Identification of two discrete ribonucleoprotein particles within the monomer population of rat liver nuclear RNPs

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30–50 S RNP particles (monoparticles) isolated from rat liver nuclei were submitted to electrophoresis in native 0.5% agarose gels. Two RNP fractions were thus separated, a minor one remaining closer to the top of the gel (MI) and a more abundant one migrating further into the gel (MII). SDS-polyacrylamide gel electrophoresis revealed that MII contains the major monoparticle (M_r 30000–40000 or 'core') polypeptides and higher molecular weight proteins, whereas MI contains several minor proteins of M_r >40000. Some proteins are common to both particle classes. Urea-acrylamide gel electrophoresis revealed that HnRNA is mainly present in MII, whereas snRNA is confined to the MI particle class.

Nuclear RNP *HnRNP* *snRNP* *snRNA* *Agarose gel electrophoresis* *Rat liver*

1. INTRODUCTION

The existence of 30–50 S RNP-structures (monoparticles) obtained during HnRNP isolation, as a result of limited nuclease digestion of in vivo pre-existing large ribonucleoprotein complexes, has been well documented [1]. These structures contain HnRNA, snRNA and a heterogeneous set of non-histone proteins. The structure–function relationship of their RNA and protein constituents is not well understood. Any information concerning the organization of RNA and protein within the RNP structure would be important for defining the functional role of nuclear RNPs, such as their postulated participation in RNA processing [2–7].

A size heterogeneity of monoparticles has already been demonstrated [8]. However, reports suggesting the existence of biochemically distinct monoparticle populations, on the basis of salt and nuclease sensitivity of the RNP, have been disputed as representing rearrangements of monoparticles following RNase treatment and resedimentation on sucrose gradients [9,10]. Nevertheless, reports exist that strongly suggest a heterogeneity of 30–50 S RNP structures [11–15].

We have further investigated the question of monoparticle heterogeneity employing electrophoretic techniques that have been used in the analysis of heterogeneity of other protein–RNA complexes; i.e., polynucleosomes [16,17] and polyribosomes [18]. By analyzing monoparticles on 0.5% agarose gels under conditions of minimal nuclease action, we have separated two monomer populations (M I and M II) that have distinct and characteristic protein and RNA compositions. These complexes do not appear to represent rearrangements of RNP components. Therefore, we believe that their biological significance should be considered in respect to the intranuclear fate of HnRNA.

Abbreviations: snRNA, small nuclear RNA; RNP, ribonucleoprotein; DEP, diethylpyrocarbonate; SDS, sodium dodecyl sulphate

2. MATERIALS AND METHODS

30–50 S monoparticles from rat liver were prepared as in [19]. To label HnRNA, [^{3}H]orotic

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acid (Amersham; spec. act. 21 Ci/mmol) was injected intraperitoneally to rats 2 h prior to the removal of the livers. Ribosomal subunits were isolated as in [20] from polysomal pellets obtained from the post-mitochondrial fraction of cytoplasmic extracts prepared as in [21]. Fractions of the sucrose gradient containing the 40 and 60 S ribosomal subunits were pooled separately and concentrated by high-speed centrifugation (48000 rev./min, 20 h in the 50 Ti rotor at 4°C). Similarly, sucrose gradient fractions containing the 30–50 S monoparticles, as well as the 10–20 S and the >60 S structures, were pooled and pelleted (38000 rev./min, 17 h in the 50 Ti rotor at 4°C). For agarose gel electrophoresis, the pellets were well-drained, resuspended in a small volume (about 100 μl/5 A_{260} units of monoparticles) of 10 mM Tris–HCl (pH 7.6) containing 30% glycerol and clarified by low-speed centrifugation. Bromophenol blue was then added as indicator and 25 μl were applied per slot.

