Structural/functional properties of a mammalian multi-component structure containing all major spliceosomal small nuclear ribonucleoprotein particles

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An approx. 40 S multi-component structure, consisting of all major spliceosomal small nuclear ribonucleoprotein particles (snRNP) (U1, U2, U4/U6 and U5) in stable association with a large number of polypeptides, mainly in the range 50–210 kDa, has been reported to exist within rat liver nuclear extracts [Guialis, Moraitou, Patrinou-Georgoula and Dangli (1991) Nucleic Acids Res. **19**, 287–296]. Using a new polyclonal antibody recognizing a 63 kDa protein component of the complex, this multi-snRNP assembly was detected within rat liver nuclear extracts as efficiently as with the antibody for the U2 snRNP-specific B″ polypeptide. The 63 kDa protein was found to correspond to the 66 kDa subunit of the splicing factor SF3a, a known integral component of the HeLa 17 S U2 snRNP. Anti-2,2,7-trimethylguanosine affinity chromatography was an easy and efficient way of purifying the multi-snRNP complex from rat liver 40 S heterogeneous nuclear ribonucleoprotein particle (hnRNP)-containing sucrose gradient fractions. By subsequent glycerol-gradient sedimentation, all known snRNP forms active in RNA splicing were identified among its constituents. A complex structurally similar to the rat multi-snRNP was also identified in HeLa nuclear extracts. Preservation of hnRNP–snRNP interactions was observed within HeLa 40 S fractions. Moreover, these fractions were capable of restoring splicing activity when applied in reconstitution studies to supplement a micrococcal nuclease-treated splicing extract.

INTRODUCTION

The complex molecular events taking place during pre-mRNA maturation in eukaryotes have been experimentally approached following the establishment of *in vitro* systems able to carry out accurate and efficient RNA splicing. These studies permitted the identification of a multi-component 40–60 S ribonucleoprotein particle (RNP) active in RNA splicing, termed spliceosome (reviewed in [1]). Characterized components of the spliceosome are the major U small nuclear RNP (snRNP) complexes (U1, U2, U4/U6 and U5), referred to as spliceosomal snRNPs, each consisting of a single small nuclear RNA (snRNA) molecule (with the exception of U4/U6 snRNP) in stable association with a number of polypeptides, some common and others unique to each snRNP complex [2]. In addition to the spliceosomal snRNPs, an increasing number of snRNP-associated polypeptides, functioning in RNA splicing, have recently been identified, mainly in human and yeast cells (for a recent review see [3]). Moreover, the existence of the spliceosomal snRNP complexes in different states of association with specific protein components and with each other has recently been demonstrated in HeLa nuclear extracts. Thus, in addition to the free pool of 12 S U snRNP complexes, the 20 S U5 snRNP [4], the 25 S U4/U6.U5 tri-snRNP [5], as well as the 17 S U2 snRNP complex [6], have been reported. Each of these larger than 12 S snRNP assemblies contains a specific set of snRNP-associated polypeptides and represents the *in vitro* active RNA splicing form of the corresponding spliceosomal snRNP entity (reviewed in [3]). The precise composition of the spliceosome and the way in which this dynamic, multi-component RNP structure assembles and functions during pre-mRNA splicing is currently a matter of active investigation.

We have previously reported the identification in rat liver nuclear extracts of a 40 S multi-snRNP complex containing all major spliceosomal snRNP complexes together with a large number (over 20) of snRNP-associated polypeptides in the range of 50–200 kDa [7]. Initial evidence supporting the presence of such an snRNP assembly, named MI multi-snRNP, originated from native gel electrophoresis of the 40 S material from sucrose-gradient-fractionated rat liver nuclear extracts [8]. In these fractions, the MI multi-snRNP was found to co-exist with a distinct RNP entity, the monomeric 40 S hnRNP structure (MII), shown to contain the bulk of heterogeneous nuclear RNA (hnRNA) and hnRNP polypeptides. The existence of such a multi-snRNP complex within rat liver nuclear extracts, prepared under physiological salt conditions, was later verified immunochemically. This was shown by the ability of the monoclonal antibody (mAb) 4G3, with its established specificity for the B″ polypeptide of U2 snRNP [9], to co-precipitate all major spliceosomal snRNP complexes [7]. As pointed out then, a distinct structural feature of the MI multi-snRNP was the large assortment of stably associated polypeptides, mainly of 50–200 kDa, among which the largest 200 kDa protein species, often appearing as a doublet, was identified as the rat homologue of the yeast PRP8 splicing factor [10]. In the present work we have extended those immunochemical studies in order to characterize further the structural composition of the rat MI multi-snRNP assembly. Moreover, we present evidence supporting the existence in HeLa cell extracts of an assembly structurally similar to the rat multi-snRNP, which can function in RNA splicing *in vitro*.

MATERIALS AND METHODS

Preparation of nuclear extracts

Rat liver nuclear extracts were prepared as previously described [11]. The mains steps involved extraction of nuclei in 10 mM

Abbreviations used: RNP, ribonucleoprotein particle; hnRNP, heterogeneous nuclear ribonucleoprotein particle; hnRNA, heterogeneous nuclear RNA; snRNP, small nuclear ribonucleoprotein particle; snRNA, small nuclear RNA; m_3G, 2,2,7-trimethylguanosine; m^7G, 7-methylguanosine; MN, micrococcal nuclease; mAb, monoclonal antibody; GPI serum, guinea pig pre-immune serum; EtBr, ethidium bromide.

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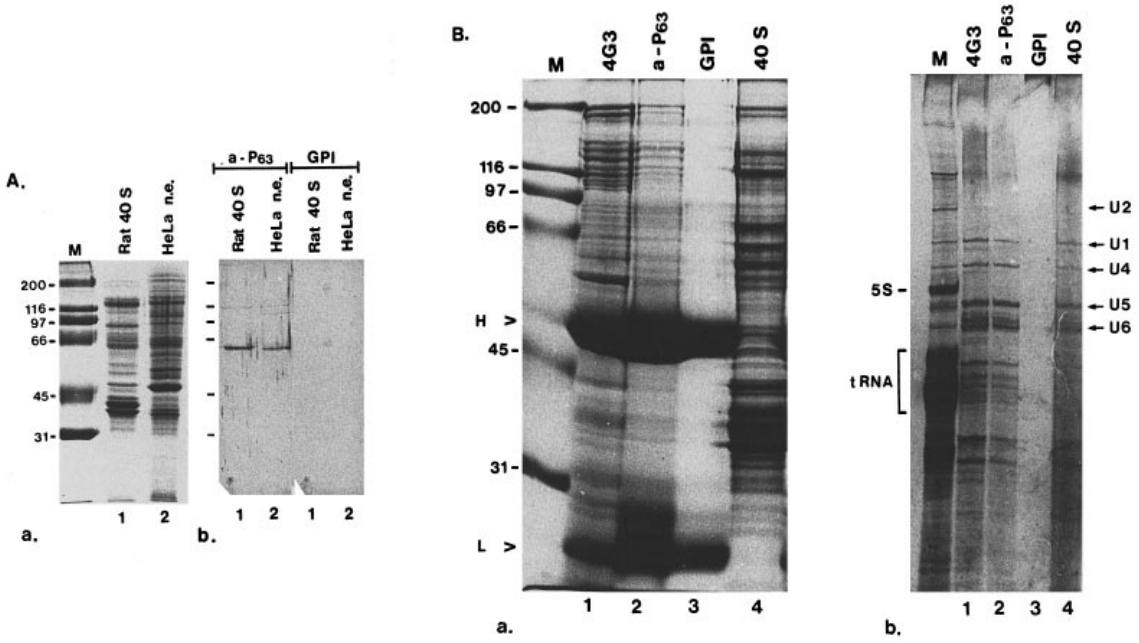

