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Isolation and visualization of large compact ribonucleoprotein particles of specific nuclear RNAs

(RNA processing/β-actin ribonucleoprotein/dihydrofolate reductase ribonucleoprotein/small nuclear RNP/electron microscopy)

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ABSTRACT **We have previously shown that nuclear transcripts of the multifunctional enzyme, carbamoyl-phosphate synthetase, aspartate transcarbamylase, dihydroorotase RNA can be released from nuclei of Syrian hamster cells as compact ribonucleoprotein (RNP) particles that sediment at the 200S region in a sucrose gradient. The 200S nuclear RNP particles contain U1, U2, and U6 small nuclear RNPs, which are known to be required for splicing of pre-mRNA, as integral components of the particles. In this study we demonstrate that nuclear transcripts of dihydrofolate reductase in Syrian hamster cells and of β-actin in both Syrian hamster and human cells are also released from the respective nuclei as 200S particles—despite the difference in length of these RNAs. Electron microscopy of the 200S particles revealed discrete compact composite structures with a cross section of ≈50 nm. Finding that two more nuclear RNAs from two different cell types and two different species are released as 200S RNP particles suggests a general mode for packaging of heterogeneous nuclear RNA in large compact RNP particles the size of which is independent of the RNA length.**

In eukaryotic cells, the synthesis of heterogenous nuclear RNA (hnRNA) is followed by rapid assembly of nascent RNA transcripts into heterogenous nuclear ribonucleoprotein (hnRNP) particles (1, 2). Studies, both *in vivo* and *in vitro*, indicate that the processing of hnRNA occurs on such complexes (for reviews, see refs. 3 and 4). Therefore, the isolation of hnRNP particles in a native form, amenable to biochemical characterization, should help reveal the components involved in these processes. In our studies of nuclear RNP particles, we pursue abundant specific RNA transcripts with a view to the possible isolation of homogeneous material.

In an earlier paper (5) we demonstrated the release of intact nuclear transcripts of the gene for a multifunctional enzyme designated CAD (for carbamoyl-phosphate synthetase, aspartate transcarbamylase, dihydroorotase) in an RNP form that sediments as a peak at the 200S region in a sucrose gradient. We have further shown that U1, U2, and U6 small nuclear RNPs (snRNPs), which are known to be required for splicing of pre-mRNA (refs. 4 and 6 and the references therein), are integral components of the 200S particles (7). These observations, in conjunction with the proposed role for snRNPs in hnRNA processing (4, 6), raised the possibility that the 200S particles represent the hnRNP complexes on which the processing of hnRNA presumably occurs *in vivo*. However, association with CAD RNA alone could not account for the high abundance of snRNPs in the 200S RNP particles. Therefore the packaging of other species of the hnRNA population in 200S particles has been hypothesized.

In this study we demonstrate that nuclear transcripts of dihydrofolate reductase (DHFR) in Syrian hamster cells and of β-actin in both Syrian hamster and human cells are also released from the respective nuclei as 200S particles—despite the difference in length of these RNAs. Electron micrographs of particles sedimenting in the 200S peak region reveal discrete compact composite structures with a cross section of ≈50 nm, similar to the Balbiani ring RNP particles seen *in situ* (8–10).

MATERIALS AND METHODS

Cell Growth and RNP Fractionation. 165-28 and BT-6 are both mutant Syrian hamster fibroblast cell lines, provided by G. R. Stark, Imperial Cancer Research Fund, London. 165-28 cells contain an amplified CAD gene (11) and BT-6 cells have both the CAD and DHFR genes amplified (G. R. Stark, personal communication). Monolayers of 165-28 or HeLa cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (Biological Industries, Beth Haemek, Israel). BT-6 cells were grown similarly except that methotrexate at 24 mM was included in the growth medium.