In our initial studies, several electrophoretic systems were tested, including low acrylamide–agarose composite gels, either slab or disc, as well as running buffers of different ionic strength. The horizontal slab gel electrophoretic system of 0.5% agarose (a modification of the method described in [16] for the electrophoretic analysis of polynucleosomes) was finally adopted as giving the best reproducible results. 100 ml of 0.5% agarose (agarose T, Behringwerke AG, Marburg/Lahn, low EEO) in a low ionic strength buffer of 6.4 mM Tris–HCl (pH 8.3), 0.2 mM sodium acetate and 0.32 mM EDTA were layered onto a 20 × 20 cm glass plate. Electrophoresis took place in the same buffer at 4°C and 40 V for 18 h. Care was taken that samples and buffers were kept cold at all times. After electrophoresis, the gel was stained either for protein in 0.1% Coomassie blue or for RNA in 0.2% methylene blue. For protein analysis of the monoparticles separated on agarose gels the briefly stained gel was washed thoroughly in H_2O, and a strip containing the material was cut and placed in the SDS-sample buffer as in [22] for 20 min. It was then either frozen at −20°C or immediately transferred onto a 10% SDS–acrylamide slab gel with a 4.5% stacking gel. Electrophoresis was at 120 V for 3–4 h. Silver staining of the gel was performed as in [23].

RNA was extracted from the agarose gel by cutting the corresponding areas of monoparticle populations and mechanically mincing the gel by passing it through a medium-sized syringe in a buffer consisting of 10 mM Tris–HCl (pH 7.5), 2 mM EDTA and 0.1% DEP (TE-buffer). Proteinase K and SDS were then added to final concentrations of 200 μg/ml and 1%, respectively. The suspension was then placed in a waterbath at 37°C and mechanically stirred for 1 h. The agarose was removed by centrifugation (5000 rev./min, 10 min in the HB-4 rotor) and the supernatant was phenol-extracted at room temperature (1.5 vol. phenol, saturated with TE-buffer, without DEP). The aqueous phase was extracted once more in 1 vol. chloroform–isoamylalcohol (99:1, v/v) and the RNA precipitated in 0.4 M LiCl and 2.5 vol. ethanol at −20°C overnight. The RNA pellet was washed once in 80% ethanol, dried well and analysed on the 5% urea–acrylamide system as in [24].

RNA extraction from the pelleted monoparticles was done by immediately resuspending the pellet in TE buffer and following the same procedure described above.

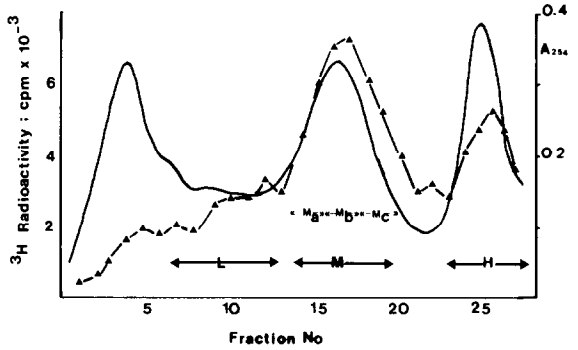

Fig.1. Sucrose gradient fractionation of monoparticles from rat liver nuclei. Nuclear extract was prepared from rats that received [^{3}H]orotic acid 2 h before sacrifice. Sucrose gradients, 15–30%, 17 h centrifugation at 25000 rpm in the SW27 Beckman rotor. 5% Trichloroacetic acid insoluble radioactivity was determined from aliquots of the gradient fractions (▲—▲). The solid line indicates the absorbance at 254 nm. L, M and H refer to the light structures (10–20 S), monoparticles (30–50 S) and heavier structures (> 60 S), respectively. M_a, M_b and M_c indicate the three subregions of the monoparticle peak (see also fig.2B).

3. RESULTS

3.1. *Separation of the 30–50 S monoparticle into two RNP populations by native agarose gel electrophoresis*

Treatment of purified rat liver nuclei with a pH 8 buffer and mild sonication, yields an extract which subjected to centrifugation on 15–30% sucrose gradients gives the previously well characterized pattern with predominance of the 30–50 S monoparticle population (see fig.1). When relatively short pulses of [^{3}H]orotic acid were used to label HnRNA molecules, the majority of radioactivity in the gradient was recovered in the monomer peak. Some radioactivity was also present in the lighter (10–20 S) as well as in the heavier (>60 S) fractions of the gradient. The monoparticles and a large proportion of the lighter and heavier structures gave the characteristic density of 1.4 g/ml upon banding of formaldehyde fixed material on CsCl gradients (not shown). The 10–20 S structures contain snRNPs, whereas the