Figure 1 Detection of the rat MI multi-snRNP complex by two distinct antibody populations

(**A**) Specificity of guinea pig anti-P_{63} antibodies. (a) Rat liver 40 S nuclear fractions (lane 1) and HeLa nuclear extracts (lane 2) were resolved on a 10% SDS/PAGE gel, transferred to a nitrocellulose membrane and stained with Ponceau S. (b) The same membrane was blotted using guinea pig anti-P_{63} (a-P_{63}) and GPI sera. (**B**) Immunoprecipitation ability of the mAb 4G3 and anti-P_{63} antibodies. Protein components (a) and RNA species (b) were recovered from rat liver 40 S hnRNP-containing sucrose-gradient fractions following precipitation with either the mAb 4G3 antibody (lane 1) or the guinea pig anti-P_{63} (lane 2) and pre-immune (lane 3) sera. In lane 4, a portion (10%) of the 40 S material used per immune reaction was analysed. The protein gel was stained with Coomassie Blue and the RNA gel with silver nitrate. Lanes M in (**A**) (panel a) and (**B**) (panel a) represent marker polypeptides used for the estimation of molecular mass. The heavy (H) and light (L) IgG antibody chains are marked in (**B**) (panel a). The RNA species recovered from rat liver cell extracts, used to define the position of migration of the U snRNAs, are shown in (**B**) (panel b) (lane M). The absence of intact U2 snRNA in the 40 S fractions used in these assays was due to extensive RNA degradation.

Tris/HCl, pH 8.0/140 mM NaCl/1 mM $MgCl_2$, followed by sonic disruption and brief centrifugation to remove nuclear residues. 40 S hnRNPs were obtained by fractionating the nuclear extracts on 15–30% (w/v) sucrose gradients at 55000 ***g*** for 17 h at 4 °C, followed by pelleting the 40 S hnRNP-containing fractions at 70000 ***g*** for 18 h at 4 °C.

HeLa nuclear extracts were prepared according to the method of Choi and Dreyfuss [12] and splicing extracts by the method of Dignam et al. [13]. Where applied, both HeLa extract preparations were fractionated on sucrose gradients as described for the rat liver nuclear extracts.

Radiolabelled HeLa extracts were obtained from cells grown in the presence of [^{35}S]methionine as described previously [14].

Purified HeLa 17 S U2 snRNP and 25 S U4/U6.U5 tri-snRNP complexes were provided by Dr. R. Lurhmann (Philipps Univ., Ist. für Molekularbiologie Tumorforsch, Marburg, Germany).

Antibodies

The mAb 4G3 [9] was a gift from Dr. W. van Venrooij (Department of Biochemistry, University of Nijmegen, Nijmegen, The Netherlands). The mAbs Y12 [15] and 4F4 [16] were provided by Dr. J. Steitz (Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, U.S.A.) and Dr. G. Dreyfuss (University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.), respectively. The human anti-Sm, with specificity for B, B′ and D polypeptides of snRNPs, was a standard serum from the Centers for Disease Control, Atlanta, GA, U.S.A. Non-immune sera were from healthy individuals and a control mAb (co-mAb) recognizing the rat glucocorticoid receptor was provided by Professor C. E. Sekeris (National Hell. Res. Foundation, Inst. of Biol. Res. Biotech., Athens, Greece). The two antibodies specific for protein components of the HeLa SF3a splicing factor of the 17 S U2 snRNP [17,18] were provided by Dr. A. Kramer (Biologie Cellulaire, Univ. de Genève, Geneva, Switzerland).

The polyclonal anti-P_{63} antibodies were produced in guinea pigs as follows: material corresponding to rat liver 40 S hnRNPs was used in large-scale immunoprecipitation reactions with mAb 4G3 and the proteins contained in the immune pellets were resolved by SDS/PAGE. Guinea pigs were injected with a protein band of 63 kDa using the immunization protocol previously outlined [14].

Immunoblotting and immunoprecipitation

Protein transfer after SDS/PAGE, blocking and incubation conditions for immunoblotting were as described [19]. Before immunostaining, the proteins were stained with Ponceau S. The usual serum dilution for the guinea pig pre-immune (GPI) and anti-P_{63} sera was 1:100.

Immunoprecipitation reactions were performed in NET-2 buffer [150 mM NaCl/10 mM Tris/HCl, pH 7.5/0.05% (v/v) Nonidet P40] at 4 °C, as described in [7]. RNAs were phenol-extracted from the immune pellets and resolved on denaturing 10% polyacrylamide/7 M urea gels. Protein components were dissolved in SDS sample buffer and separated by SDS/PAGE on an 8–15% gradient gel.

Anti-2,2,7-trimethylguanosine (m_3G) (anti-cap) affinity chromatography

The immunoaffinity column (CNBr-activated Sepharose 4B, Pharmacia) containing immobilized mAb H20 [20] was a gift from Dr. R. Luhrmann. All steps in the procedure were carried out at 4 °C, essentially as described in [21]. The material applied (per 1.0 ml of packed column volume) was rat liver 40 S hnRNPs (1–8 mg of protein), HeLa 40 S hnRNPs prepared from 1×10^9 HeLa cells, as well as unfractionated HeLa splicing extracts (14 mg of protein). Analysis of the proteins was by 10% polyacrylamide SDS/PAGE and the RNAs were analysed on a 10% polyacrylamide/7 M urea gel.

Glycerol-gradient sedimentation

The eluate of the anti-m_3G affinity column, originating from rat liver 40 S hnRNPs, was further fractionated on an 11 ml, 10–40% (v/v) glycerol gradient prepared as in [5]. The gradient was centrifuged in a Beckman SW 41 rotor at 30000 rev./min for 18 h, at 4 °C. Eighteen fractions, 0.65 ml each, were harvested from the top of the gradient and assayed for RNA content by phenol extraction and resolution on a 10% polyacrylamide/7 M urea gel, followed by staining with silver nitrate [22].

Hybridization studies

Following RNA gel analysis, the RNA species were electrophoretically transferred on to a nylon membrane (Hybond-N, Amersham) and immobilized by UV cross-linking, according to the manufacturer's protocol.

Labelled anti-sense RNA probes, corresponding to U2, U4, U5 and U6 snRNAs, were synthesized using appropriate plasmid constructs (provided by Dr. I. Mattaj, EMBL, Heidelberg, Germany) and used in the hybridization of the immobilized snRNAs as described in [23].

In vitro splicing

The pre-mRNA substrate used in *in vitro* RNA splicing was transcribed from a DNA template corresponding to the rabbit β-globin gene (pBSAL4; constructed by Dr. A. Lamond, EMBL) [24]. Pre-mRNA transcription and subsequent purification were carried out according to protocols described in [25] and [24].

Splicing reactions were performed at 30 °C for 1 h in a final volume of 40 μl, essentially as described in [24]. The splicing extract was pre-incubated for 20 min at 30 °C before being added to the reaction mixture at 25% (v/v). The RNAs were analysed on a denaturing 7% polyacrylamide RNA gel, followed by X-ray autoradiography.

