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Isolation and initial characterization of a specific premessenger ribonucleoprotein particle

(transcription product/ribonucleoprotein transport/spliceosome/electron microscope tomography)

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ABSTRACT **A specific type of premessenger ribonucleoprotein (RNP) particle, Balbiani ring (BR) granules, has been isolated from heterogeneous nuclear RNP (hnRNP) in the salivary glands of the dipteran *Chironomus tentans*. A BR granule contains a single 75S RNA molecule coding for a large secretory protein (Sp1). The isolation procedure is based on the abundance and exceptional size of the BR granules: in EDTA-containing sucrose gradients they sediment as a sharp 300S peak ahead of the remainder of the hnRNP population. The isolated BR granules were identified on the basis of both ultrastructural and biochemical criteria: large spherical particles that contain 75S RNA and BR sequences. A three-dimensional reconstruction of isolated particles by electron microscope tomography further supported the identification of the isolated particles as BR granules. In contrast to the entire hnRNP population, the BR granules exhibited a sharp peak in CsCl gradients with a buoyant density of 1.45 g/cm^3. This result indicates that a BR granule consists of 40% RNA and 60% protein by weight, corresponding to a 75S RNA molecule of 12 megadaltons and a total protein content of 18 megadaltons, or about 500 average-sized protein molecules.**

During synthesis, growing premessenger RNA molecules are immediately coupled to proteins and form ribonucleoprotein (RNP) complexes (1, 2). The structure of the RNP particles can to a large extent be understood as a chain of beads, a polyparticle (ref. 3; for recent reviews, see refs. 4 and 5). The beads, the monoparticles, contain a simple set of 5–10 proteins, which constitute the so-called core proteins of the polyparticles. Ultrastructural and biochemical studies indicate, however, that premessenger RNP particles also exhibit gene-specific features (for references, see ref. 6). Therefore, for full understanding of the functional behavior of the particles during processing and transport, specific premessenger particles have to be studied as individual entities.

It has proven very difficult to isolate specific RNP particles from the complex premessenger RNP population [heterogeneous nuclear (hn) RNP] in the cell nucleus. Apart from the general problem of RNA and protein degradation (for discussion, see, e.g., ref. 7), there are several biological reasons for this difficulty. First, most premessenger RNP species are present in low amounts, usually at less than 1% of all hnRNP (8). Second, a large proportion of the particles are in a nascent state and therefore constitute a heterogeneous population as to size (5). Third, most of the premessenger RNP particles are being spliced and dramatically reduced in size, often in many steps and not necessarily in one and the same order (9). It is, therefore, not surprising that the isolation of a well-defined premessenger RNP particle has not yet, to our knowledge, been accomplished.

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In the present paper we report on the isolation of a specific type of premessenger RNP particle, the Balbiani ring (BR) granules in the salivary glands of the dipteran *Chironomus tentans*. These are conspicuous spherical RNP structures of extraordinary size appearing at high concentrations in the nucleoplasm (10). At their discovery (11) it was proposed that they are generated in two heavily expanded regions, BR 1 and 2, on chromosome IV. This conclusion was based on the observation that the nucleoplasmic granules are strikingly similar to the RNP observed within the BRs. Further studies of BR transcription units *in situ* and after spreading have revealed that the RNP particles are formed on long, highly active transcription units (12). The ultrastructural analysis of the growing particles strongly indicated that each particle contains a single RNA transcript. The size of the transcript, 75S RNA, has been determined to be 37 kilobases (kb) (13). After the release from the template, the BR granules seem to migrate through the nucleoplasm and pass through the nuclear pores (14, 15). In the cytoplasm the 75S RNA is a predominant messenger RNA species (16) and directs the synthesis of the main export proteins (Sp1) of the salivary gland (17, 18).

The three-dimensional (3D) structure as well as the formation and transport of the BR granules is now being analyzed *in situ* by electron microscope tomography (6). We started the isolation of BR granules to be able to combine biochemical information with our ultrastructural data. Since splicing occurs on BR RNA (19), we believe that the BR system can contribute to our understanding of both the processing and transport of premessenger RNA.

