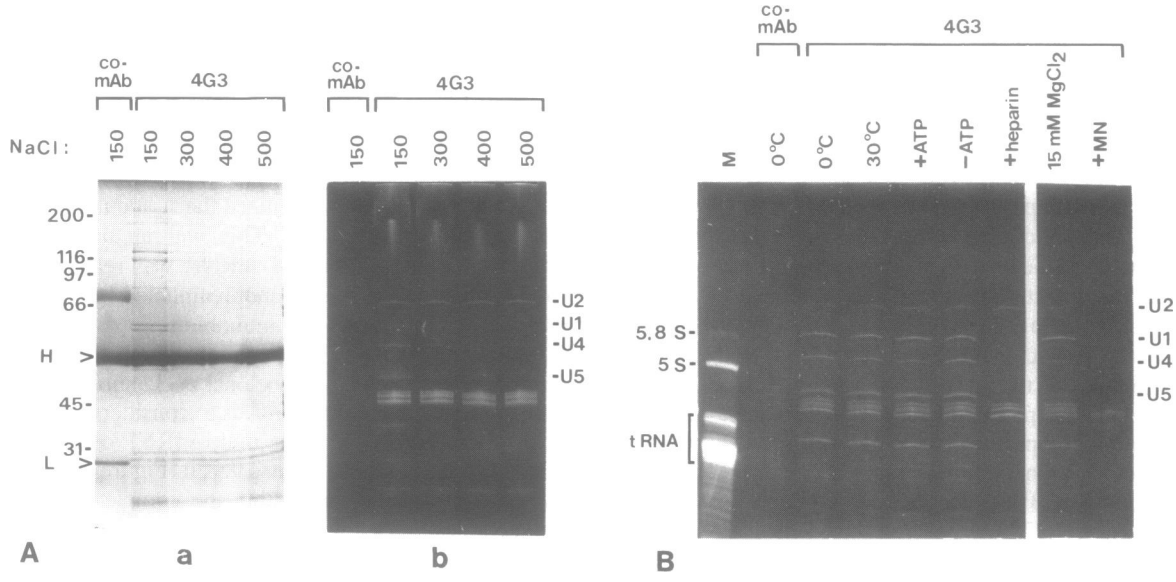

Figure 9. Stability of the multi-snRNP complex. Panel A. Same aliquots of 40S hnRNP-containing material were immunoprecipitated by monoclonal 4G3 antibodies in NET-2 buffer containing either the standard 150 mM or 300, 400 and 500 mM NaCl concentration. co-mAb is control monoclonal antibody. Protein and RNA species present in the immune pellets were resolved, respectively, on an 10% SDS-polyacrylamide gel stained with Coomasie blue (a) and on an RNA gel stained with EtBr (b). Panel B. Identical aliquots of 40S fractions in splicing buffer (see Materials and Methods) were either kept on ice (0°C) or incubated at 30°C for 15 min (30°C). Additional samples incubated at 30°C were in the presence of 1.5 mM ATP / 5 mM creatine phosphate (+ATP), 0.1 u/μl hexokinase / 100 mM glucose pH 8.0 (−ATP), 5 mg/ml heparin, 15 mM $MgCl_2$, or 250 u/ml micrococcal nuclease / 1 mM $CaCl_2$ (MN). All samples were subsequently immunoprecipitated using monoclonal 4G3 antibodies, then the RNA species of the immune pellets resolved on an RNA gel and EtBr-stained.

thus far available, MII represented a major RNP structure and corresponded to the actual hnRNP complex within the 40S sucrose gradient fractions of rat liver nuclear extracts. Moreover, by its protein and RNA composition MII resembled the hnRNP particle described in HeLa nuclear extracts, as defined by UV cross-linking and immunoprecipitation experiments using specific anti-core antibodies (3, 4). MI, on the other hand, represented the minor RNP component of the 40S fraction which was distinctively recognized by the unique presence of all major U-snRNAs (U1, U2, U4, U5, and U6) found in 40S sucrose gradient fractions and by a complex protein composition representing an assortment of polypeptides of mainly 50−210KD.

Significantly, the immunoselection experiments presented here prove that the exclusive to MI U-snRNAs and protein species were components existing within a single multi-snRNP unit of approx. 40S. This conclusion was substantiated by the finding that the snRNAs and most of the proteins included in MI could be immunoselected conjointly from 40S sucrose gradient fractions by a monoclonal antibody recognizing solely the U2-specific B″ polypeptide.