For the antibody inhibition studies, the IgG fraction of the serum that was added to the splicing reaction was first affinity-purified using Protein A–Sepharose (Pharmacia), 25 mg/100 μl of serum. The bound antibodies were eluted in a buffer containing 100 mM glycine, pH 3/500 mM NaCl/0.05% (v/v) Tween-20/0.1% (w/v) BSA, and immediately neutralized with 1 M Tris/HCl, pH 7.5, followed by dialysis against splicing buffer D [20 mM Hepes, pH 7.9/0.1 M KCl/0.2 mM EDTA/0.5 mM dithiothreitol/5% (v/v) glycerol] [13]. Splicing extracts were pre-incubated with the IgG fraction for 30 min at 0 °C before the splicing reaction proceeded.

Immunodepletion of splicing extracts was achieved by three cycles of the immunoprecipitation assay using the Protein A–Sepharose-purified IgG fraction of the serum applied.

In the *in vitro* reconstitution assays, the splicing extract had been inactivated by prior incubation with 350 units/ml micrococcal nuclease (MN) for 20 min at 30 °C in the presence of 1 mM $CaCl_2$, followed by addition of 2.5 mM EGTA. The supplementary material was pre-incubated for 20 min at 30 °C and then added to the splicing reaction mixture at 25% (v/v) of the final reaction volume. The reaction mixtures contained polyvinylalcohol at a final concentration of 2.5% (w/v).

RESULTS

Protein pattern of the rat MI multi-snRNP: identification of spliceosomal components

As previously demonstrated [7], mAb 4G3 with specificity for the U2 snRNP B″ polypeptide [9] could be used as a specific probe for the isolation of the MI multi-snRNP complex from rat liver nuclear extracts. To characterize better the protein composition of the complex, we performed scaled-up immunoprecipitation reactions, followed by protein resolution of the immune pellets by SDS/PAGE. This approach proved the feasibility of obtaining fairly large amounts of polypeptide components of the rat multi-snRNP complex and led us to attempts at antibody production, both monoclonal and polyclonal, against individual polypeptides. We succeeded, in one case, in producing in guinea pigs an antibody population recognizing a polypeptide of the multi-

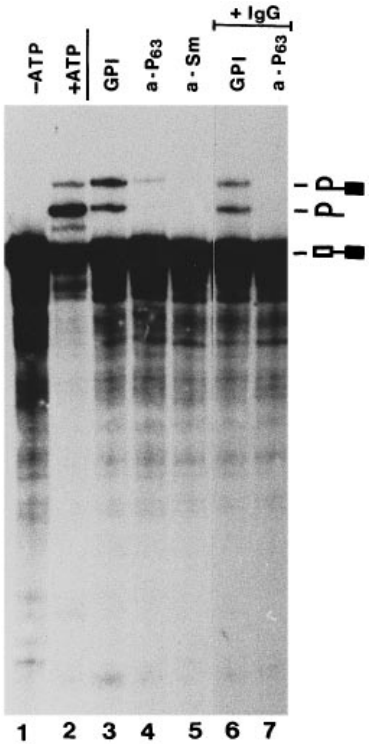

Figure 2 Ability of the anti-P_{63} antibodies to inhibit pre-mRNA splicing *in vitro*

HeLa splicing extracts, that had been immunodepleted using GPI (lane 3) and anti-P_{63} (a-P_{63}) (lane 4) or human anti-Sm (lane 5) sera were used to support RNA splicing. Splicing reactions in which immune depletion was combined with antibody inhibition, by pre-incubating the splicing extract in the presence of 150 μg IgG (+IgG) of either GPI or a-P_{63} antibodies, are shown in lanes 6 and 7 respectively. Lanes 1 and 2 (−ATP, +ATP) refer to negative and positive control splicing reactions respectively. The positions of migration of the pre-mRNA and the products of the splicing reaction are shown schematically.

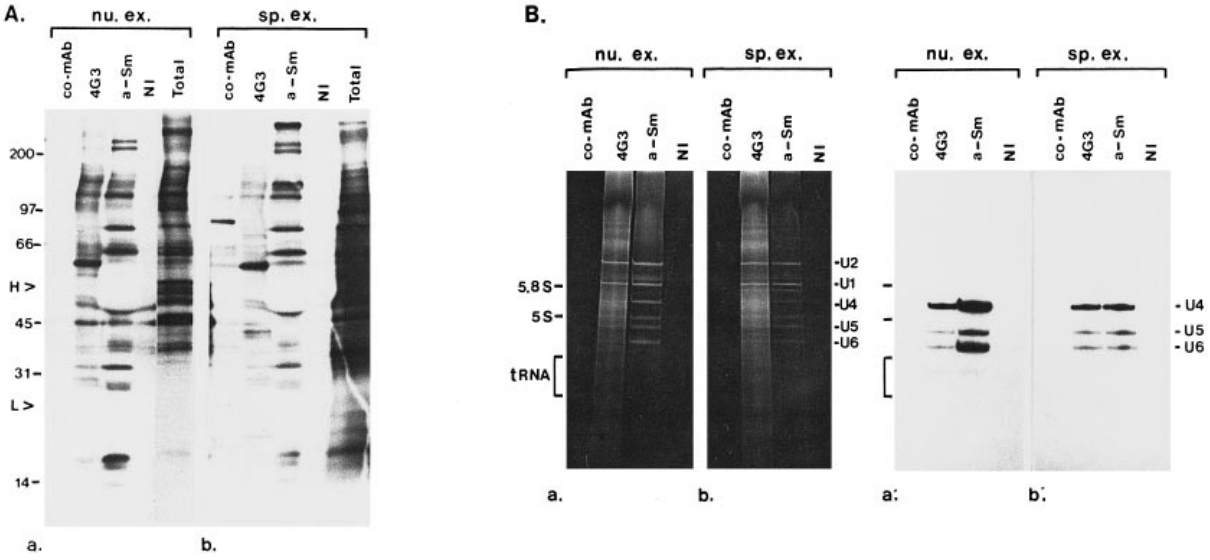

Figure 3 Immunochemical identification of an MI-type multi-snRNP complex in HeLa cells

HeLa nuclear extracts obtained according to the method of Choi and Dreyfuss [12] or splicing extracts [13] were prepared from [^{35}S]methionine-labelled cells and used in immunoprecipitation assays employing human anti-Sm serum and mAb 4G3, in parallel with control antibodies [non-immune (NI) and co-mAb respectively]. (**A**) The proteins recovered in the immune pellets from either the nuclear extract (nu. ex.) (a) or the splicing extract (sp. ex.) (b) were resolved by SDS/PAGE and visualized by fluorography. Total refers to a portion (10%) of the extract used per immune reaction. (**B**) The RNA species co-precipitating in the same immune reactions shown in (**A**) were resolved on a denaturing RNA gel and stained with ethidium bromide (EtBr) (a and b). The positions of migration of marker 5.8 S, 5 S and tRNA, as well as of the U snRNA species are indicated. Identification of the U4, U5 and U6 snRNAs was achieved after electrophoretic transfer of the RNAs to nitrocellulose and subsequent hybridization using a mixture of the corresponding ^{32}P-labelled anti-sense RNAs (a′ and b′).

snRNP complex with an apparent molecular size of 63 kDa (P_{63} protein). The specificity of these antibodies (anti-P_{63}) in rat liver 40 S fractions, as well as in HeLa nuclear extracts, is demonstrated in Figure 1(A). As can be seen, anti-P_{63} antibodies reacted mainly with a single protein in both mammalian species. Also, as shown in Figure 1(B), these antibodies were able to immunoprecipitate from the rat liver 40 S hnRNP-containing nuclear fractions an RNP complex which, in its protein (Figure 1B, a) and RNA (Figure 1B, b) composition, was indistinguishable from the 4G3-immunopurified multi-snRNP. Thus, the same RNP entity could be identified in rat liver 40 S fractions by two distinct antibody specificities, each one recognizing a single protein component of the complex. Moreover, these data proved that P_{63} protein was indeed an authentic component of the multi-snRNP.

