Isolation and Characterization of a Novel Ribonucleoprotein Particle: Large Structures Contain a Single Species of Small RNA

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Abstract. Rat liver coated vesicle preparations were frequently found to contain small ovoid bodies, which resembled coated vesicles in morphology. We have purified these bodies to homogeneity using sucrose density gradients and preparative agarose gel electrophoresis. When negatively stained and viewed by electron microscopy, the purified structures display a very distinct and complex morphology, resembling the multiple arches which form cathedral vaults. They measure 35 × 65 nm and are therefore considerably larger than ribosomes. When subjected to SDS PAGE, these structures, which we refer to as vaults, appear to contain several minor and five major species: M_r 210,000, 192,000, 104,000, 54,000, and 37,000. One of these (M_r 104,000) greatly predominates, accounting for >70% of the total Coomassie Brilliant Blue–staining protein. Another major species of M_r 37,000 has been identified as a species of small RNA of unusual base composition (adenosine 12.0%, guanosine 29.7%, uridine 30.9%, and 27.4% cytidine), which migrates as a single species in urea PAGE between the 5S and 5.8S ribosomal standards, containing ~140 bases. Although the RNA constitutes only 4.6% of the entire structure, the large size of the particle requires that each one contains ~9 molecules of this RNA. Antibodies prepared against the entire particle are largely specific for the major (M_r 104,000) polypeptide species. Although they do not directly react with the RNA constituent on Western blots, these antibodies immunoprecipitate a ^{32}P-labeled RNA of identical size from metabolically-labeled rat hepatoma cells. Vaults are observed in partially purified fractions from human fibroblasts, murine 3T3 cells, glial cells, and rabbit alveolar macrophages. It therefore appears that these novel ribonucleoprotein structures are broadly distributed among different cell types. The function of vaults is at present unknown.

RIBONUCLEOPROTEIN structures have recently been demonstrated to be of greater diversity and significance than previously realized. Although the importance of the ribosome in cellular metabolism is well established, the functional roles of the small nuclear ribonucleoprotein particles are only currently being explored (2, 12; for review see references 6 and 9). Recently it has also been demonstrated that the targeting of nascent secretory polypeptides to the endoplasmic reticulum is mediated by an 11S structure termed signal recognition particle (29) which contains a 7S RNA essential for its function (30). Another small (10S) cytoplasmic ribonucleoprotein particle is apparently involved in the regulation of protein synthesis (25). Each of these small ribonucleoprotein particles contains multiple polypeptides and a single species of RNA.

We describe the purification of a novel ribonucleoprotein particle from rat liver microsomal extracts. This structure is unusual in its size, molecular composition, and morphology. It is considerably larger than the ribosome and is therefore the largest ribonucleoprotein body reported to date. Its protein composition is greatly dominated by a single polypeptide species of M_r 104,000, which comprises >70% of its total protein as measured by Coomassie staining. The particle has a unique morphology composed of a complex series of highly regular intertwined arches.

Materials and Methods

Materials

2-(N-Morpholino)ethane sulfonic acid (MES)[1] was purchased from Research Organics, Inc., Cleveland, OH. Ultrapure sucrose and Immuno-Precipitin was obtained from Bethesda Research Laboratories, Gaithersburg, MD. Ficoll-70, EGTA, 4-chloro-1-napthol, ribonuclease A (RNase), human placental RNase inhibitor, and bovine serum albumin (BSA) were obtained from Sigma Chemical Co., St. Louis, MO. Sprague-Dawley rats were obtained from our own breeding colony. "Isogel" agarose was obtained from FMC BioProducts, Rockland, ME. Redistilled phenol was purchased

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1. *Abbreviation used in this paper*: MES, 2-(N-morpholino)ethane sulfonic acid.

from International Biotechnologies, Inc., New Haven, CT. All other reagents were obtained from commercial suppliers.