Thus, by two entirely different approaches, electrophoretic separation and specific immunoselection, the presence of a novel multi-snRNP complex pre-existing in 40S hnRNP-containing nuclear structures has been substantiated. We wish to stress at this point the particularly gentle conditions employed during immunoprecipitation and gel electrophoresis that did not favour major rearrangements of the components of endogenous RNP complexes. Moreover, we have recently been able to identify this multi-snRNP assembly on larger than 40S hnRNP particles, namely on 60 to 200S RNP complexes, by fractionating nuclear extracts prepared under conditions that minimized endogenous nuclease action (unpublished observation).

Of the U-snRNAs recovered in MI, U5 was the one detected at the highest relative proportion. This may simply reflect its greater stability to nuclease degradation (38) when compared to the abundant in the cell nucleus U1-and U2-snRNAs. Nonetheless, the prominent presence of the U5-snRNA in MI was accompanied by the concomitant finding of the high mol. wt. polypeptides of 50−210K. Amongst these proteins we could identify polypeptides that corresponded by relative mol. mass to those characteristic for the 20S U5-snRNP complex from HeLa splicing extracts (29). We believe, therefore, that the respective rat 20S U5-snRNP is encompassed in the multi-snRNP entity described here. It should be noted that U5-snRNA (see Figure 4), as well as the Sm-precipitable 50−210KD proteins (data not shown) were recovered from rat liver nuclear extracts exclusively in larger than 30S RNP structures. It is also interesting to note in this connection the decisive role played by the use of HeLa splicing extracts in the identification of the 20S U5-snRNP particle. Nuclear extracts prepared from HeLa cells extracted with buffers containing 0.5 M NaCl and 50 mM $MgCl_2$ yielded, instead, the ~10S U5-snRNP complex lacking the U5-associated 40−200KD polypeptides of the 20S particle (29). We believe, therefore, that the conditions employed by us for nuclear extract preparation (low salt and $MgCl_2$ concentration) do not dissociate pre-existing U5-snRNP-containing multi-snRNP complexes.

While U5 was clearly a major snRNA constituent of MI, this was not true for U1-snRNA. Nevertheless, the subset of U1 recovered in the 40S sucrose gradient fractions (shown in Figure 4D) was a true component of the multi-snRNP complex as judged by its immunoprecipitability by 4G3. The association of U1-snRNA to the complex seems, however, to be unstable as suggested by the immunoprecipitation experiments using the anti-U1-snRNP antibodies and as also evident by the finding that most of U1-snRNA was moving to lighter sucrose gradient fractions during fractionation of agarose-eluted MI (Figure 5b). A loose association of U1-snRNP to multi-component structures has also been considered to explain the absence of U1-snRNA from affinity-purified (33) and electrophoretically analysed active spliceosomes (33, 37, 40), unless EDTA is added to the electrophoresis buffer (36, 41). Nonetheless, U1-snRNA has been found associated to the mammalian spliceosome purified by gel filtration chromatography (13) and to the affinity-purified spliceosome from yeast (42).

As shown, MI multi-snRNP contained a complex set of Sm-associated 50−210KD protein species. Of these, the 200/210KD doublet was identified as the rat homologue of the yeast PRP8 protein, a U5-associated splicing component (25, 30), as well as of the ~200KD doublet of the HeLa 20S U5-snRNP (31). It should be noted here, parenthetically, that neither the 200/210KD protein doublet nor any of the other rat multi-snRNP high mol. wt. proteins were found upon immunoblotting to give any reproducibly significant reaction with the anti-Sm antibodies or the Y12 monoclonals, although such reactions have been reported for the respective HeLa proteins (29, 39). We have pointed out above the presence of polypeptides that, together with the 200/210KD doublet, are most probably U5-snRNP-associated within the multi-snRNP entity. Clearly, more work is needed to establish the identity of the other polypeptides reproducibly recovered in the multi-snRNP complex. It is likely that, in addition to the 200/210KD doublet, other so far known protein components of the splicing machinery, such as the 70/100KD U5-associated intron binding protein (43, 44) and the U2AF protein factor (45), are components of the MI multi-snRNP.

As also indicated in the present study, the association in MI of all U-snRNAs (including U1-snRNA) was stable at physiological salt conditions. The complex could also sustain incubation at 30°C and was not affected by the presence of 15 mM $MgCl_2$, as well as by drastic changes in ATP concentration. It was, however, amenable to micrococcal nuclease digestion and sensitive to heparin treatment. More experiments are required to understand the type of interactions involved in holding together the snRNA and the protein components within this multi-snRNP entity.