Since, as pointed out before [7], the multi-snRNP resembled structurally the *in vitro* spliceosome, we investigated the extent to which anti-P_{63} antibodies could inhibit RNA splicing *in vitro*. From the results obtained (see Figure 2), it was clear that immunodepletion of HeLa splicing extracts with anti-P_{63} antibodies (lane 4) drastically reduced splicing activity, in contrast to the GPI serum (lane 3). In agreement with published work [26], anti-Sm antibodies could completely block splicing (lane 5). A complete inhibition of RNA splicing could also be achieved with anti-P_{63} antibodies when immunodepletion was combined with antibody addition during splicing (lane 7). A significant inhibition of RNA splicing by anti-P_{63} antibody addition alone could be seen at rather high antibody concentrations (150 μg of IgG/assay; results not shown).

The protein composition of the recently identified 17 S U2 snRNP [6] and 25 S U4/U6.U5 tri-snRNP [5] complexes in HeLa cell extracts has been reported. To investigate the extent to which polypeptides of the rat 4G3-immunopurified 40 S multi-snRNP complex could correspond to protein components of the HeLa snRNP complexes, the protein patterns of the rat and HeLa snRNP components were directly compared by SDS/PAGE (results not shown). This comparison revealed that the overall protein pattern of the rat multi-snRNP resembled that of the HeLa 17 S U2 snRNP, whereas the polypeptides of the tri-complex were under-represented. Furthermore, immunoblotting with the anti-P_{63} antibodies identified the presence of the P_{63} protein in the 17 S U2 snRNP, but not in the 25 S tri-snRNP, complex. During the course of this work, antibodies recognizing the SF3a66 and SF3a60 spliceosomal components associated with the 17 S U2 snRNP became available [17,18]. Using those antibodies we were able to detect their respective antigens within the rat multi-snRNP complex (results not shown). Moreover, crossing-over experiments proved that the P_{63} polypeptide was the rat homologue of the human SF3a66 protein. All of the above findings indicated that the components of the 17 S U2 snRNP constituted a major part of the rat multi-snRNP.

Detection of an MI-type multi-snRNP complex in HeLa nuclear extracts

To investigate the feasibility of detecting in other mammalian species a multi-snRNP assembly structurally similar to the rat MI complex, we analysed HeLa nuclear extracts that are primarily used in *in vitro* RNA splicing. As for the rat, the main criterion for the presence of a multi-snRNP complex in HeLa nuclear extracts was the co-precipitability by 4G3 antibodies of all spliceosomal snRNP complexes (U1, U2, U4/U6 and U5). In these experiments, two different HeLa nuclear extract preparations were tested in parallel. The first, referred to as 'nuclear extract', was prepared by extracting nuclei in an isotonic buffer containing 140 mM NaCl according to the protocol of Choi and Dreyfuss [12]. The second was a splicing extract preparation

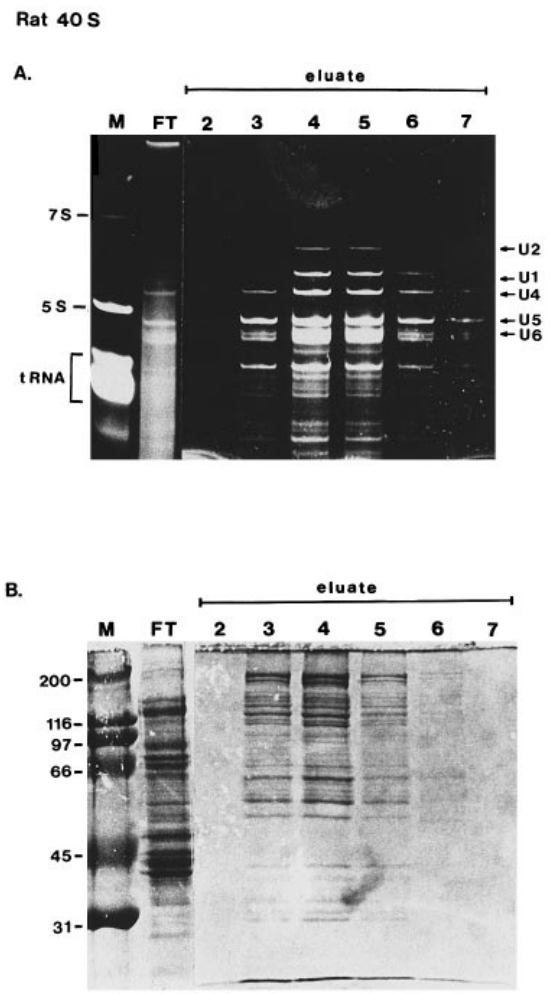

Figure 4 Affinity purification of the rat MI multi-snRNP complex

Rat liver 40 S hnRNP-containing nuclear factors were loaded onto an anti-m_3G immunoaffinity column and the bound material was eluted using m^7G competitor. The flow-through (FT) and the eluate (fractions 2–7) were analysed with respect to the RNA (**A**) and protein (**B**) content. The RNA gel was stained with EtBr and the protein gel with Coomassie Blue. Degraded U snRNA species appearing on the RNA gel run mainly at the level of, or below, U6 snRNA.

obtained by the standard protocol of Dignam et al. [13], which involved extracting nuclei at high salt concentration (0.4 M NaCl), followed by dialysis of the nuclear extract in 0.1 M KCl. Specific immunoprecipitation reactions were performed with both extract preparations, followed by analysis of the RNA and protein components of the immune pellets. Since a low concentration of the snRNP complexes relative to the rat nuclear extract was anticipated, nuclear extracts prepared from [^{35}S]-methionine-labelled HeLa cells were used in the detection of immunoprecipitated polypeptides. Similarly, the unequivocal identification of snRNA species in the immune pellets was done by performing specific hybridization assays.

In Figure 3(A), the pattern of the polypeptides specifically recovered in the 4G3 or anti-Sm immunoprecipitates from nuclear extracts (Figure 3A, a) and splicing extracts (Figure 3A, b) of [^{35}S]methionine-labelled HeLa cells is presented. Although a comparison, on a one-to-one basis, between the HeLa and rat protein species was not easy, it was evident that, as in the case of the rat extract (Figure 1B), a large number of proteins in the range 50–210 kDa specifically appeared in the HeLa 4G3 immunoprecipitate when compared with the control immune reaction. The protein pattern of the 4G3 precipitate was very similar in the two extract preparations, as was that of the anti-Sm immune pellets. However, the 4G3 and anti-Sm protein patterns were distinct when compared with each other.

In addition to the co-precipitated polypeptides, the spliceosomal snRNA species of the same immune pellets were analysed and compared. As seen in Figure 3(B), parts a and b, the most abundant U1 and U2 snRNA species could be easily identified in the anti-Sm and the 4G3-immunoprecipitates by EtBr-staining alone. The unequivocal presence, in the same immune pellets, of the less-abundant U4, U5 and U6 snRNAs was shown by hybridizing a Northern blot of the gel with specific anti-sense RNA probes (Figure 3B, part a′ and Figure 3B, part b′). There were no snRNA species detected in the control immune pellets.

In agreement with the results obtained using rat liver nuclear extracts, immunoprecipitation studies in HeLa nuclear extracts, as well as splicing extracts, employing the polyclonal anti-P_{63} antibodies, provided similar findings as when using the mAb 4G3 (results not shown). Therefore, the specific co-precipitation of the spliceosomal snRNAs (U1, U2, U4, U5 and U6), together with a large number of polypeptides, strongly suggests the presence of an MI-type multi-snRNP complex in HeLa cells.