Purification of Vaults

The purification scheme, derived from a procedure previously described for coated vesicle purification (3), is summarized in Fig. 2. Sprague-Dawley rats (15–25 per preparation) were fasted overnight, anesthetized with chloroform, and decapitated. Livers were removed and immediately chilled on ice in MES buffer (0.09 M MES, 0.01 M sodium phosphate, 1.0 mM MgCl₂, 0.5 mM EGTA, 0.02% sodium azide [pH 6.5], containing 0.25 M sucrose (MES–sucrose). All subsequent operations were performed at 4°C. Rinsed livers were minced and homogenized in 1 vol of MES–sucrose buffer using four strokes of a loose-fitting Teflon-glass homogenizer. The homogenate was forced through two layers of medium mesh cheesecloth, diluted 1:1 with MES–sucrose buffer, and centrifuged for 10 min at 2,000 rpm in a Sorvall GSA rotor. The supernatant was filtered through cheesecloth and reserved on ice. The pellet was re-extracted in two times its volume of MES–sucrose using four strokes of tight-fitting Teflon-glass homogenizer and centrifuged at 2,000 rpm (650 g) for 15 min in a Sorvall GSA rotor. The two supernatants were pooled, and centrifuged at 11,000 rpm (20,000 g) for 30 min. The supernatant from this centrifugation was filtered through cheesecloth and centrifuged for 2 h at 20,000 rpm in a Beckman 21 ultracentrifuge rotor. Pellets were resuspended in MES buffer (50–75 ml) using a glass-glass dounce homogenizer. Resuspended material was diluted 1:1 with MES buffer containing 14% Ficoll-70 and 14% sucrose, mixed thoroughly, and centrifuged in an SS-34 Sorvall rotor at 19,000 rpm (40,000 g) for 40 min. The supernatant was diluted 1:5 with MES buffer (no sucrose), and centrifuged at 20,000 rpm in a Beckman T21 rotor. The clear, reddish pellets were resuspended in MES buffer using a glass-glass dounce homogenizer, layered over step gradients containing 5/10/20/30/40% sucrose in MES buffer (7/7/6/6/6 ml in each layer, respectively, in Beckman SW-28 ultraclear ultracentrifuge tubes, with interface positions marked for reference), and centrifuged at 25,000 rpm for 60 min. The 5% layer (reddish with ferritin) was removed, diluted 1:1 with MES buffer, and centrifuged

2 h at 20,000 rpm in the Beckman T21 rotor to concentrate the vaults. This velocity-purified material (Pool A) was then applied to a 30/40/45/50/55/60% sucrose step gradient in MES buffer (containing 6/6/6/6/6/2 ml in each layer, respectively) and centrifuged at 25,000 rpm in a Beckman SW-28 rotor for 16 h. Vaults were present in the red ferritin-rich band present just below the original 40/45% sucrose interface mark, extending to mid-way between the 40/45 and 45/50 marks. A syringe was used to harvest this material through the side of the tube (16). This material was diluted fourfold with MES buffer, and centrifuged for 90 min at 100,000 g. The purification scheme is summarized in Fig. 2.

Gradient-purified material was subjected to agarose gel electrophoresis as described by Rubenstein et al. (24), with slight modifications: "Isogel" agarose was used due to its low electroendosmosis, and gels contained 0.25% rather than 0.15% agarose. The same buffer system (50 mM MES, pH 6.5) was used in both analytical and preparative agarose gel separations. The modifications of the agarose system to enable preparative recoveries are described in detail elsewhere (11).

Electron Microscopy

Carbon films were prepared using a Varian glow-discharge vacuum evaporator to coat freshly cleaved mica surfaces. Carbon films were floated off the mica into distilled water and transferred to 400-mesh copper grids (Ted Pella, Inc., Tustin, CA). Samples were adsorbed onto the carbon-coated grids, stained with 1% uranyl acetate, air dried, and viewed on a Zeiss EM109 electron microscope.