Pure nuclei were prepared from subconfluent monolayers as described previously (5, 7, 12), except that the lysed cells were layered on a 25% glycerol cushion and centrifuged at 750 × *g* for 5 min at 4°C. RNP was released from the purified nuclei and fractionated in a sucrose gradient as described (5). For refractionation of the 200S nuclear RNP particles, fractions corresponding to the 200S peak region of the 165-28 cell nuclear RNP gradient were combined, dialyzed, concentrated as described by Sperling *et al.* (7), and rerun on a second 15–45% sucrose gradient in an SW41 rotor (Beckman) for 19 hr at 11,400 rpm and 4°C. The gradient was collected in 20 fractions (0.55 ml each) starting from the bottom.

Analysis of RNAs. RNA was recovered from sucrose-gradient fractions as described (5, 7). For slot blot analysis, RNA was resuspended in 6× SSC (1× SSC = 0.15 M NaCl/0.015 M sodium citrate), aqueous formaldehyde was added to 7.5%, and the mixture was applied to nitrocellulose filters and hybridized with the respective DNA probe as described (7). For blot analyses of snRNA, the ethanol-precipitated RNA samples were denatured by heating for 2 min in 80% formamide, fractionated on 10% polyacrylamide gels [acrylamide/methylenebis(acrylamide), 27:1] containing 7 M urea, 45 mM Tris borate (pH 8.3), 1.25 mM EDTA, transferred electrophoretically to Zetabind membrane, and hybridized to nick-translated probes as described (7). Blot hybridization of specific RNAs was performed as described (5). Densitometry of autoradiograms was carried out as described (7).

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Abbreviations: RNP, ribonucleoprotein; hnRNA and hnRNP, heterogeneous nuclear RNA and RNP, respectively; snRNP, small nuclear RNP; CAD, carbamoyl-phosphate synthetase, aspartate transcarbamylase, dihydroorotase; DHFR, dihydrofolate reductase.

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Plasmids. The cloned DNA plasmids used for hybridization were the following: CAD, a 2.3-kilobase (kb) *Pst* I–*Pvu* I fragment of Syrian hamster cDNA isolated from pCAD41 (11) (provided by G. R. Stark); DHFR, pDHFR11—a mouse cDNA cloned into pBR322 (13) (given to us by R. T. Schimke, Stanford University); β-actin, pAc18.1—a pBR322 recombinant plasmid that contains a 4.4-kb genomic insert of the rat cytoplasmic β-actin DNA (14) (a gift from U. Nudel, Weizmann Institute). U1 snRNA—a 0.54-kb *Bam*HI fragment of human U1 DNA—was prepared from plasmid pHU1-ID (15) (contributed by J. E. Dahlberg, University of Wisconsin).

Isolation of UV-Crosslinked Polyadenylylated [Poly(A)$^+$] Nuclear RNP Complexes. Subconfluent 165-28 cells, in 85-mm tissue culture dishes, were incubated with [^{35}S]methionine (1220 Ci/mmol; 1 Ci = 37 GBq) at 50 μCi/ml for 2 hr in 2 ml of Dulbecco's modified Eagle's medium without unlabeled methionine. The medium was replaced by 2 ml of phosphate-buffered saline (10 mM sodium phosphate, pH 7.0/140 mM NaCl), and the labeled cells were then irradiated *in situ* at 4°C with short-wave UV light at 1200–1400 μW/cm^2 for 2 min. Nuclear RNPs were released from the irradiated cells and fractionated in a 15–45% sucrose gradient as described. Fractions across the gradient were treated with Sarkosyl (1%); the solution was heated for 10 min at 65°C and then chilled on ice. NaCl was added to 0.5 M, and the poly(A)$^+$ fractions were then selected by chromatography on oligo(dT)-cellulose (16). The amount of ^{35}S-labeled proteins in the poly(A)$^+$ RNP fractions was determined by liquid scintillation counting.