Immunoaffinity purification of the MI multi-snRNP complex from 40 S nuclear fractions

Immunoprecipitation assays using 4G3 or anti-P_{63} antibodies, although decisive in defining the presence of the MI multi-snRNP complex in nuclear extracts, did not allow its recovery in a native form. Resolution of the complex could be done following native gel electrophoresis [8]. However, this biochemical method has serious drawbacks, such as possible contamination with co-migrating nuclear components, extensive RNA degradation and some loss of associated protein species. Therefore, we attempted affinity purification of the multi-snRNP complex from the rat 40S hnRNP-containing nuclear fractions, using anti-m_3G (anti-cap) antibodies coupled with Sepharose beads, in conjunction with the 7-methylguanosine (m^7G) competitor for the specific recovery of the bound snRNP complexes. This method has been successfully applied in the past for the recovery from HeLa nuclear extracts of the individual 12 S U snRNP complexes, as well as of the larger snRNP assemblies [4–6,20]. In the present study, a sucrose-gradient-fractionation step of the nuclear extract preceeded affinity purification, in order to obtain the multi-snRNP from the 40 S material free of 10–12 S and < 30 S snRNP complexes [11].

In Figure 4, the RNA (Figure 4A) and protein (Figure 4B), components detected in the flow-through (FT) and the m^7G eluate, after anti-m_3G affinity purification of the rat 40 S hnRNP-containing sucrose-gradient fractions, are presented. While the bulk of the hnRNA molecules appeared as a smear in the flow-through fraction, the great majority of the snRNA species were retained in the column and were subsequently recovered in the m^7G eluate. The same was observed in the case of the protein composition of the two column fractions. As seen in Figure 4(B), the flow-through fraction contained almost exclusively the protein species that had previously been found in the monomeric 40 S hnRNP structure MII [7]. This refers to the 32–45 kDa core hnRNP proteins and polypeptides of 60–80 and 110–130 kDa, which have also been found highly enriched in hnRNP complexes immunopurified from HeLa nuclear extracts

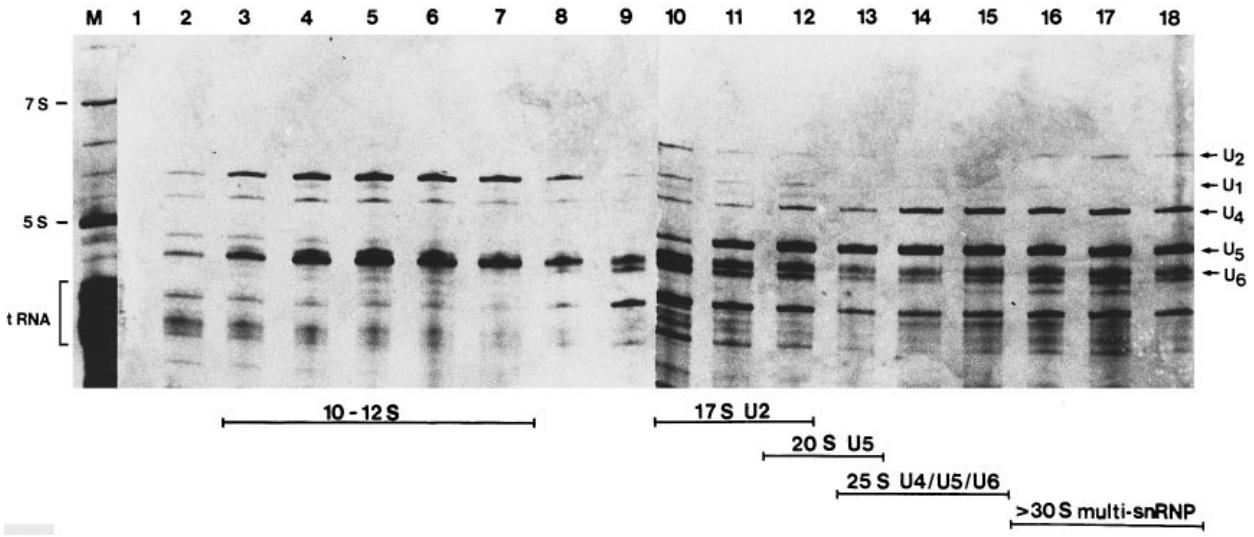

Figure 5 Glycerol gradient sedimentation of the affinity-purified rat MI multi-snRNP

Eluate fraction number 4, shown in Figure 4, was fractionated on a glycerol gradient. The RNA species, present in each of the 18 gradient fractions, were phenol-extracted, resolved on a denaturing RNA gel and identified by silver staining. The positions of migration of the 10–12 S snRNPs, as well as of the 17 S U2, 20 S U5, 25 S U4/U6.U5 tri-snRNP and > 30 S multi-snRNP complexes are indicated in brackets, as deduced from the relative intensities of the intact U snRNAs along the gradient.

[12,27]. The protein pattern of the m^7G eluate was clearly distinct from that of the flow-through fraction and had a great resemblance to that of the multi-snRNP complex immunopurified by 4G3 and anti-P_{63} antibodies, as shown in Figure 1(B), part a. Thus, affinity purification proved to be an easy and reproducible method of obtaining fairly large amounts of the rat 40 S multi-snRNP complex.

In order to study the stability of the affinity-purified multi-snRNP complex and to determine its specific composition in U snRNP assemblies, we further fractionated the m^7G eluate (fraction 4, shown in Figure 4) by glycerol-gradient centrifugation. The snRNA species recovered in every fraction of the gradient were resolved by RNA gel electrophoresis (Figure 5). As deduced from the distribution pattern and the relative amount of each of the U1, U2, U4, U5 and U6 snRNAs along the glycerol gradient, all the assemblies corresponding to the known HeLa 10–25 S snRNP forms functioning in RNA splicing *in vitro* (reviewed in [3]) were identified within the affinity purified rat multi-snRNP. These included the 17 S U2 snRNP structure, which appeared enriched in fractions 10–12, as well as the 25 S U4/U6.U5 tri-snRNP complex that was mainly detected in fractions 13–15. Moreover, as seen from the relative amounts of the U5 snRNA alone, the 20 S snRNP was detected in fractions 12 and 13. The heavier than 25 S material (fractions 16–18) contained the remaining 40 S multi-snRNP complex. As expected from previous reports ([17] and references therein), the loosely associated U1 snRNP complex was recovered in the light 10–12 S fractions, as shown by the presence of the U1 snRNA and its degraded species. These findings indicated an extensive destabilization of the MI multi-snRNP complex that took place during the affinity purification step, which was also confirmed by its poor immunoprecipitability using 4G3 antibodies (results not shown). Nonetheless, the presence in the affinity-purified rat 40 S multi-snRNP complex of the 17 S U2 and 25 S U4/U6.U5 snRNP assemblies, the HeLa counterparts that are active in RNA splicing *in vitro*, strongly suggests a relevant functional association of all these components *in vivo*.