MATERIALS AND METHODS

Organ Culture and Release of RNP. Salivary glands were dissected from *C. tentans* fourth-instar larvae. Six glands were incubated for 90 min at 18°C in 50 μl of modified Cannon's medium supplemented with 4 μl of [α-^{32}P]ATP (NEN; 10 mCi/ml; 800 Ci/mmol; 1 Ci = 37 GBq). The glands were then transferred into a plastic tube containing 250 μl of 0.3% Nonidet P-40 (Sigma)/TKE (0.1 M KCl/0.01 M EDTA/0.01 M triethanolamine hydrochloride, pH 7.0)/with 0.5 mg of *Escherichia coli* tRNA (Boehringer Mannheim) per ml and were sonicated with a Branson sonifier equipped with a microtip (setting 2; five pulses of 20% duty cycle). The contents of the plastic tube were transferred into a cooled 2-ml tissue grinder (Kontes) and homogenized by 10 strokes with a loose-fitting pestle.

Sucrose Gradient Sedimentation. The homogenate was layered on a 5-ml linear sucrose gradient of 15–40% sucrose (BDH) with a 200-μl cushion of 60% sucrose; the sucrose

Abbreviations: RNP, ribonucleoprotein; hnRNP, heterogeneous nuclear RNP; BR, Balbiani ring; cRNA, complementary RNA; 3D, three-dimensional.

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solutions were made in TKE. The gradient was centrifuged in an AH 650.1 rotor (Sorvall) at 0°C and 40,000 rpm for 55 min and subsequently collected in 28 fractions plus a detergent-containing top fraction. The radioactivity was determined by scintillation counting of trichloroacetic acid-precipitable material or by measuring Cerenkov counts in preparative experiments.

Electrophoresis of RNA. The RNP in pooled sucrose gradient fractions (see legend to Fig. 2) was precipitated in ethanol, dissolved in an SDS/proteinase K solution, and electrophoresed in a 0.75% agarose slab gel according to Case and Daneholt (13). Pelleted material in the gradient was dissolved in SDS/proteinase K, precipitated in ethanol, and studied in parallel.

RNase Protection Assay. RNP from selected sucrose gradient fractions (300S and 100S) was precipitated in ethanol. RNA was dissolved in an SDS/proteinase K solution, precipitated in ethanol, redissolved, and analyzed by an RNase A and RNase T1 protection procedure as described by Lendahl and Wieslander (20). The BR complementary RNA (cRNA) probes used are specified in the legend to Fig. 3.

Electron Microscopy of RNP Particles. RNP was deposited onto electron microscope grids after high-speed centrifugation through a sucrose solution in a Lucite plastic microchamber (21). Sucrose gradient fractions (300S and 100S) were diluted with 2 vol of TKE. A sample (150 μl) of each fraction was layered on 100 μl of 0.5 M sucrose in TKE and containing 2% (wt/vol) glutaraldehyde. Centrifugation took place for 2 hr (300S fraction) or 4 hr (100S fraction) at 25,000 rpm and 4°C in an AH 650.1 rotor. Grids were washed in 0.4% Photo-flo (Kodak), stained with 1% phosphotungstic acid in 75% ethanol, rinsed in 95% ethanol, and dried in air (1). The specimens were examined with a JEOL Tem-Scan 100 CX electron microscope.

Electron Microscope Tomography. Grids with RNP particles were prepared as above and colloidal gold was deposited onto the grids. Electron microscope tomography was applied as specified (6).

Isopycnic Centrifugation of RNP Particles. Sucrose gradient fractions (300S and 100S) were made 4% (wt/vol) as to formaldehyde, and about 1 A_{260} unit of isolated ribosomes was added. The samples were dialyzed in the cold for 20 hr against 4% formaldehyde in TKM. Linear 5-ml CsCl gradients were formed from a heavy solution (1.6 g/cm^3) and a light solution (1.2 g/cm^3). Both solutions contained 4% formaldehyde in TKM, and the light solution also contained the sample. The gradients were spun at 36,000 rpm and 20°C for 20 hr in an AH650.1 rotor. They were fractionated into plastic tubes containing 10 μl of 0.1% gelatin solution. Each fraction was examined for absorbance at 260 nm (to identify the ribosome peak), and every third was examined for refractive index. The radioactivity was determined by scintillation counting of trichloroacetic acid-precipitable material.

RESULTS

Isolation of BR Granules. Salivary glands were incubated for 90 min *in vitro* in the presence of [α-^{32}P]ATP, which is readily taken up by these cells and incorporated into newly synthesized RNA (22). During a 90-min pulse, mainly chromosomal, nucleolar, and nucleoplasmic RNA are labeled (16). The RNP particles were released by sonication and subsequent homogenization of the glands in a Nonidet P-40-containing medium. The combined action of mild sonication and treatment with Nonidet P-40 made it possible to release the nuclear contents directly from whole salivary glands upon application of weak shearing forces. The homogenate was immediately loaded onto a 15–40% sucrose gradient.