By all its features, the multi-snRNP entity presented here is an endogenous multi-component structure distinct to those so far described in the literature. The latter include snRNP assemblies of 15 to 25S detected in HeLa splicing extracts (27, 37, 40). Special reference is made to the reported U5/U4/U6 complex of 25S. Association of U5, U4, U6 in this complex appears to be ATP-dependent, although contradictory data exist when the stability of the complex is tested under different experimental conditions (see ref. 27 for relevant discussion). As already mentioned, we could not observe any ATP effect on our multi-component structure as tested by its specific immuno-precipitability. Nonetheless, it is very probable that the U5/U4/U6-snRNP complex is a major domain of the 40S multi-snRNP assembly described in this study. Moreover, our complex does not relate to the recently identified 'pseudospliceosome', an approx. 28S multi-snRNP assembly containing U4, U5, U6 and U2 snRNAs (37). The 'pseusospliceosome', in contrast to MI, does not contain U1-snRNA, is heparin-resistant, does not pre-exist in nuclear extracts and it is only formed under high salt and Mg^{++} concentrations. In conclusion, we believe that the approx. 40S multi-snRNP complex presented here, most likely represents a higher state of U-snRNP association than so far

presented in the literature and that it might constitute an important functional domain of the spliceosome.

As regards the association of the MI multi-snRNP to the MII hnRNP entity, it is clear from the results presented in Figures 6 and 8 that MII specific components are barely detected above background level in the immunoselected multi-snRNP complex. This, when considered together with the fact that MI and MII can be isolated as separate entities upon native gel electrophoresis, led us to conclude that these RNP entities were not tightly associated to each other in the 40S sucrose gradient fractions. It is worth mentioning at this point that we have not so far succeeded in co-precipitating components of MI and MII even from larger (60 to 200S) polymeric RNP structures which contain the RNA and protein components specific for either MI or MII (unpublished observation). We believe, therefore, that the interactions between these complexes which should exist *in vivo* are vulnerable to experimental manipulations and that special conditions (e.g. cross-linking) are needed for their preservation.

In direct support of our findings on the existence in animal cells of two discrete MI- and MII-like RNP entities is recent evidence originating from the analysis of endogenous RNP complexes of sucrose gradient fractionated splicing extracts from HeLa cells (46). In this study, two types of RNP complexes, both larger than 30S, have been assayed immunologically by employing snRNP- as well as hnRNP-specific antibodies. One RNP type is identified as an snRNP-containing structure with associated high mol. wt. polypeptides, while the other is an hnRNP complex. Moreover, in agreement to our findings, no interaction between the two complexes could be detected immunologically. Although direct proof for the existence of all U-snRNPs in a single multi-snRNP complex (MI-like) was lacking from this study and the associated high mol. wt. polypeptides have not been analysed any further, it is very likely that the MI and MII RNP structures identified in rat liver nuclear extracts are closely related to those reported for the HeLa splicing extracts.

As to the biological significance of the findings presented in this study, it is logical to conclude that MII RNP containing the abundant core polypeptides is mainly responsible for the packaging of hnRNA molecules, whereas MI RNP, a multi-snRNP structure with the associated proteins of 50 to 210KD, would provide the necessary functional elements for pre-mRNA processing.