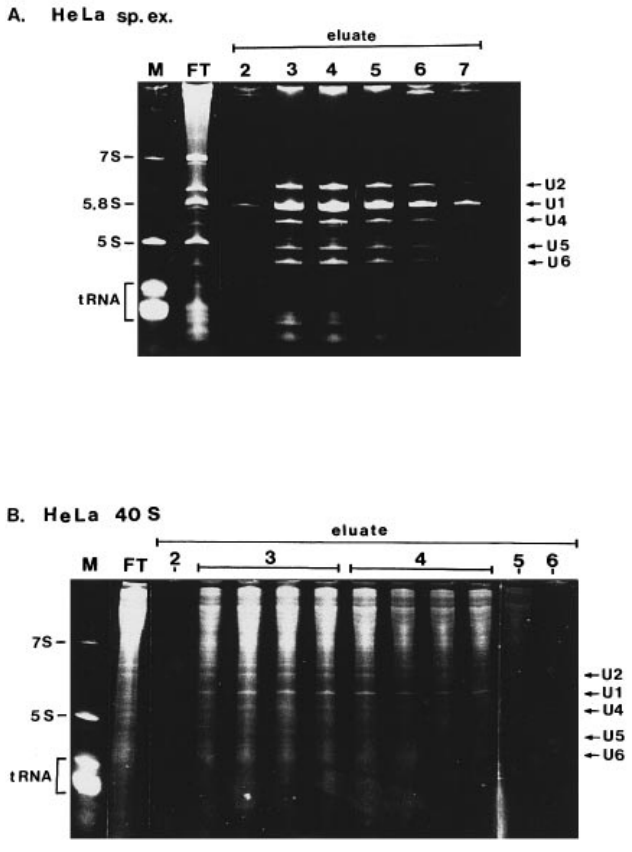

Figure 6 Anti-m_3G affinity chromatography of HeLa snRNP complexes

Either a total splicing extract (**A**) or a subfraction corresponding to 40 S material (**B**) was applied to an anti-m_3G column. The RNA species present in the flow-through (FT) and in the eluate fractions were analysed as in the case of the rat 40 S material (Figure 4A). In (**B**) (HeLa 40 S), eluate fractions 3 and 4 were collected in smaller aliquots (125 μl instead of the usual 500 μl).

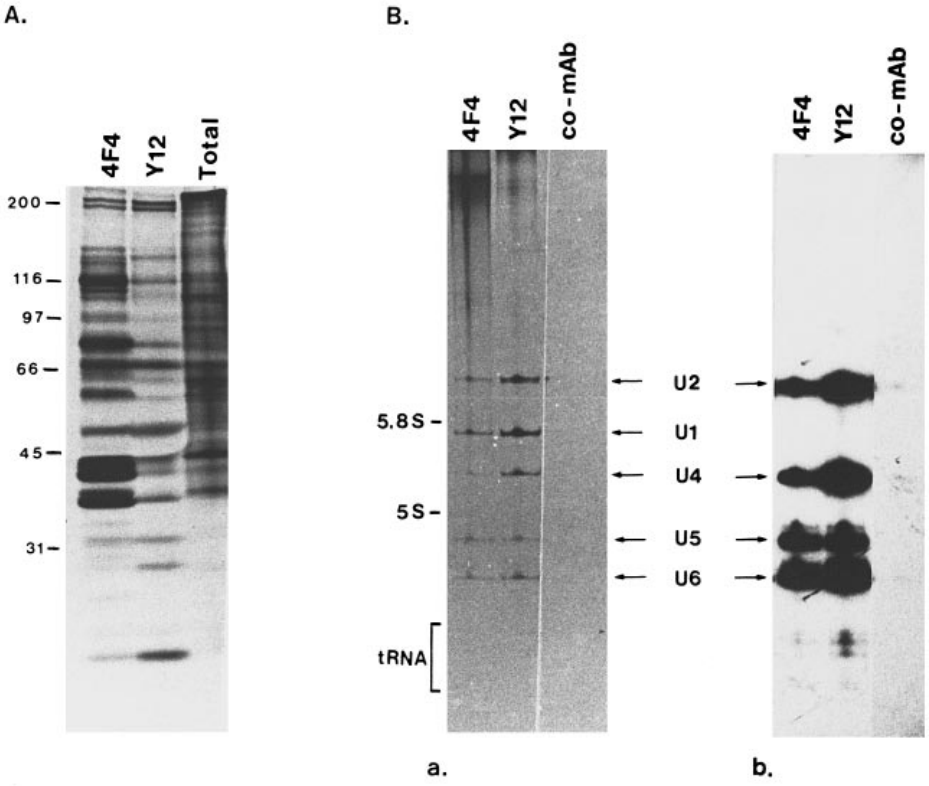

Figure 7 Detection of hnRNP–snRNP interactions in HeLa nuclear extracts

Immunoprecipitation assays, using the mAbs 4F4 and Y12, were performed on nuclear extracts prepared from [^{35}S]methionine-labelled cells, followed by the parallel analysis of the protein and RNA composition of the immune pellets. The proteins (**A**) were visualized by fluorography of the SDS/polyacrylamide gel. Total represents an aliquot (10%) of the extract applied per immune assay. The RNAs (**B**) were detected by EtBr staining (a) and further identified by hybridizing a Northern blot with a mixture of ^{32}P-labelled anti-sense U2, U4, U5 and U6 snRNAs (b).

As in the case of the rat MI multi-snRNP, affinity purification of the complex from HeLa cells was also performed. In these studies, 40 S hnRNP-containing fractions, obtained following sucrose-gradient centrifugation of a HeLa splicing extract, were applied onto the anti-m_3G affinity column. This 40 S material contained all spliceosomal snRNAs, as well as hnRNA, similar to a HeLa nuclear extract prepared according to Choi and Dreyfuss [12] shown below in Figures 8(A) and 8(B). Analysis of the RNA species recovered in the flow-through and the m^{7}G eluate fractions clearly showed that a significant amount of hnRNA molecules contained in the HeLa 40 S fractions was retained on the column and specifically eluted together with the bound U snRNA species (Figure 6B). The same observation was made when 40 S fractions were from HeLa nuclear extracts prepared according to Choi and Dreyfuss [12]. This was in contrast to what was observed in the rat 40 S fractions (Figure 4A), where a clear separation of the snRNA species from the hnRNA was achieved in the m^{7}G eluate. In fact, in HeLa cells the smear of the hnRNA in the eluate largely obscured the clear identification of the U snRNAs, the latter being verified by subsequent specific hybridization. This presumptive association of hnRNP and snRNP complexes in the m^{7}G eluate fractions was specific, since it could not be disturbed by prior treatment of the 40 S fractions with heparin (5 mg/ml). The difference observed in the RNA pattern of the rat and of the HeLa m^{7}G eluates could possibly be explained by the preservation of pre-existing associations between hnRNP and snRNP complexes in the HeLa 40 S fractions. This was not the case in the rat 40 S fractions, as deduced from the observed extensive degradation of both hnRNA and U snRNA species (compare corresponding FT and m^{7}G eluate fractions in Figures 4 and 6). In accordance with published reports [4,5], when unfractionated HeLa splicing extracts were applied onto the anti-m_3G affinity column, a clear resolution of the snRNAs in the m^{7}G eluate could be seen (Figure 6A). These snRNA species presumably represented the free pool of spliceosomal snRNP complexes in HeLa splicing extracts.