PAGE

One-dimensional polyacrylamide slab gels were prepared using the SDS containing buffer system described by Laemmli (14). Routinely, gels were cast with two separating gels of 8 and 12% acrylamide, using a commercially available minislab apparatus (Idea Scientific, Corvallis, OR). The two separating gels provided better overall resolution of both high (>80,000 D) and low molecular mass peptides, and were easier to prepare than casting
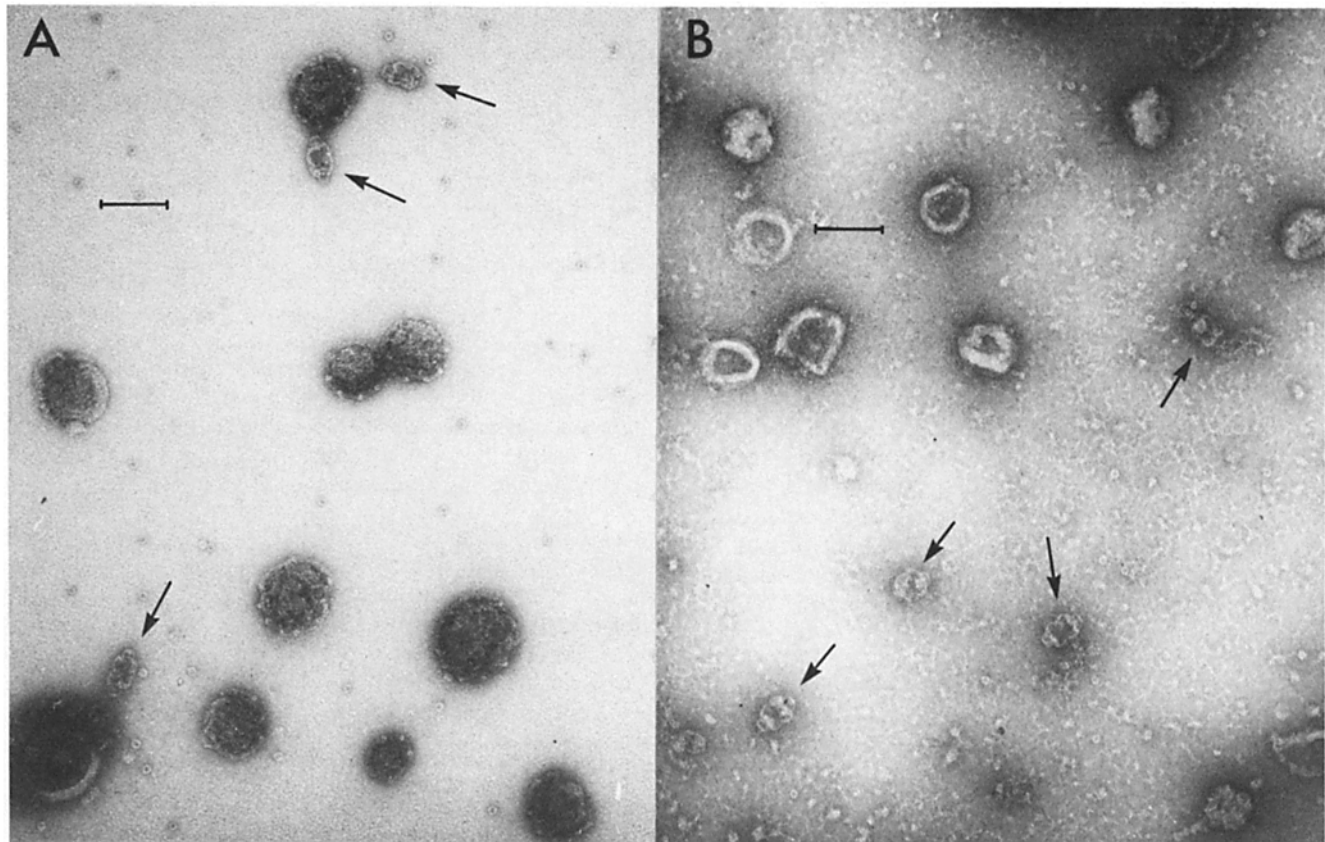

Figure 1. Electron microscopy of negatively stained coated vesicle preparations showing vault-like structures as contaminants. (*A*) Purified coated vesicles showing vaults (*arrows*) and some ferritin as contaminants. (*B*) Same after treatment for 12 h with 2 M urea. Bar, 100 nm.