Electron Microscopy. Aliquots from each fraction of the sucrose gradient were fixed in 1% glutaraldehyde for 5 min at 4°C. A drop of the fixed specimen solution was placed upon a carbon-coated grid and washed with several drops of 10 mM Tris·HCl/100 mM NaCl/2 mM $MgCl_2$ (pH 8.0) and then with a few drops of 1% solution of uranyl acetate. Excess liquid was withdrawn with the edge of a filter paper. A Phillips EM 300 electron microscope operating at 80 kV and 42,000-fold magnification was used. To construct Fig. 4*c*, duplicate negatively stained EM grids were prepared from each fraction of the sucrose gradient. The abundance of 200S particles on each grid was determined by averaging the number of particles found in 8–10 different fields on the grid—each field corresponding to an area of 4 μm^2.

RESULTS

DHFR and β-Actin RNAs Are Released from Nuclei of Syrian Hamster Cells as 200S RNP Particles. In previous studies we have demonstrated the association of U snRNPs with nuclear CAD RNP in 200S nuclear RNP particles (7). Because association with CAD RNP alone could not account for the relatively high abundance of U snRNPs in the 200S peak region, the packaging of other hnRNA species has been hypothesized. To test this prediction we compared the distribution in fractionated RNP particles of nuclear transcripts of genes that differ widely in size. Thus, transcripts of DHFR [pre-mRNA of 36 kb (17) and a predominant mature mRNA of 1.6 kb (13) in mouse] and β-actin [pre-mRNA of 5.5 kb and mature mRNA of 1.8 kb (14), in rat] were compared with transcripts of CAD RNA [pre-mRNA of 25 kb (18) and mature mRNA of 7.9 kb (11) in Syrian hamster]. First, nuclear RNPs were released from pure nuclei of mutant (BT-6) Syrian hamster cells, in which the genes for both CAD and DHFR were amplified (G. R. Stark, personal communication), by mild sonication as described (5, 7, 12). After pelleting the chromatin, the RNP-enriched supernatant was fractionated in a sucrose gradient. Hybridization of fractions across the gradient with the respective cDNA probes revealed peaks of DHFR RNA (Fig. 1*A*) and CAD RNA (Fig. 1*B*), both sedimenting at ≈200S in the gradient.

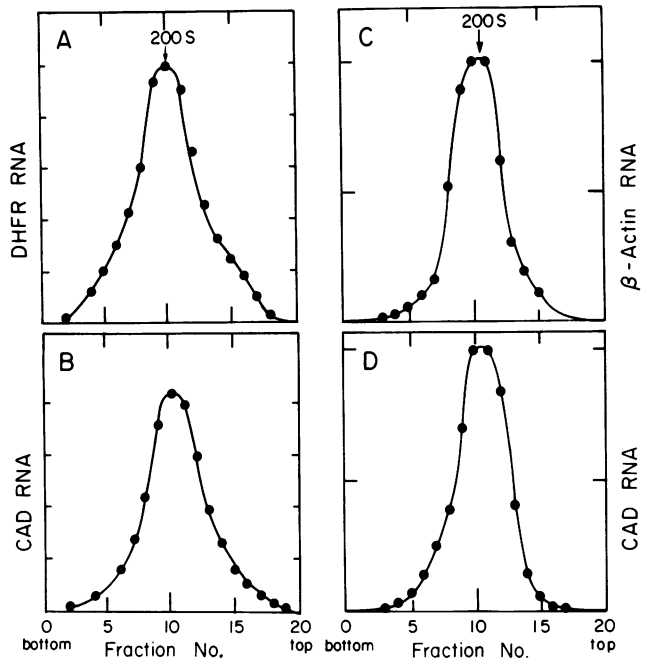

FIG. 1. Nuclear transcripts of DHFR, β-actin, and CAD RNAs are released from Syrian hamster cells as 200S RNP particles. Distribution of the RNA transcripts in sucrose gradient-fractionated RNPs was analyzed by RNA-blot hybridization of aliquots from each fraction across the gradient with the respective nick-translated probe and autoradiography. Relative amounts of each transcript (in arbitrary units) were estimated by densitometry of the autoradiograms. (*A*) Distribution of DHFR RNA in nuclear RNPs released from BT-6 cells. (*B*) Distribution of CAD RNA transcripts in nuclear RNPs released from BT-6 cells. (*C*) Distribution of β-actin RNA transcripts in refractionated 200S nuclear RNPs released from 165-28 cells. (*D*) Distribution of CAD RNA transcripts in refractionated 200S nuclear RNPs released from 165-28 cells. The gradients were calibrated with tobacco mosaic virus particles (200S) and bacterial ribosomes (70S) run in parallel gradients.