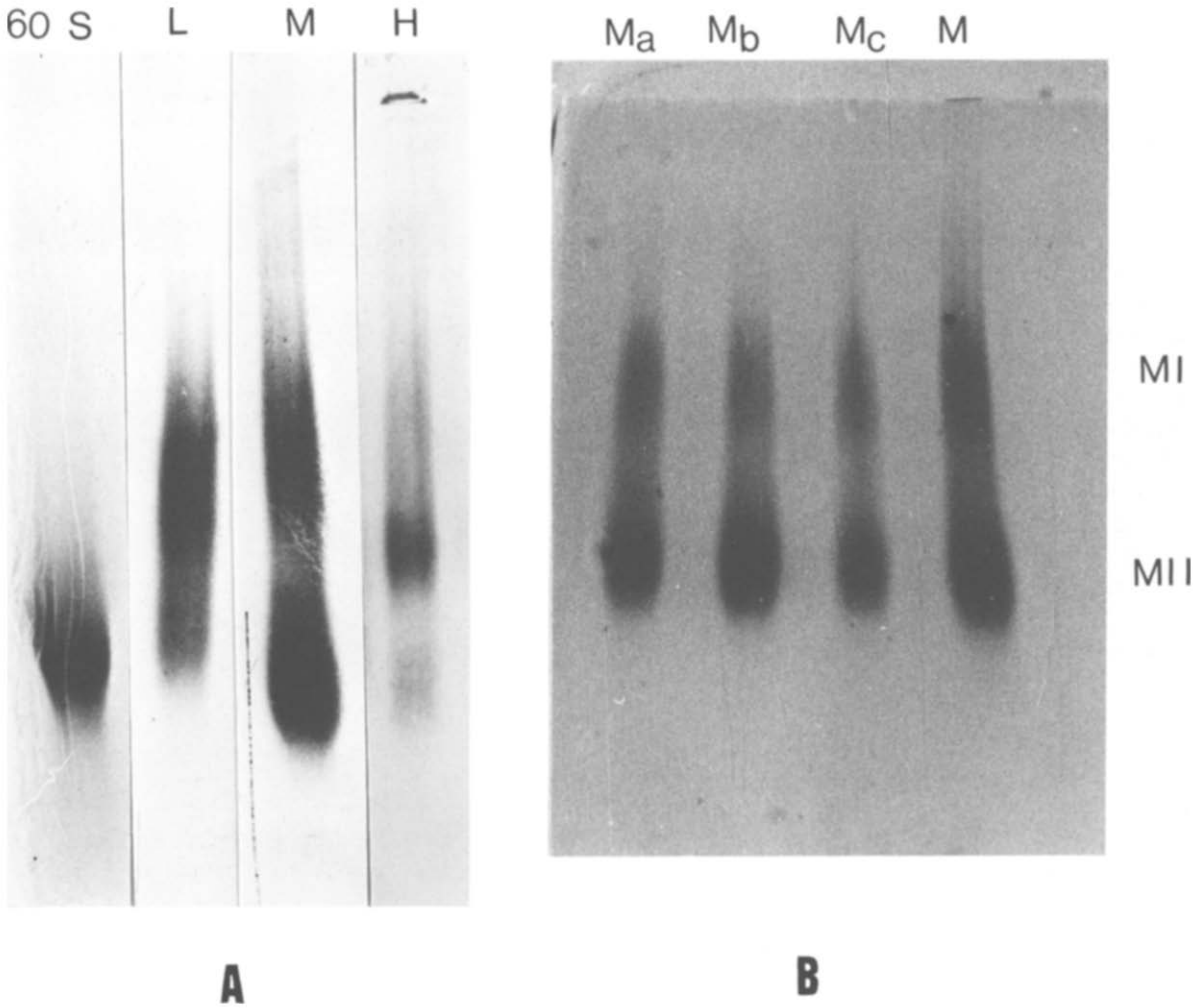

Fig.2. Agarose gel, 0.5%, electrophoresis of nuclear RNP obtained from sucrose gradients: (A) Pelleted material corresponding to L, M and H regions of fig.1 were submitted to electrophoresis in 0.5% agarose gels, as in section 2. The electrophoretic separation of 60 S ribosomal subunits is also included for comparison; (B) Electrophoretic separation of the three subregions, M_a, M_b and M_c, of monoparticles, as shown in fig.1. Also shown is the electrophoresis of the total monoparticle population (M). The gels were stained with methylene blue.