The ability to detect pre-existing hnRNP–snRNP interactions in HeLa nuclear extracts was also demonstrated by the results presented in Figure 7. Extracts prepared according to Choi and Dreyfuss [12] were used in immunoprecipitation studies with the monoclonal antibodies 4F4 and Y12, which are specific for hnRNP [16] and Sm-precipitable snRNP [15] complexes respectively. After analysis of the protein components recovered in the 4F4 and Y12 immune pellets (Figure 7A), the expected distinct protein patterns of hnRNP and snRNP complexes respectively, were observed. Nonetheless, the sharing of some polypeptides of similar apparent molecular size was also evident, the most prominent being the protein doublet of approx. 200 kDa, known as a U4/U6.U5 tri-snRNP-associated splicing factor [5,10]. The RNA species present in the same immune pellets are shown in Figure 7(B). As can clearly be seen, all the spliceosomal snRNAs (U1, U2, U4, U5 and U6) could be easily identified, not only in the Y12 but also in the 4F4 immuno-

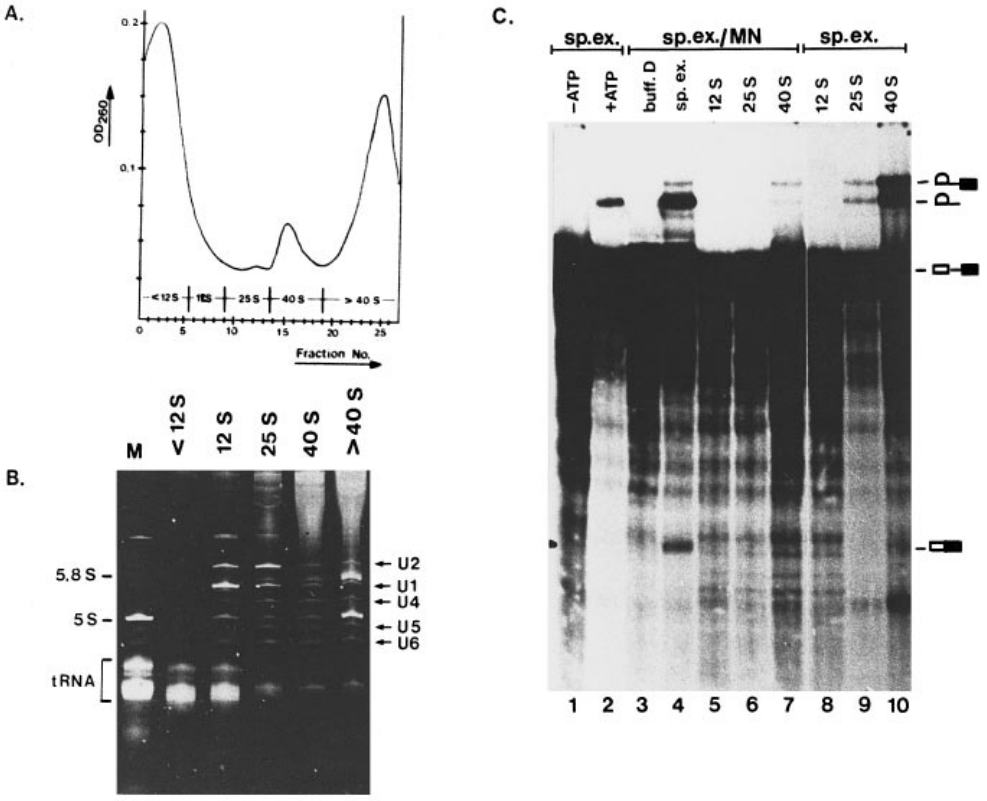

Figure 8 Test of the ability of a sucrose-gradient-fractionated HeLa splicing extract to supplement an MN-treated splicing extract

(**A**) Absorbance profile (A_{260}) of a HeLa nuclear extract after sedimentation on a 15–30% sucrose gradient. (**B**) RNA composition of the pooled sucrose-gradient fractions corresponding to material of < 12S, 12S, 25S, 40S and > 40S, as indicated in (**A**). The extracted RNAs were resolved on a 10% polyacrylamide/7 M urea RNA gel and visualized with EtBr. (**C**) The material of 12S, 25S and 40S was used to supplement an RNA splicing reaction with, or without, prior MN treatment of the splicing extract (sp. ex./MN lanes 3–7 and sp. ex. lanes 8–10 respectively). Control assays included reactions −ATP and +ATP, as well as splicing assays where the MN-treated splicing extract was supplemented with buffer D [13] alone (lane 3) or with untreated splicing extract (lane 4).

precipitate. The above findings, when combined with the data obtained from the affinity purification studies, strongly suggest the preservation of hnRNP–snRNP interactions *in vivo*.

Ability of the multi-snRNP complex contained within HeLa 40 S nuclear fractions to function in RNA splicing *in vitro*

All structural characteristics of the 40 S multi-snRNP complex presented above strongly suggest that it functions in RNA splicing *in vivo*. In an effort to identify its specific role in RNA splicing, we performed reconstitution experiments to test the ability of the spliceosomal snRNPs contained in the multi-snRNP complex to supplement an MN-treated HeLa nuclear extract. In these *in vitro* reconstitution assays, HeLa 40 S sucrose-gradient fractions were used as the source of the MI-type multi-snRNP complex, without prior anti-m_3G immunoaffinity purification. This was so because, as discussed above (see Figure 6), the multi-snRNP complex was in close association with hnRNP components in the HeLa 40 S sucrose-gradient fractions and could not be separated by immunoaffinity purification. At the same time, we wished to eliminate RNA degradation, as much as possible, by avoiding any further unnecessary treatment of the material under investigation. Material corresponding to 40 S, as well as to lighter (< 12 S, 12 S and 25 S) and heavier (> 40 S) fractions, was obtained following sucrose-gradient fractionation of HeLa nuclear extracts prepared by the method of Choi and Dreyfuss [12], as shown in Figure 8(A). The pattern of the RNA species contained in the different regions of the sucrose gradient is presented in Figure 8(B). The bulk of the spliceosomal snRNAs (U1, U2, U4, U5 and U6) was recovered in the 12 S and 25 S fractions, but they could also be seen within the 40 S and > 40 S material, together with a large excess of hnRNA molecules running at the top of the RNA gel. The material of > 40 S was found to be enriched in addition in 5 S and 5.8 S ribosomal RNA, while the light < 12 S fractions were devoid of snRNAs.

Sucrose-gradient fractions corresponding to 12 S, 25 S and 40 S material were subsequently used in an *in vitro* RNA splicing assay. Since none of these fractions was found to be active by itself (results not shown), they were tested for their effect on RNA splicing when added to a HeLa splicing extract. As deduced from the analysis of the RNA products of the splicing reaction (Figure 8C), combining an active splicing extract with 40 S material (lane 10) did not affect splicing. This was not the case with the 25 S and 12 S material (lanes 9 and 8 respectively), the latter being totally inhibitory. The simplest interpretation of this result is the presence of an inhibitory factor(s), mainly enriched in the 12 S sucrose-gradient material. In order to identify the nature of this putative inhibitor, we subjected the 12 S fractions to several pre-treatments, such as boiling, MN or *N*-ethyl-

maleimide treatment. As the only treatment that alleviated inhibition of splicing activity was boiling (results not shown), we concluded that the inhibitory factor(s) was not a nucleic acid but, most likely, proteinaceous in nature and not involving S–S interactions.

The ability of the 40 S material, as well as that of 12 S and 25 S, to supplement a HeLa splicing extract that had been inactivated by MN treatment, due to the destruction of the endogenous snRNA molecules, was then investigated. As seen in Figure 8(C), the 40 S fractions, in contrast to the 12 S and 25 S material, were, indeed, able to reconstitute splicing activity (compare lane 7 with lane 3, where buffer D alone was used to supplement the MN-treated extract). Under the experimental conditions used, this was a partial reconstitution effect when compared with that of supplementing with active splicing extract (lane 4). Nonetheless, this finding proved the ability of the spliceosomal snRNAs included within the HeLa 40 S nuclear fractions to function in RNA splicing *in vitro*.