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REFERENCES

1. Jacob, M., Devilliers, G., Fuchs, J-P., Gallinaro, H., Gattoni, R., Indes, and Stevenin, J. (1981) In Busch, H. (ed), Nuclear Particles, Part A, The Cell Nucleus, Academic Press, Inc., New York, Vol. III, pp 193–246.
2. Beyer, A.L., Christensen, M.E., Walker, B.W. and LeStourgeon, W.M (1977) Cell **11**, 127–138.
3. Dreyfuss, G., Choi, Y.D. and Adam, S.A. (1984) Mol. Cell. Biol.**4**, 1104–1114.
4. Choi, Y.D. and Dreyfuss, G. (1984) Proc. Natl. Acad. Sci. USA **81**, 7471–7475.
5. Pinol-Roma, S., Choi, Y.D., Matunis, M.J. and Dreyfuss, G. (1988) Genes Dev. **2**, 215–227.
6. Stevenin, J.H., Gallinaro-Matringe, R., Gattoni, R. and Jacob, M. (1977) Eur. J. Biochem. **74**, 589–602.
7. Stunnenberg, H.G., Louis, C. and Sekeris, C.E. (1978) Expt. Cell Res. **112**, 335–351.
8. Gattoni, R., Stevenin, J., Devilliers, G., Jacob, M. (1978) Febs Lett. **90**, 318–323.
9. Steitz, J.A., Black, D.L., Gerke, V., Parker, K.A., Kramer, A., Frendewey, D. and Keller, W. (1988) In Birnstiel, M.L. (ed), Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles, Springer-Verlag, Berlin, pp 115–154.
10. Pikielny, C.W. and Rosbash, M. (1986) Cell **45**, 869–877.
11. Pikielny, C.W., Rymond, B.C. and Rosbash, M. (1986) Nature **324**, 341–345.
12. Konarska, M.M. and Sharp, P.A. (1986) Cell **46**, 845–855.
13. Reed, R., Griffith, J. and Maniatis, T. (1988) Cell **53**, 949–961.
14. Clark, M.W., Goelz, S. and Abelson, J. (1988) EMBO J. **7**, 3829–3836.
15. Guialis, A., Arvanitopoulou, A., Patrinou-Georgoula, M. and Sekeris, C.E. (1983) Febs Lett. **151**, 127–133.
16. Guialis, A., Dangli, A. and Sekeris, C.E. (1987) Molec. Cell. Biochem. **76**, 147–162.
17. Laemmli, U.K. (1970) Nature **227**, 680–685.
18. O'Farell, P.Z., Goodman, H.M. and O'Farell, P.H. (1977) Cell **12**, 1133–1142.
19. Schenborn, E.T. and Mierendorf, R.C. (1985) Nucl. Acids Res.**13**, 6223–6236.
20. Maniatis, T., Efstratiadis, A. (1980) Methods Enzymol. **65**, 299–305.
21. Merill, C.R., Goldman, D., Sedman, S.A. and Ebert, M.H. (1981) Science **211**, 1437–1438.
22. Habets, W.J., Hoet, M.H., de Jong, B.A.W., van der Kemp, A. and van Venrooij, W.J. (1989) J. Immunol. **143**, 2560–2566.
23. Bochnig, P., Reuter, R., Bringmann, P. and Luehrmann, R. (1987) Eur. J. Biochem. **168**, 461–467.
24. Lerner, E.A., Lerner, M.R., Janeway, C.A. and Steitz, J.A. (1981) Proc. Natl. Acad. Sci. USA **78**, 2737–2741.
25. Lossky, M., Anderson, G.J., Jackson, S.P. and Beggs, J. (1987) Cell **51**, 1019–1026.
26. Petterson, I., Hinterberger, M., Mimori, T., Gottlieb, E. and Steitz, J.A. (1984) J. Biol. Chem. **259**, 5907–5914.
27. Black, D.L. and Pinto, A.L. (1989) Mol. Cell. Biol. **9**, 3350–3359.
28. Fritz, A., Parisot, R., Newmeyer, D. and DeRobertis, E.M. (1984) J. Mol. Biol. **178**, 273–285.
29. Bach, M., Winkelmann, G. and Luehrmann, R. (1989) Proc. Natl. Acad. Sci. USA **86**, 6038–6042.
30. Jackson, S.P., Lossky, M. and Beggs, J.D. (1988) Mol. Cell. Biol. **8**, 1067–1075.
31. Anderson, G.J., Bach, M., Luehrmann, R. and Beggs, J.D. (1989) Nature, **342**, 819–821.
32. Reuter, R. and Luehrmann, R. (1986) Proc. Natl. Acad. Sci. USA **83**, 8689–8693.
33. Grabowski, P.J. and Sharp, P.A. (1986) Science **233**, 1294–1299.
34. Bindereif, A. and Green, M.R. (1987) EMBO J. **6**, 2415–2424.
35. Reveillaud, I., Lelay-Taha, M-N, Sri-Widada, J., Brunel, C. and Jeanteur, P. (1984) Mol. Cell. Biol. **4**, 1890–1899.
36. Zillmann, M., Rose, S.D. and Berget, S.M. (1987) Mol. Cell. Biol. **7**, 2877–2883.
37. Konarska, M.M. and Sharp, P.A. (1988) Proc. Natl. Acad. Sci. USA **85**, 5459–5462.
38. Chabot, B., Black, D.L., Le Master, D.M. and Steitz, J.A. (1985) Science **230**, 1344–1349.
39. Pinto, A.L. and Steitz, J.A. (1989) Proc. Natl. Acad. Sci.USA **86**, 8742–8746.
40. Konarska, M.M. and Sharp, P.A. (1987) Cell **49**, 763–774.
41. Zillmann, M., Zapp, M.L. and Berget, S.M. (1988) Mol. Cell. Biol. **8**, 814–821.
42. Ruby, S.W. and Abelson, J. (1988) Science **242**, 1028–1035.
43. Tazi, J., Alibert, C., Temsamani, J., Reveillaud, I., Cathala, G., Brunel, C. and Jeanteur, P. (1986) Cell **47**, 755–766.
44. Gerke, V. and Steitz, J.A. (1986) Cell **47**, 973–984.
45. Ruskin, B., Zamore, P.D. and Green, M.R. (1988) Cell **52**, 207–219.
46. Conway, G.C., Krainer, A.R., Spector, D.L. and Roberts, R.J. (1989) Mol. Cell. Biol. **9**, 5273–5280.