>60 S region of the gradient is partly composed of larger HnRNP complexes [1], as demonstrated by comparing the protein composition of the fractions to those published in the literature (not shown).

The monoparticles were concentrated by high-speed centrifugation and subsequently run on a 0.5% agarose gel, under native conditions of electrophoresis, as in section 2. Two focusing areas were thus reproducibly obtained (fig.2A). Some streaking of material between the two areas was present. However, the formation of two focusing centers was clearly seen. Under the same conditions of electrophoresis, ribosomal subunits each focused as a single spot, as expected for such homogeneous material. Therefore, as indicated by the use of ribosomal subunits to standardize the method, electrophoresis by itself does not cause artefactual disaggregation or rearrangement of the monomer population. Consequently, the electrophoretically observed heterogeneity of monoparticles should be an intrinsic property of these RNP structures. Some rearrangements of the monoparticles due to endogenous nucleases acting during electrophoresis can not be totally excluded, but are very unlikely, as suggested by experiments in which monoparticles were incubated at 37°C for 1 h prior to pelleting and agarose electrophoresis. Under these conditions favoring endogenous nuclease action the same pattern of electrophoresis was obtained as with monoparticles not subjected to the incubation.

The material focusing closer to the top of the gel

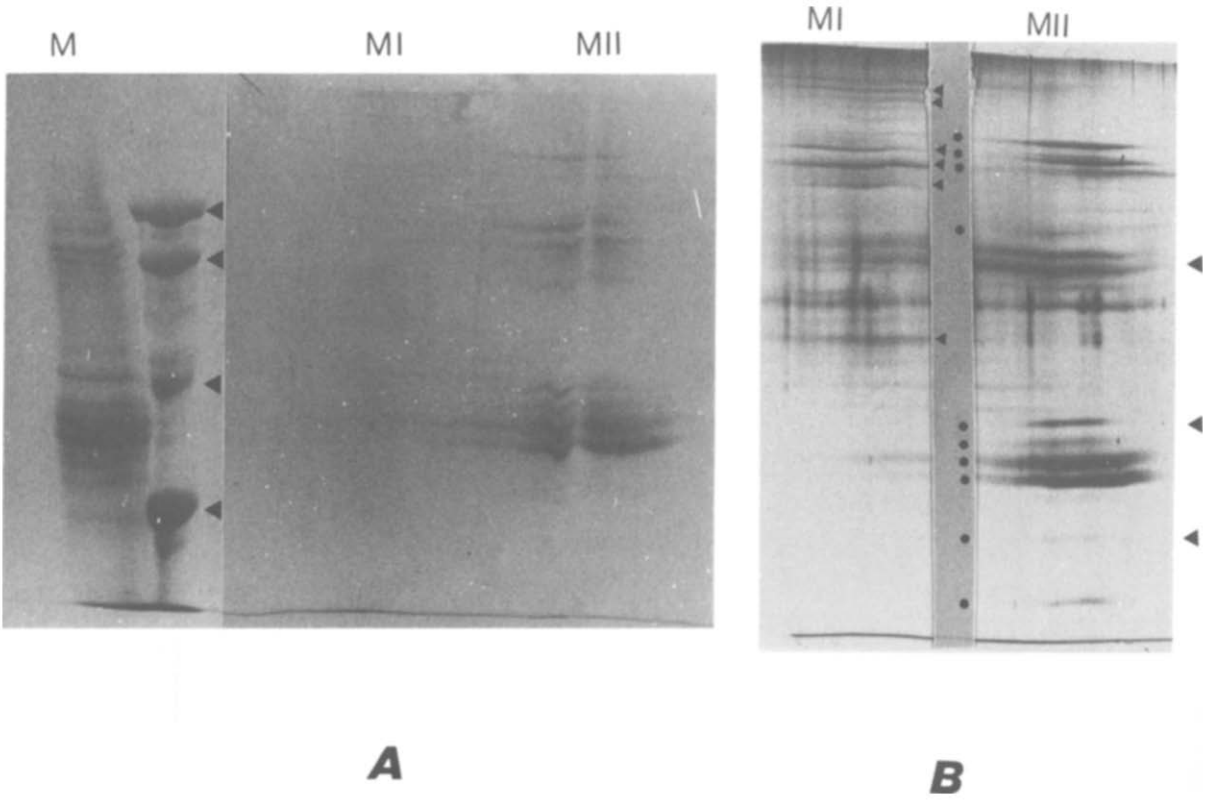

Fig.3. Protein composition of M I and M II. A strip containing the monoparticle population after electrophoresis in 0.5% agarose gel (fig.2) was horizontally applied and subsequently submitted to electrophoresis on a 10% SDS–polyacrylamide slab gel: (A). Coomassie blue staining of the slab gel. M refers to the protein pattern of the monoparticles. The proteins used as M_r standards are: phosphorylase *b* from rabbit muscle (M_r 94400), bovine serum albumin (M_r 66000), egg albumin (M_r 45000) and carbonic anhydrase from bovine erythrocytes (M_r 29000); (B) Silver staining of the slab gel. (●) Protein bands present exclusively in M II, while arrows point to proteins found in M I; (►) position of migration of the proteins used as M_r standards (bovine serum albumin, egg albumin and carbonic anhydrase).