In our standard procedure, the RNP particles were released into, and fractionated in, a 0.01 M EDTA-containing buffer. Under these conditions polysomes are disassembled, while hnRNP remains essentially intact. A typical sucrose gradient profile of labeled RNP particles is presented in Fig. 1. The most prominent feature is a leading peak around fraction 10. All criteria applied in the following confirmed that the radioactivity of this peak was due to the presence of BR granules (see below). Its sedimentation coefficient was determined to be 300 S, since the granules sedimented four times faster than isolated *Chironomus* ribosomes (75 S); the ribosomes sedimented with constant velocity from the top of the gradient to beyond fraction 8.

The gradient profile did not change when 1 mM $MgCl_2$ replaced EDTA throughout the procedure (not shown). However, as polysomes appear in the 300S fraction in the presence of magnesium, the EDTA method was chosen as the standard procedure. Extraction from isolated nuclei also yielded a 300S peak, but the recovery was low (about 10%) (not shown).

Analysis of RNA. If the 300S RNP particles do represent BR granules, they should contain 75S RNA. To test this prediction, RNP in the various sucrose gradient fractions was treated with SDS/proteinase K, and the labeled RNA was electrophoresed in nondenaturing agarose gels. The results are presented in Fig. 2; the positions of 75S RNA and ribosomal 28S and 18S RNA have been indicated to the left in the figure. We found that most radioactivity from the 300S peak migrated as 75S RNA (lanes 4–6). Furthermore 75S RNA not only appeared in the 300S fraction, it also seemed to be restricted to this fraction. The 75S RNA was usually resolved into one major and two minor bands. In a separate study the three bands have been related to the products of the specific genes in BR 1 and BR 2: the major band has been identified as the BR 2.1 gene transcript and the minor bands as the BR 1 and BR 2.2 transcripts (N. Kirov, T.W., and B.D., unpublished results).

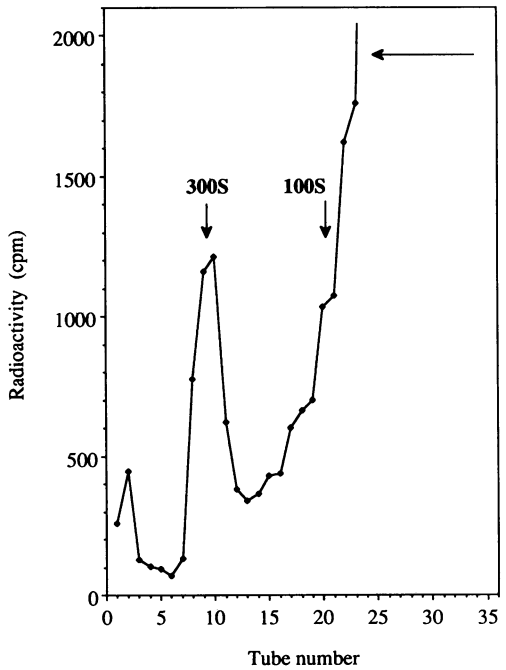

FIG. 1. Sucrose gradient analysis of ^{32}P-labeled RNP particles from salivary glands of *C. tentans*. To estimate the S values, isolated *Chironomus* ribosomes (75 S) were centrifuged for different times in equivalent gradients. The direction of sedimentation is indicated by an arrow.

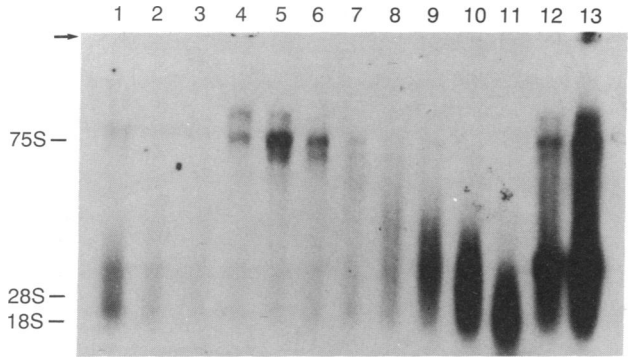

FIG. 2. Agarose gel electrophoresis of pulse-labeled RNA from RNP particles separated in a 15–40% sucrose gradient. The RNP sample was prepared and sedimented in a sucrose gradient as shown in Fig. 1. Three (in the 300S peak, two) consecutive fractions were pooled for RNA analysis: lanes 1 and 2 correspond to fractions 1–6; lanes 3–6, to fractions 7–14; lanes 7–11, to fractions 15–29; lanes 12 and 13, to the pellet (20% and 60% of the pelleted material, respectively). The 300S peak corresponded to lane 5 and the 100S peak to lane 9 in this experiment. The positions of 75S, 28S, and 18S RNA have been indicated to the left; the arrow denotes the start.