gradient gels. Isoelectric focusing was performed using the method of O'Farrell (19) modified as follows: sample dissociation buffer contained 1% SDS, 8% Nonidet P-40, 5% (vol/vol) β-mercaptoethanol, 2% pH 3-10 ampholytes, and 9 M urea. Gels contained 8 M urea, 2% Nonidet P-40, 4% acrylamide, 2% pH 3-10 ampholytes, 0.8% pH 5-7 ampholytes, 0.2% pH 2.5-4 ampholytes, and 10 mM each lysine and arginine. The second dimension SDS polyacrylamide gels were prepared according to Laemmli (14) and contained linear 5-15% gradients of polyacrylamide with 2.7% bisacrylamide. Gels were stained either with silver according to the procedure of Merril et al. (17) or with 0.25% Coomassie Brilliant Blue in 30% methanol, 10% acetic acid, and destained in 10% each methanol and acetic acid. Urea gels were prepared using a standard Tris-borate-EDTA buffer system (0.05 M Tris, 0.05 M boric acid, 1 mM EDTA, pH 8.3) as described for RNA sequencing gels (7). Gels contained 8.3 M urea, 7.6% acrylamide, 0.4% bisacrylamide, and 0.1% SDS, and were prerun to achieve a temperature of 60°C which was maintained throughout the run.

Antibody Preparation

For the two initial injections, 120 µg of purified vault particles were mixed with 0.8 mg keyhole limpet hemocyanin (Sigma Chemical Co.) in phosphate-buffered saline (total vol, 2 ml). The material was cross-linked by the addition of glutaraldehyde (Ted Pella, Inc., Tustin, CA) to achieve a final concentration of 0.25%, incubated for 3 h at room temperature, and dialyzed against several changes of phosphate-buffered saline. Three-fourths of the conjugated antigen was emulsified in Freund's complete adjuvant (Gibco, Grand Island, NY) and injected subcutaneously. The rabbit was boosted with the remaining conjugate in Freund's incomplete adjuvant after 2 wk and bled 2 wk later. Subsequent boosters were administered monthly using purified vaults in Freund's incomplete adjuvant.

Immunoprecipitation

Microsomal extracts were prepared in MES buffer diluted 1:1 in 0.2 M sodium phosphate, 0.3 M NaCl, 1.0% Triton X-100 (pH 7.2). *Staphylococcus aureus* cells (pretreated with 0.1% [wt/vol] formalin and washed) were added to the extracts and allowed to incubate at 4°C for 1 h. The *S. aureus* cells were removed by a 2-min centrifugation in an Eppendorf microfuge. The supernatants were then treated with various amounts of immune or non-immune sera and incubated for 1 h at 4°C with constant agitation. Washed protein A-Sepharose beads were added (2 mg per 0.5 ml sample) and the incubation continued for at least 2 h further. The samples were microfuged for 2 min and washed four times with buffer (0.1 M sodium phosphate, 0.15 M NaCl, 0.5% Triton X-100 [pH 7.2] containing 0.1% SDS). Samples for SDS PAGE were treated as follows: the washed beads were incubated in slab sample buffer (14) containing 5% β-mercaptoethanol for 10 min at 80°C, centrifuged, and the supernatant applied to SDS PAGE according to the method of Laemmli (14). Immunoprecipitates to be phenol extracted were treated differently: the beads were suspended in 0.1 M Tris-HCl (pH 7.5) containing 0.15 mM EDTA, 0.15 M NaCl, 1% SDS, and 25 µg of proteinase K, and digested at 37°C for 1 h. Supernatants were removed and the beads washed with fresh buffer. The pooled extracts were phenol-chloroform extracted (16). All solutions were prepared using sterile water and precautions were taken against RNase contamination.

³²P Labeling and Immunoprecipitation of H4 Cells

Six 175-cm² flasks of confluent H4 cells (a rat hepatoma line) were incubated for 2 d in Dulbecco's modified Eagle's medium containing 1% of the normal phosphate concentration and 10% dialyzed fetal calf serum. The media was removed and replaced with fresh media supplemented with 10 mCi (0.18 mCi per ml) of [³²P]phosphate, and the cells were incubated for 16 h at 37°C. The flasks were rinsed with PBS, the cells trypsinized, washed twice with serum-containing media to remove the trypsin, and centrifuged. The cell pellet was homogenized on ice in 2.5 ml of buffer (0.1 M phosphate, 0.15 M NaCl, 0.5% Triton X-100 [pH 7.4]) containing 250 µg/ml human placental RNase inhibitor using 10 strokes of a teflon glass homogenizer.