Second, the distribution of β-actin RNA, an abundant nuclear transcript in fibroblasts, was analyzed in fractionated nuclear RNPs released from mutant (165-28) Syrian hamster cells in which only the gene for CAD was amplified (11). We found that nuclear β-actin RNA was released in RNP particles sedimenting at about 200S, as was previously found for the nuclear transcripts of CAD RNA (5). The fractions corresponding to the 200S region of the gradient were combined and refractionated in another sucrose gradient. Hybridization revealed that both β-actin RNA (Fig. 1*C*) and CAD RNA (Fig. 1*D*) sediment as 200S RNP particles.

β-Actin and U snRNAs Are Released from Nuclei of Human (HeLa) Cells as 200S RNP Particles. To further demonstrate the validity of the 200S particle as a general mode of packaging for hnRNA, we analyzed a human epithelial-like cell line (HeLa) for the distribution of β-actin RNA and U1 snRNA in fractionated nuclear RNPs. Here again, nuclear β-actin RNA and a major fraction of U1 snRNA cosedimented as 200S particles (data not shown). Upon refractionation of the combined fractions corresponding to the 200S region of the first gradient, we found by RNA-blot analysis a peak for β-actin RNA (Fig. 2*A*) and a peak for U1 snRNA (Fig. 2*B*), both sedimenting at about 200S in the second gradient. Similar analyses (data not shown) indicated the presence of U2 and U6 snRNAs in the 200S peak region, as was previously found for 200S nuclear RNP particles of Syrian hamster cells.

To examine the possibility that RNA is nonspecifically adsorbed or aggregated onto the 200S particles, a set of experiments aiming to exchange exogenous RNA for the

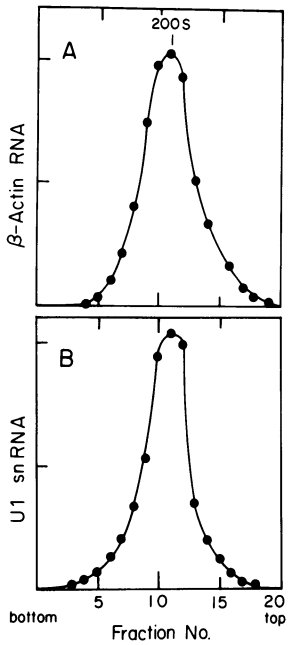

FIG. 2. β-Actin RNA and U1 snRNA are released from nuclei of human (HeLa) cells as 200S particles. Distribution of the transcripts was determined by repeated fractionation of nuclear RNPs in sucrose gradients followed by RNA-blot hybridization and autoradiography, as described in Fig. 1 for 165-28 cells. The relative amounts of the RNA in arbitrary units were estimated by densitometry of the autoradiograms. (*A*) Distribution of β-actin RNA transcripts in refractionated 200S nuclear RNPs released from HeLa cells. (*B*) Distribution of U1 snRNA as in *A*.