The yield of 75S RNA in the 300S peak was estimated to be at least 50% of the total radioactive 75S RNA in the homogenate. Most of the nonpeak 75S RNA was pelleted in the centrifugation tube and was probably not released from the nuclei. This pellet contained most of the total radioactive RNA (Fig. 2; lanes 12 and 13), including the precursors of ribosomal RNA (23); structurally the pellet consists of polytene chromosomes with the nucleoli attached, cellular debris, and unbroken nuclei and whole cells. We conclude that we have obtained an efficient release of the 75S RNA-containing particles and that these band almost exclusively at 300 S.

For various comparisons with 300S RNP (see below), we chose the 100S region as a representative fraction of hnRNP (cf. Fig. 1; lane 9 in Fig. 2). It contains heterogeneous RNA (15–50S) and is likely to be mainly of nuclear origin, since more than 80% of the radioactive non-75S RNA is present in the nucleus after a 90-min pulse (16). Furthermore, the length of labeled RNA in the 100S fraction did not change significantly when EDTA was replaced by $MgCl_2$ during isolation of RNP particles, indicating that the EDTA-mediated disassembly of the polysomes did not result in a transfer of labeled RNP from the polysomal region of the gradient to the 100S region. Finally, it should be noted that the RNA size distribution in the 100S material is clearly different from that of the main preribosomal RNA species, which exhibit characteristic 38S, 30S, and 23S peaks (23). Thus, the 100S fraction represents an hnRNP fraction with RNA in the 15–50S size range.

We next tested whether the radioactive RNA from the 300S and 100S particles contained BR sequences. Using an RNase protection procedure (20), we prepared RNA from the two RNP fractions and hybridized it with nonradioactive cRNA corresponding to BR 1 and BR 2 sequences; we had selected a BR 1γ and a BR 2α sequence, representing two major blocks of repeated sequences within the BR 1 and BR 2.1 genes, respectively (24, 25). When hybridized with salivary gland RNA, the BR 1γ probe is known to protect three sequences in the 60- to 70-base range, and the BR 2α probe protects two sequences in the 70- to 80-base range (20); the occurrence of the multiple bands is caused by imperfect homology of single repeat units. Fig. 3 shows that RNA from the 300S peak (lanes 2 and 3) but not from the 100S region (lanes 5 and 6) gave the expected patterns. Omission of nonradioactive cRNA in the hybridization mixture did not result in protected bands in either the 300S (lane 1) or the 100S

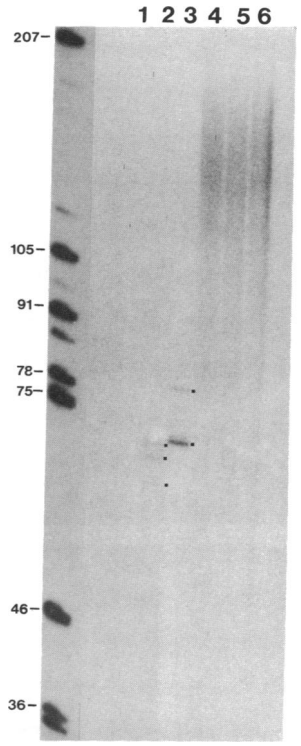

FIG. 3. RNase A and RNase T1 protection of RNA from 300S and 100S RNP particles. Lanes 1–3, 300S region; lanes 4–6, 100S region. Hybridization to BR 1γ cRNA (lanes 2 and 5) and to BR 2α cRNA (lanes 3 and 6); lanes 1 and 4 are controls with no added cRNA. DNA length standards (in bases) are shown to the left. Dots mark cRNA-protected fragments.

sample (lane 4). RNA from the 100S region gave resistant fragments of heterogeneous lengths (100–150 bases) regardless of the presence of cRNA. These fragments disappeared upon RNase A and RNase T_2 treatment, suggesting that they consist of poly(A) (not shown). The hybridization analysis of RNA therefore indicated that the radioactivity in the 300S peak represented 75S RNA transcribed from BR genes.