The homogenate was spun for 5 min in an Eppendorf microfuge (12,000 g) to remove the nuclei; the nuclear pellet was washed once and immediately subjected to hot phenol/detergent extraction (16). The postnuclear supernatant was further centrifuged 15 min at 12,000 g to remove mitochondria, lysosomes, and other large structures. The remaining supernatant, containing microsomes and cytosol components, was divided into three unequal aliquots; one 50-µl aliquot was immediately phenol extracted, another 50-µl aliquot was incubated at 4°C for the duration of the immunoprecipitation procedures, and the remainder was immunoprecipitated.

For immunoprecipitation, the aliquot was incubated for 30 min at 4°C with 50 µl of formalin-fixed *S. aureus* beads (Immuno-precipitin), precoated with non-immune antibody. The beads were removed by centrifugation and discarded. The supernatant was incubated with 50 µl of protein A-Sepharose beads precoated with anti-vault (B7) antibodies for 2 h at 4°C, pelleted, washed, and processed for urea PAGE as described above.

Other Procedures

Stock solutions (10 mg/ml) of RNase A, DNase I, and proteinase K were prepared in distilled water, aliquoted, and stored frozen at −20°C. Working stock solutions (500 µg/ml) were made by dilution into MES buffer just before use. Reactions were performed in MES buffer at pH 6.5. Phenol/chloroform extractions and ethanol precipitations were performed as described by Maniatis et al. (16). Rat liver poly A⁻ RNA standards were a gift from Dr. James Paulson (University of California at Los Angeles). Western blot analysis was performed according to Towbin et al. (27), using a secondary antibody conjugate (goat anti-rabbit-horseradish peroxidase) which was visualized using 4-chloro-1-napthol (10).

Results

Detection of Vaults in Coated Vesicle Preparations

Rat liver coated vesicles were purified according to a modification of the procedure of Blitz et al. (3) and separated by preparative agarose gel electrophoresis as previously described (11). We consistently observed structures that were
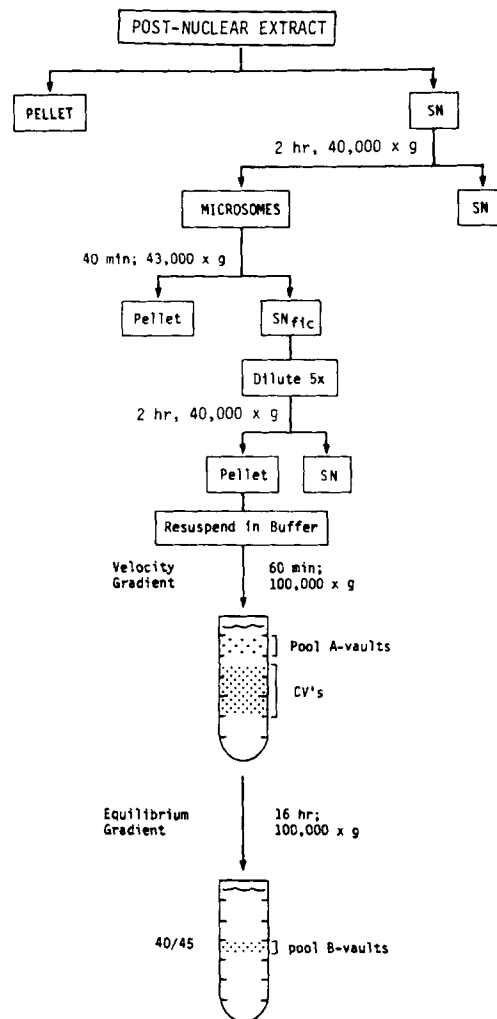

Figure 2. Purification scheme used to prepare crude vaults.

ovoid, somewhat smaller than most coated vesicles, and possessed a complex, knot-like morphology (Fig. 1 *A*, arrows). These structures were also present in preparations of coated vesicles that had been treated with 2 M urea in order to remove the clathrin triskelions from the vesicles (Fig. 1 *B*), suggesting that they were more stable than coated vesicles. Fractions shown by electron microscopy to be enriched in these structures appeared to be coincidentally enriched in protein with an M_r of 104,000 on SDS PAGE; this species co-migrated with the largest of the coated vesicle–associated 100-kD polypeptides. As these polypeptides are required for the binding of triskelions to the underlying membrane vesicle (28), the greater stability and apparent polypeptide composition of these unknown particles suggested that they might constitute a coated vesicle–related organizing center or nucleation site.