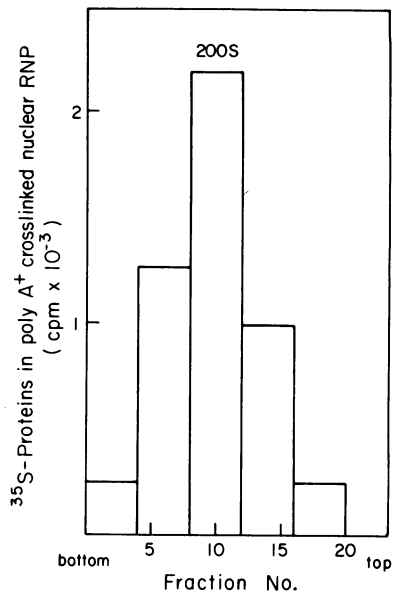

FIG. 3. Crosslinked poly(A)$^+$ nuclear RNP complexes sediment as 200S particles in a sucrose gradient. Subconfluent 165-28 cells were labeled with [^{35}S]methionine as described. The labeled cells were then irradiated *in situ* at 4°C with short-wave UV light. Nuclear RNPs were released from the irradiated cells and fractionated in 15–45% sucrose gradient as described. Fractions across the gradient were combined as indicated and denatured as described. Selection of the poly(A)$^+$ RNP was then carried out by chromatography on oligo(dT)-cellulose (16), and the amount of ^{35}S-labeled proteins in the bound fractions was determined by liquid scintillation counting.

constitutive RNA components of the particles were done. Nuclear supernatants enriched for RNPs were prepared from pure nuclei as described, incubated with various ^{32}P-labeled RNA species, including SP6 RNA polymerase transcripts of globin pre-mRNA, and then fractionated on sucrose gradients. In all these cases the input radioactivity migrated at the top of the gradients, and no radioactivity was associated with the RNA peak at 200S (data not shown). Exogenous RNA could be incorporated into the RNP only when the ionic environment was changed, indicating the necessity of unwinding of the compact RNP particles for an exchange of RNA to occur. This observation rules out the possibility of adsorption or aggregation of RNA onto compact 200S complexes.

UV-Crosslinked Nuclear Poly(A)$^+$ RNPs Are Released as 200S Particles. We employed UV-induced crosslinking of proteins to RNA to demonstrate the persistence of poly(A)$^+$ nuclear RNP in 200S particles. The experiment is based on earlier observations by Havron and Sperling (19, 20) demonstrating that upon UV irradiation of protein–nucleic acid complexes, covalent crosslinks occurred only between interacting neighboring residues in the native complex. This approach was subsequently used to determine histone–DNA contacts in the nucleosome (21) and protein–RNA interactions in hnRNP *in vivo* (22–25). In the experiment reported here, 165-28 cells were grown in the presence of [^{35}S]-methionine and irradiated by UV light *in situ*. Nuclear RNPs were then released from purified nuclei by mild sonication and fractionated in a sucrose gradient as described (5). The UV-induced crosslinking rendered the nuclear RNP complexes less sensitive to degradation during isolation and fractionation, resulting in higher yields of 200S particles (data not shown). This result accords with previous observations indicating the stabilization of hnRNP complexes by UV-induced crosslinking (22). Poly(A)$^+$-crosslinked RNP was then selected from fractions of the sucrose gradient by oligo(dT)-cellulose, and the amount of ^{35}S in the bound material was recorded (Fig. 3). We found that ≈90% of the ^{35}S-labeled proteins that had been crosslinked to poly(A)$^+$ RNA sedimented at the 200S region, indicating the persistence of general poly(A)$^+$ hnRNA in 200S complexes.

Visualization of 200S RNP Particles by EM. Electron micrographs of fixed and negatively stained RNPs sedimenting in the 200S peak reveal compact composite particles with a cross section of ≈50 nm (Fig. 4 *a* and *b*). To verify the assignment of these complexes as the 200S particles, we determined the distribution of the 50-nm particles across the sucrose gradient by counting particles in electron micrographs taken from each fraction. We found that the distribution of the 50-nm particles (Fig. 4*c*) corresponds to the distribution of the specific RNAs as determined by hybridization. Also demonstrated is the apparent similarity in size and general appearance between the 200S RNP particles released from nuclei of Syrian hamster cells (Fig. 4*a*) and those released from nuclei of HeLa cells (Fig. 4*b*).