Electron Microscopy and Electron Microscope Tomography. To morphologically analyze the material, we sedimented the 300S and 100S fractions at high speed onto electron microscope grids. In the 300S fraction we observed abundant, densely stained granules with a size and gross structure similar to those of BR granules observed *in situ* (Fig. 4 *A* and *C* vs. *D*). Solitary clumps of fibrous material could also be recognized. In the 100S fraction small particles of approximately ribosome size and fibrils constituted the predominant material; bigger granules were only occasionally seen (Fig. 4*B*). The putative BR granules in the 300S fraction were investigated in detail in two additional ways to further assess their identity.

First, the relative frequency of large granules in the 300S peak and in fractions flanking the peak was determined. Equal volumes from selected sucrose gradient fractions were loaded into the microchamber, the material was collected onto the electron microscope grids, and the large granules were counted. The flanking fractions contained 11% (corresponding to fraction 7 in Fig. 1) and 12% (fraction 13) of the number of granules in the peak fraction (fraction 10). This close correlation between granule number and radioactivity clearly indicates that the granules contain the radioactive 75S BR RNA.

Second, the 3D structure of isolated granules was compared with the structure of BR granules *in situ* (Fig. 4 *E* vs. *F*). Electron microscope tomography of BR granules *in situ*

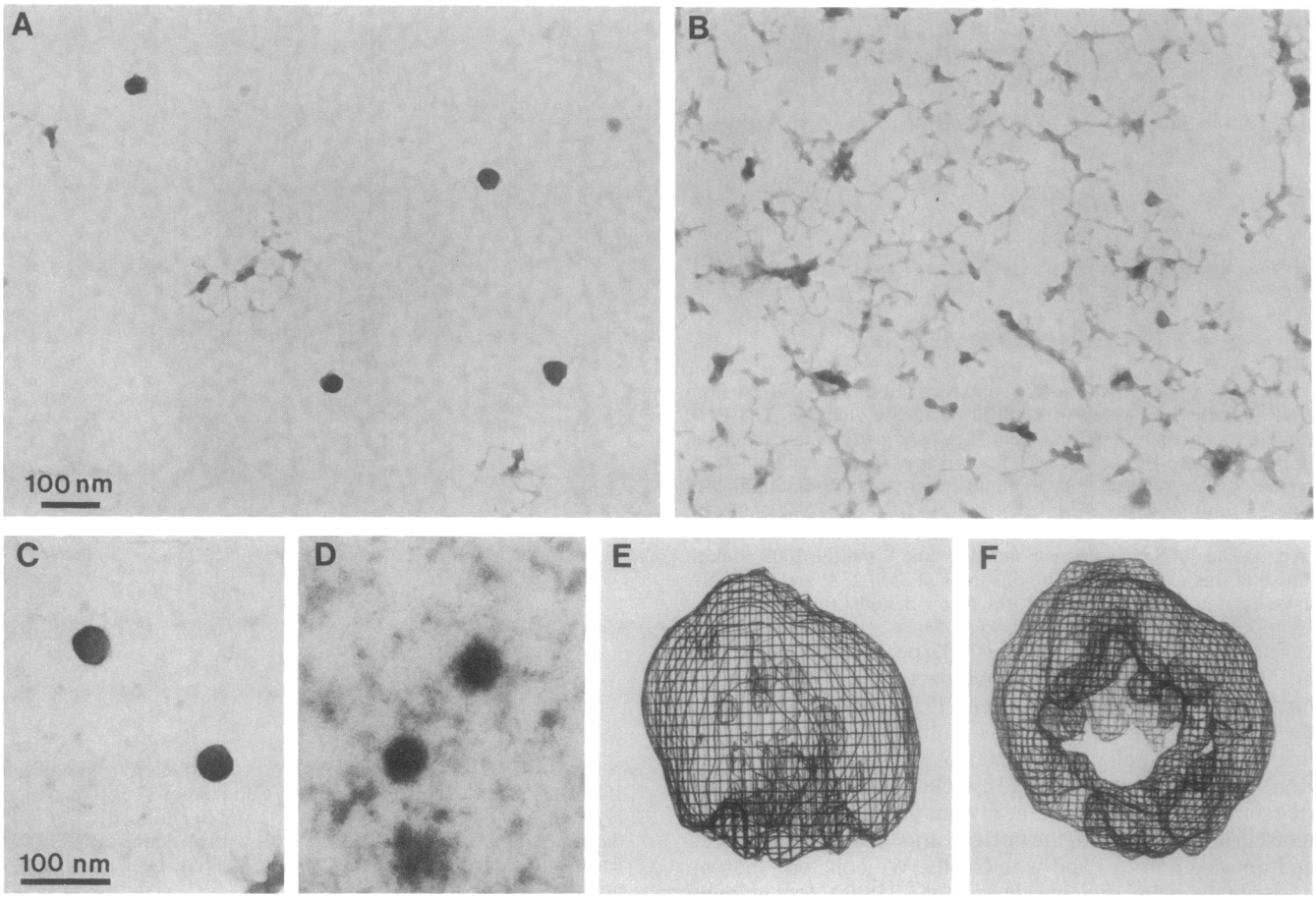

FIG. 4. Electron microscope analysis of 300S and 100S RNP particles. The 300S fraction is presented in *A* and the 100S fraction in *B*. RNP particles from the 300S fraction are displayed at a higher magnification in *C*. For comparison, BR granules observed *in situ* are shown in *D*. Granules corresponding to those in *C* and *D* were reconstructed in three dimensions by electron microscope tomography, and the results are presented in *E* and *F*, respectively. The isolated particles were about 20% smaller in diameter than the BR granules *in situ* (*C* vs. *D*), which was compensated for in the comparison of the 3D reconstructions (*E* vs. *F*). Since this had no effect on the outer shape, it is probably caused by drying during specimen preparation for electron microscopy.