Morphology and Protein Composition of Purified Vaults

Several procedures were required to obtain homogeneous material (see Fig. 2): crude microsomes were prepared, a differential centrifugation in sucrose/Ficoll was used to remove the larger membranous species, and smaller microsomes were recovered by an additional centrifugation. Coated vesicles were largely separated from the vaults by a velocity sedimentation into a 5/10/20/30/40% sucrose gradient, in which the larger coated vesicles moved into the 10–30% layers while the vaults remained in the 5% layer. Ferritin co-purified with the vaults and served as a convenient visible reddish marker during the procedure. After pelleting, the material was applied to a 30/40/45/50/55/60% sucrose isopycnic gradient and centrifuged. Vaults and ferritin were again coincident just below the 40/45% sucrose in-
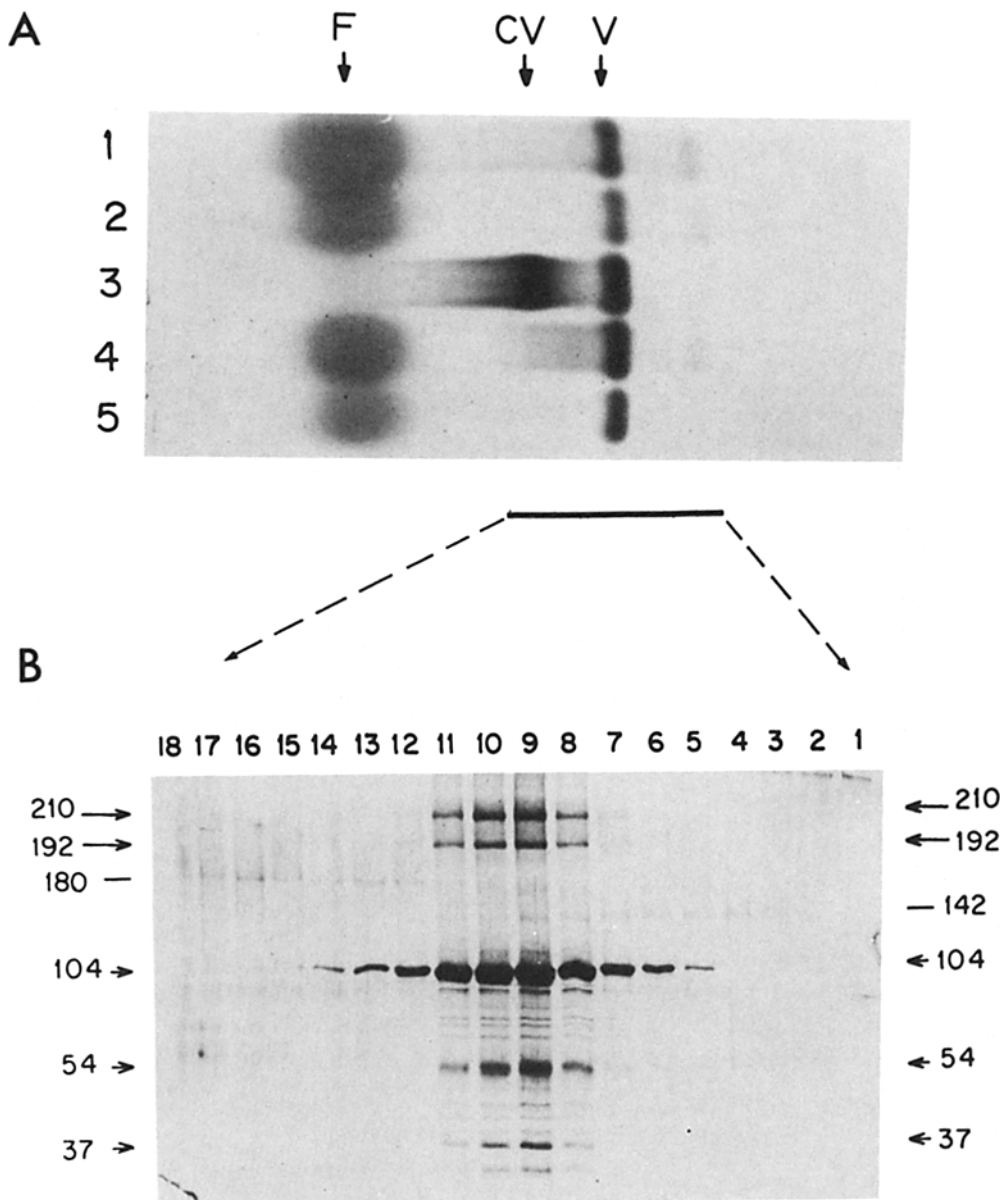
Figure 3. Agarose gel purification step of sucrose-purified crude vaults. (*A*) Analytical agarose gel stained with Coomassie Brilliant Blue. Different preparations (prepared using the same procedure on different days) of sucrose-purified material were applied to lanes *1*, *2*, *4*, and *5*. Lane *3* contains a mixture of vaults and coated vesicles. The migration positions of the various species are indicated; *V*, vaults; *CV*, coated vesicles; *F*, ferritin. Origin is at the left. (*B*) SDS PAGE stained with silver, showing the composition of material eluted from a preparative agarose gel similar to that shown in *A*. Material equivalent to lane *5* in *A* was applied to a preparative gel, eluted during the run, and fractions were collected. Equal aliquots were applied to the SDS gel. The region marked with a bar in *A* represents the region which was eluted and whose molecular composition is shown in *B*. The lanes are numbered according to their order of elution (i.e., the fastest-migrating fraction was applied to lane *1*).