It should be noted that contaminating material, smaller or larger than 50 nm, was observed only in a very minor fraction of the EM fields used to construct Fig. 4*c*. Most fields are faithfully represented in Fig. 4 *a* and *b*. The larger particles were only rarely seen and appeared as two 50-nm particles close to one another. The smaller particles were seen in a minor fraction of the fields and amounted to no more than ≈20–30% of the total particles. The appearance of the smaller particles in fractions across the gradient is randomly dispersed and does not follow any particular pattern. We can therefore attribute their existence to an accidental breakdown of 50-nm particles during the specimen preparation for the EM, rather than to their pre-existing as RNP degradation products in the nuclear RNP preparation before sucrose-gradient fractionation.

DISCUSSION

In previous studies, the isolated hnRNP of the nucleoplasm displayed a heterodisperse sedimentation profile between

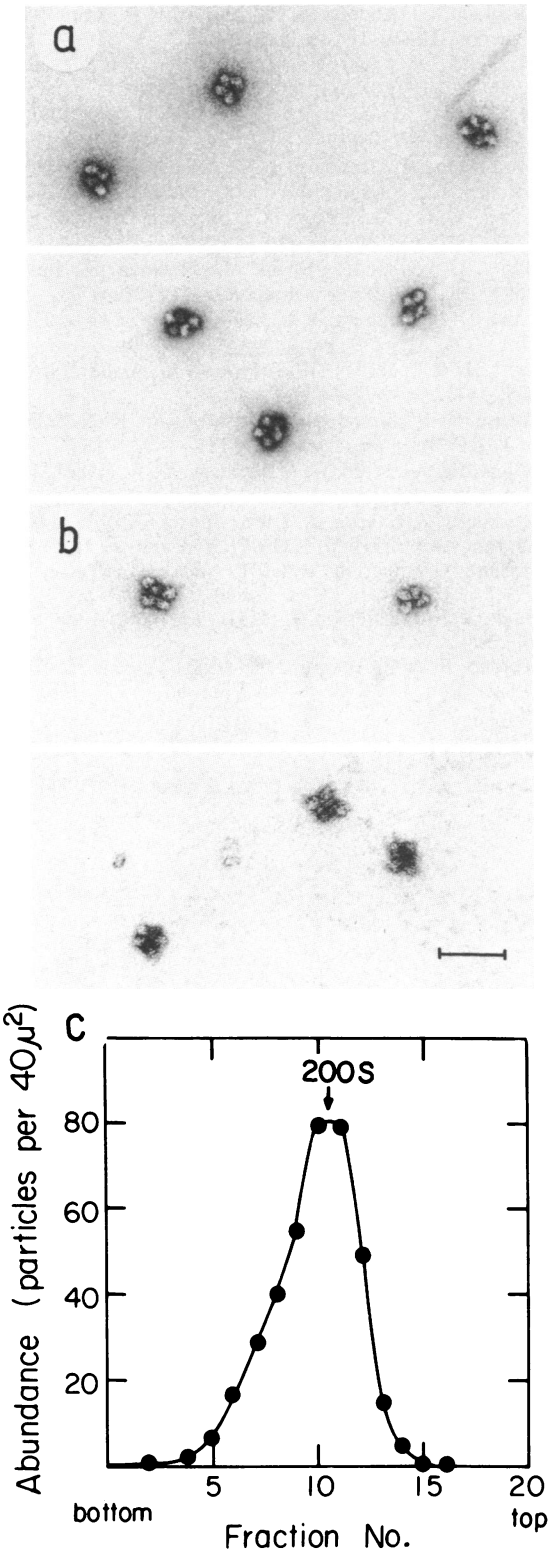

FIG. 4. EM of 200S RNP particles. Electron micrographs of particles from the 200S peak of fractionated RNPs released from mutant Syrian hamster cells (*a*) and HeLa cells (*b*); distribution of 200S particles from HeLa cells in electron micrographs of fractions across the sucrose gradient (*c*). (Bar, 100 nm.) Distribution of the nuclear 200S RNP particles from the Syrian hamster cells is identical to that from HeLa cells.