in sections of embedded glands has previously allowed structural characterization at a resolution of 85 Å: the granules can be looked upon as a ribbon bent into a ring-like structure (6). The same procedure was used to analyze isolated granules centrifuged onto electron microscope grids. We obtained relevant information on their contour, which even in detail resembled that of the granules *in situ*; all structural asymmetries in those parts of the granules which protruded from the surface of the grid (top parts in Fig. 4*E*) had counterparts in the granules *in situ* (Fig. 4*F*). The parts facing the grid (bottom part in Fig. 4*E*) seemed somewhat distorted and the hole through the particle was partly filled with stain, probably due to the fact that the conditions for exclusively positive staining are not as favorable on a grid as in a plastic section. As the 3D reconstructions could easily be fitted into each other, we conclude that the electron microscope tomography data further support the view that the 300S particles represent BR granules.

CsCl Gradients. The 300S and 100S fractions were analyzed with isopycnic centrifugation to study their homogeneity and their RNA and protein compositions. It has been shown by Perry and Kelley (26) that the relative RNA and protein contents of formaldehyde-fixed RNP particles can be calculated from the buoyant density of the particles. As

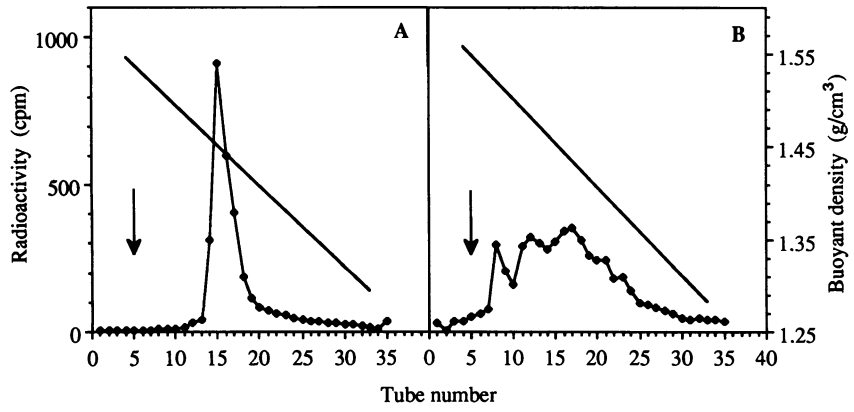

FIG. 5. Isopycnic centrifugation of 300S and 100S RNP particles in CsCl gradients. (*A*) 300S particles; (*B*) 100S particles. The position of *Chironomus* ribosomes has been indicated by arrows.

internal standard and carrier we used ribosomes from *C. tentans*, which band at 1.55 g/cm^3 (27). Fig. 5*A* shows that the 300S peak exhibited a narrow density range around 1.45 g/cm^3, which corresponds to 40% RNA and 60% protein. The 100S material was distributed between 1.3 and 1.5 g/cm^3 (Fig. 5*B*). Thus, the BR granules band as a well-defined population of particles, while hnRNP of 100S size exhibit a pronounced heterogeneity. As a BR granule is known to contain a single 75S RNA molecule of 12 megadaltons, the proteins of the particle should amount to a total of 18 megadaltons, and hence the particle as a whole is 30 megadaltons.