DISCUSSION

In this paper, an extensive analysis concerning the structural features of the rat MI multi-snRNP complex of approx. 40 S has been presented. The complex had been shown previously to contain all major spliceosomal snRNP particles (U1, U2, U4/U6 and U5) in stable association with a large assortment of polypeptides, mainly in the range 50–210 kDa [7]. In this group of proteins, the largest 200/210 kDa protein species had, at that stage, been identified as the U5-associated $\sim$ 200 kDa spliceosomal protein ([28] and references therein). In the present study, we have been able to identify additional splicing factors, as well as detect the presence of the active *in vitro* RNA splicing forms of snRNP assemblies (17 S U2 and 25 S U4/U6.U5 tri-snRNP), as major constituents of the MI multi-snRNP. In addition, we have provided evidence supporting the presence of an MI-type multi-snRNP complex in HeLa nuclear extracts and have presented functional studies showing its participation in RNA splicing *in vitro*.

As pointed out earlier [7], the use of moderate salt concentrations (100–140 mM NaCl) during rat liver nuclear extract preparation played a decisive role in the detection of the MI multi-snRNP complex by preventing the dissociation of its components that occurred at higher ($>$ 300 mM NaCl) salt concentrations. As shown here, this was also the case for the HeLa nuclear extracts prepared according to the method of Choi and Dreyfuss [12]. Moreover, the detection of an MI-type multi-snRNP complex in HeLa splicing extracts as well, indicated the ability of the complex to re-assemble, at least partially, during the dialysis step that followed high-salt (0.4 M NaCl) extraction of nuclei [13]. It should be mentioned that the salt concentration during HeLa nuclear extract preparation had also been a critical factor in the identification of the 25 S U4/U6.U5 tri-snRNP complex [5], as well as the 17 S U2 snRNP [6] and the 20 S U5 snRNP [4], each one containing a specific set of snRNP-associated polypeptides. All these findings strongly suggest the involvement of electrostatic interactions in the stabilization of the multi-snRNP assembly.

In the present study we employed a new polyclonal antibody population specific for a polypeptide with apparent molecular mass of 63 kDa (P_{63} protein), in parallel with the mAb 4G3 which recognizes the U2 snRNP-specific B″ polypeptide [9]. By virtue of its stable association with the 4G3-immunopurified multi-snRNP complex, P_{63} represents an authentic protein component of the complex. Moreover, P_{63} was found to correspond to the 66 kDa subunit of the recently identified SF3a HeLa splicing factor, a component of the 17 S U2 snRNP [17,18]. Thus, both the anti-P_{63} and mAb 4G3 antibodies applied in this study recognized a distinct, single-protein component residing in the U2 snRNP complex. The ability of the two different antibody populations to immunoselect from both the rat and the HeLa nuclear extracts a structurally similar multi-snRNP complex, containing all known major spliceosomal snRNPs in addition to U2, provides further strong support for the existence *in vivo* of such a macromolecular assembly. Based on the above findings, the observed over-representation of polypeptides belonging to the 17 S U2 snRNP (polypeptide species of 35, 53, 60, 66, 92, 110, 120, 150 and 160 kDa) [6] within the protein constituents of the rat MI multi-snRNP complex was therefore an expected finding. The complex contained additional protein species, some of which (such as the 200/210 kDa protein doublet) corresponded to polypeptides of the U4/U6.U5 tri-snRNP complex [5]. The remaining proteins are expected to represent the rat counterparts of the large pool of HeLa spliceosomal components that have been identified *in vitro* (reviewed in [3]). As shown in the present study, the anti-m_3G immunoaffinity method provided an easy and reproducible way to obtain, from rat liver 40 S hnRNP-containing nuclear fractions, large amounts of the multi-snRNP components in their native form. Thus, the eluate fraction of the anti-m_3G column can be considered as an experimental reservoir of splicing components that could be applied in *in vitro* studies.

The rat MI multi-snRNP complex, with an estimated size of approx. 40 S [7], represents a rather large, endogenous U2 snRNP-containing assembly. U2 snRNP has also been detected as a component of the 200 S large nuclear RNP complex [29]. The latter has been reported to represent a large, homogeneous RNP population, detected within mammalian cell extracts, that contains intact pre-mRNA species in association with the spliceosomal U1, U2, U4/U6 and U5 snRNPs. A number of splicing factors, such as the SR proteins [30] and an 88 kDa polypeptide [31], have also been identified as integral components of the 200 S lnRNP. Dissociation of the 200 S complex into smaller 70 S RNP units in the absence of magnesium cations has also been reported [32]. The apparent structural resemblance (in terms of protein and RNA composition) of the 40 S multi-snRNP complex and the 200 S large nuclear RNP gives rise to the possibility that the latter represents a multimeric structure of the MI multi-snRNP unit.

In contrast to the rat, the identification of the MI-type multi-snRNP complex in HeLa nuclear extracts, especially with respect to individual polypeptide components, was not so easy. Moreover, anti-m_3G affinity chromatography provided evidence for a tight association between components of the snRNP and the hnRNP complexes within the HeLa 40 S sucrose-gradient fractions. This association was observed in 40 S material fractionated from HeLa nuclear extracts prepared under different experimental protocols, either that of Choi and Dreyfuss [12] or of Dignam et al. [13] (results presented in Figure 6). As in the case of the rat, the bulk of HeLa 40 S hnRNP polypeptides was recovered in the flow-through fraction of the anti-m_3G affinity chromatography (results not shown). However, we could not exclude the presence of a minimal amount of hnRNP polypeptides associated with the hnRNA species in the m^7G eluate fractions. The specific binding of several hnRNP proteins, such as A1, C and D, to the 3′-end of intron sequences has been demonstrated [33]. It is also interesting to note very recent findings that implicate hnRNP–snRNP interactions operating during spliceosome assembly [34]. As stated in the Results section, the snRNP–hnRNP associations deduced from our experimental system could withstand heparin treatment of the 40 S material prior to immunoaffinity purification, suggesting the

presence of specific interactions of the protein–RNA type. We cannot exclude, of course, the presence of RNA–RNA interactions also operating in such pre-existing snRNP–hnRNP assemblies, considering the well-established RNA–RNA interactions involved in the binding of snRNA molecules to intron/exon boundaries (reviewed in [35]). It remains to be seen whether the hnRNA fraction detected in the m^7G eluate represents a specific subset of hnRNA species, possibly enriched in intron sequences.

As stated above, the snRNP–hnRNP association believed to be preserved within the HeLa 40 S fractions was not observed in the corresponding rat 40 S material. The most likely cause of this finding is the different extent of RNA degradation observed between rat and HeLa nuclear extracts. In the case of the rat, early studies had indicated the existence of polymeric RNP structures when RNase inhibitors were present during nuclear extract preparation [36]. Thus, increased nuclease action could lead to an efficient release of the multi-snRNP assembly from pre-existing larger hnRNP–snRNP structures and their subsequent accumulation in the 40 S sucrose-gradient fractions. The hypothesis that in HeLa nuclear extracts such hnRNP–snRNP interactions remain, to a large extent, intact, could explain the observed low yield of the multi-snRNP complex in HeLa 40 S material.

Finally, we have presented here studies which support the notion that the multi-snRNP assembly of the spliceosomal snRNPs represents a structural entity able to function in RNA splicing *in vitro*, and that it is not simply a by-product formed during experimental manipulations. Reconstitution experiments using HeLa 40 S hnRNP-containing fractions to supplement an MN-treated splicing extract revealed their ability to restore splicing activity. Since the supplementary snRNPs were part of the multi-snRNP entity included in the 40 S fractions, these data provide direct evidence for its ability to function in pre-mRNA splicing. Thus, the finding that this endogenous multi-snRNP complex shares not only structural but also functional features with the spliceosome identified *in vitro*, strongly indicates that it corresponds to its *in vivo* counterpart.

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