30–250S in sucrose gradients (ref. 3 and references therein). This heterogeneity has been interpreted as resulting from the size variations of the individual hnRNA species. However, because most of these studies pursued ^{3}H-labeled nonspecific hnRNA, it is plausible that the heterogeneity observed can be attributed to the extreme susceptibility of RNP particles to degradation (3) and to dissociation of subcomponents during isolation (unpublished observations). The large nuclear RNPs described here have been prepared in the presence of potent RNase inhibitors and appear to be of more homogeneous nature.

Evidently the 200S peak comprises a family of particles that sediment at the 200S region in the sucrose gradient and contain mature as well as precursor RNPs. By definition, precursor RNPs are not identical to mature RNP particles, because the former contain intervening RNA sequences that are presumably associated with specific hnRNP proteins and snRNPs. CAD and DHFR RNAs, for example, each have several tens of intervening sequences. However, considering the multitude of expected RNP intermediates that can be formed after the stepwise removal of introns, it is notable that this family of RNP particles sediment as a relatively narrow peak at the 200S region in the gradient. It should also be pointed out that nuclease S1 mapping analysis of CAD RNA precursor molecules across the sucrose gradient shows a broader distribution around the 200S peak of precursor CAD RNP complexes that contain splicing intermediates (N. Cahana, J.S., and R.S., unpublished work). These observations suggest that the pathways that different RNA transcripts use for their assembly into a general RNP structure, destined for transport to the cytoplasm, are governed by common principles.

Electron micrographs of 200S nuclear RNP particles of both Syrian hamster and human (HeLa) cells reveal compact composite structure of 50-nm cross section—similar to the dimensions of the Balbiani ring RNP particles observed *in situ* by Daneholt and co-workers (8–10). The presently described RNP particles appear to be composed of several substructures. It is not clear at this stage whether the observed images display the native structure or may have resulted from the collapse of the structure on the EM grid. In any event, it is feasible that they reflect the substructural organization of the 200S RNP particles. In this context it should be pointed out that processing of hnRNA involves several activities, such as splicing and 3′-end processing, that reside in nuclear RNP particles (for reviews, see refs. 3, 4, and 26). The regulation of transport of processed RNA from the nucleus to the cytoplasm may also reside in RNP structure. It is thus reasonable to assume that the observed substructures of the 200S particles correspond to these functional activities.

The data presented is consistent with the notion that the 200S particles represent a general mode of packaging for hnRNA, the major structural features of which are probably determined by the protein components of the complex. These complexes accommodate RNA transcripts that largely differ in size and, yet, sediment as 200S RNP particles. Supporting evidence for the proposed generality of the 200S particles is provided by the fact that identical 200S particles, as reflected by the reported hybridization and EM studies, are obtained from different cell types and species.

Finally, though the specific transcripts studied so far code for both structural proteins (e.g., β-actin) and enzymes (e.g., CAD and DHFR), they are all products of RNA polymerase II. On the other hand, the nuclear transcripts of ribosomal RNA that are transcribed by RNA polymerase I migrate as a distinct peak near the top of the gradient (see figure 5 of ref. 5). This difference raises the intriguing possibility that the 200S particles serve to package and process transcripts of RNA polymerase II exclusively. Our observation that general poly(A)$^+$ RNP also sediments as 200S particles supports this supposition. Transcripts of other polymerases are excluded from these particles, unless required to function in RNA processing or transport. In this context we note that of

the known elements required for RNA processing, the 200S RNP particles contain precursor molecules [as was shown for CAD RNA by nuclease S1 mapping using intron-specific probes (N. Cahana, J.S., and R.S., unpublished work)] and U1, U2, and U6 snRNPs (7) as integral components of the particles. We therefore suggest that the 200S nuclear RNP particles represent the native apparatus for the processing of hnRNA in eukaryotic cells.

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