is referred to as M I, while that entering further into the gel as M II. The first indication that both M I and M II represent ribonucleoprotein complexes and not solely free protein or RNA, came from their ability to stain both for protein and RNA. Specific protein and RNA components were later detected (see below). The size heterogeneity of monoparticles in sucrose gradients [8] does not appear sufficiently to explain the electrophoretic segregation into two RNP populations. Both M I and M II were obtained when pelleted material from the shoulders and from the top of the monomer peak were separately submitted to electrophoresis and compared to material from the total monoparticle population (fig.2B).

We have also followed the electrophoretic behavior of the lighter (10–20 S) and heavier (>60 S) fractions. As seen in fig.2A, the lighter structures gave a diffuse picture on the gel with no obvious focusing center, whereas the heavier material focused between the M I and M II areas. Considerable material from the heavy RNPs did not enter the 0.5% agarose gel.

3.2. *Characteristics of the M I and M II RNP complexes*

To investigate whether M I and M II represent discrete RNP structures we analysed their protein and RNA compositions. Following agarose gel electrophoresis, a strip of the gel containing the segregated M I and M II populations was applied horizontally onto a SDS–acrylamide slab gel and the proteins submitted to electrophoresis. In fig.3 the results obtained after Coomassie blue staining of the gel (3A) and after the more sensitive silver staining (3B) are presented. It is evident that M I and M II have discrete protein compositions. M II contains the major monomer proteins, i.e., those with M_r 30000–40000, referrred to as 'core' proteins, as well as a group of proteins of higher M_r (60000–70000). In contrast, M I contains 6–8 minor proteins of $M_r > 40000$. A few other proteins appear to be shared by both particle classes. However, it should be stressed that the polypeptides of the M I complex represent a minor proportion of the total monoparticle proteins, as they are barely stained by Coomassie blue alone (fig.3A). The silver staining techniques, although extremely sensitive as analytical tools, do not give quantitative answers. It is worth mentioning that the most abundant 'core' proteins stain yellow with the silver nitrate method, which is indicative of glycoproteins [25]. This has been suggested elsewhere [1].

Similar studies were then undertaken in respect to the RNA composition of M I and M II. The respective areas were cut from the agarose gels and