DISCUSSION

In the present paper we describe the isolation of a specific type of premessenger RNP particle, the BR granules in the salivary glands of *C. tentans*. In other experimental systems this has not been possible to achieve, mainly due to the paucity of each premessenger RNP particle in the complex hnRNP population and the size heterogeneity caused by the presence of nascent RNP and the process of splicing (see *Introduction*). When considering these particular obstacles, the BR system offers obvious advantages. In the first place, the BR granules constitute a predominant premessenger RNP particle: the BR products have been estimated to comprise as much as 30% of the labeled high molecular weight RNA in the nucleoplasm (16). Secondly, electron micrographs demonstrate that the abundant BR granules are the largest RNP particles in the nucleoplasm (6). Third, the nascent premessenger RNP can be conveniently separated from the completed and released premessenger RNP. In our procedure, we release all nuclear RNP directly from detergent-treated and mildly sonicated glands by homogenization, and the polytene chromosomes with nascent RNP attached are subsequently pelleted in the sucrose gradient along with cellular debris. It can, therefore, be concluded that it proved feasible to isolate BR granules due to their abundance and large size coupled with the possibility of easily separating the finished premessenger RNP particles from nascent transcription products.

The abundant RNP particles sedimenting at 300 S have been identified as BR granules by several criteria. They contain 75S RNA as shown by electrophoresis of RNA from particles with different S values. Moreover, RNase protection experiments demonstrated that BR sequences (BR 1 and BR 2.2) are present in the peak. Finally, ultrastructural analysis of the particles revealed that the 300S granules are similar in size and shape to BR granules observed *in situ*; this conclusion was strengthened by a comparison of the 300S particles with BR granules as they appear within the cell by electron microscope tomography. Taken together, the electrophoresis, hybridization, and electron microscopy experiments show that the 300S particles represent BR granules.

Electron microscopic studies have shown that the RNP particles in BR 1 and BR 2 are similar in size and structure (6) and pass through the same type of packaging process during their formation (28). However, these ultrastructural results do not rule out minor differences between particles from BR 1 and BR 2. The transcripts are known to differ slightly in size (29) and they can be separated in the nondenaturing gels used here (see *Results*). In fact, the 300S particles in the present study exhibited a distinct but usually slightly asymmetric peak in both sucrose and CsCl gradients (Figs. 1 and 5, respectively), indicating subpopulations of BR particles. Furthermore, the relative proportions of the different 75S RNA species vary within the peak (Fig. 2, lanes 4–6). Thus, BR particles synthesized on different BR genes probably display subtle differences in size, structure, or both.

This method to isolate BR particles will make it possible to obtain biochemical information on the particles. In this study we have observed that the particles form a distinct peak with a buoyant density of 1.45 g/cm^3, while the 100S fraction shows a heterogeneous density distribution (range 1.3 to 1.5 g/cm^3). This result again suggests that the BR particle is a well-defined structure and supports the concept that the RNP particles constitute gene-specific products also on the RNP level.

The buoyant density value of 1.45 g/cm^3 allowed us to estimate that the BR particles are composed of 40% RNA and 60% protein, corresponding to 12 megadaltons of RNA (one 75S RNA molecule) and 18 megadaltons of protein. The latter figure would indicate that a single particle could accommodate approximately 500 average-sized protein molecules. As premessenger RNP particles in general contain a set of predominant proteins, the core proteins (4, 5), it seems likely that the BR particles contain some kind of repetitive basic structure. There should also be structural elements responsible for the specific folding of the elementary RNP fiber into the toroid structure. Finally, it should be pointed out that the large size of the object makes it particularly suited for a combined biochemical and ultrastructural analysis. The most obvious *in vivo* processes at hand for such an analysis are the formation of a spliceosome as part of a premessenger RNP complex and the translocation of an RNP particle through a nuclear pore, involving major conformational changes of the particle.

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