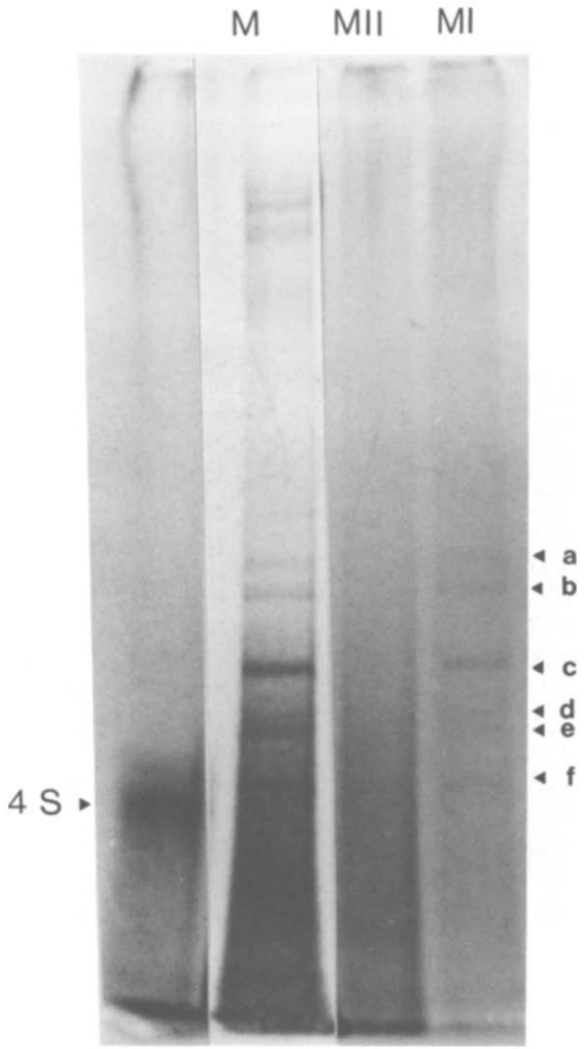

Fig.4. RNA composition of M I and M II. RNA was extracted from the agarose areas corresponding to M I and M II, as well as from pelleted monoparticles (M) and analysed on a 5% urea–acrylamide gel. The 6 most prominent snRNA species found in monoparticles and in M I are indicated by arrows and named a–f: (a) U_2; (b) U_{1B}; (c) U_{1A}; (d) 5 S RNA and (f) 4.5 S RNA [11,15]. Yeast t-RNA was used as a marker RNA.

RNA extracted as described in section 2. The obtained RNA was then analysed in denaturing 5% urea–acrylamide gels and compared to RNA obtained from the total monoparticle population (see fig.4). As expected [26], RNA from total monoparticles contained a number of snRNA species and, in addition, quite degraded HnRNA, despite the presence of DEP during RNA isolation. Higher M_r HnRNA was also present, together with 3–4 distinct RNA bands, larger in size than snRNA, that might correspond to the larger snRNA species described in [27] and named sPLRNA. RNA eluted from M II did not contain snRNA species, only degraded HnRNA. In contrast, all snRNA species found in total monoparticles could be recovered from the M I population. Some degraded HnRNA was also found in M I.

These results clearly indicate a close association of the minor protein components, detected in M I, to snRNA. In contrast, the major 'core' proteins do not show an association to snRNA, only to HnRNA.

4. DISCUSSION

We have shown that upon agarose gel electrophoresis rat liver 30–50 S monoparticles segregate into two subpopulations, referred to as M I and M II. Furthermore, that M I contains the total population of snRNA found in the monoparticles, some degraded HnRNA and a set of discrete polypeptides of $M_r > 40000$. In contrast, M II contains HnRNA, but no snRNA, and all of the 'core' proteins. One major question which confronted us was whether these RNP structures exist in vivo as well. The electrophoretic system we have used does not appear to cause dissociation of protein–nucleic acid complexes. Partial rearrangements, caused by endogenous nucleases acting during the fractionation of the monoparticles, seem very unlikely. M I contains minor protein species which show strong association to snRNA; M II on the other hand is composed of major proteins in close association to HnRNA. The M I complex does not appear to be directly related to the small snRNP species, that have been recently characterized [28,29]. These snRNP complexes contain, in addition to snRNA, a set of 4–5 proteins with M_r 10000–14000, whereas M I contains proteins of higher M_r (> 40000). In vivo, both M I and M II take part in the formation of the giant HnRNP structures and are recovered during extraction as 30–50 S monoparticles due to nuclease cleavage. We envisage the association of M I to M II by way of hydrogen-bonding between complementary snRNA and HnRNA sequences and an association of higher order complexes with nuclear matrix components, as in [14,26]. Work is now in progress to ascribe enzymic functions to the proteins of M I and M II, as a first step towards understanding their role in the post-transcriptional fate of HnRNA.

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