terface (relative to the pre-centrifugation reference marks) in a reddish band which equilibrated slightly above the opalescent coated vesicle bands in a parallel tube. Another pelleting step preceded agarose gel electrophoresis. Typically, the final gradient-purified material was dark red with ferritin.

The gradient-purified crude vault fraction was resolved into several species by agarose gel electrophoresis (Fig. 3 A). When the same material was applied to a preparative agarose gel, a group of polypeptides dominated by a species with an M_r of 104,000 eluted from the gel in a single peak (Fig. 3 B). Although coated vesicles were largely separated from this species by the preceding sucrose gradient steps (Fig. 2), the agarose gel step also effectively separated the small amount of contaminating coated vesicles from the vaults (Fig. 3 A, lane 3), as the coated vesicles migrated more slowly than the vaults. An additional contaminating species (M_r 142,000) migrated more rapidly in the agarose gel than did the vaults (see Fig. 3 B).

The purified structures were negatively stained and examined by electron microscopy, which revealed highly uniform, ovoid bodies, measuring 35 × 65 nm and possessing an elaborate substructure (Fig. 4). Although their complex "woven surface" morphology somewhat resembled that of clathrin-coated vesicles, the purified vaults contained no clathrin when examined by SDS PAGE (Fig. 5). Their protein composition was dominated by a single polypeptide of

M_r 104,000 which migrated close to the largest of the coated vesicle 100-kD family of polypeptides (Fig. 5); however, immunological studies indicate that the vault and coated vesicle polypeptides are not related (see below). The vault M_r 104,000 species constituted >70% of the Coomassie-staining polypeptides as indicated by densitometry. Other major species were seen at M_r 210,000, 192,000, and 54,000. Of interest was the major silver-staining species of M_r 37,000 which was completely unstained by Coomassie Brilliant Blue (Fig. 5), even in very overloaded gels. These major species co-purified throughout the entire procedure (not shown). Several minor species were consistently present in all preparations, with M_r 89,000, 46,000, 31,000, 30,000, 28,800, and 10,000. Western blots of the vault species were probed with concanavalin A and wheat germ agglutinin lectins (10); no reactive species were detected, suggesting that mannose and N-acetylglucosamine residues are not present (not shown). Typically 500–800 µg of purified vault protein were obtained from 150 g of rat liver.

Two dimensional electrophoresis according to O'Farrell (19) revealed that the major vault polypeptide (M_r 104,000) focused to a single species at pH 6.3, exhibiting no charge heterogeneity (Fig. 6). The M_r 210,000 species was quite basic and did not enter the isoelectric focusing gel; the M_r 192,000 polypeptide was only slightly less basic. The M_r 54,000 species was resolved into four separate species of
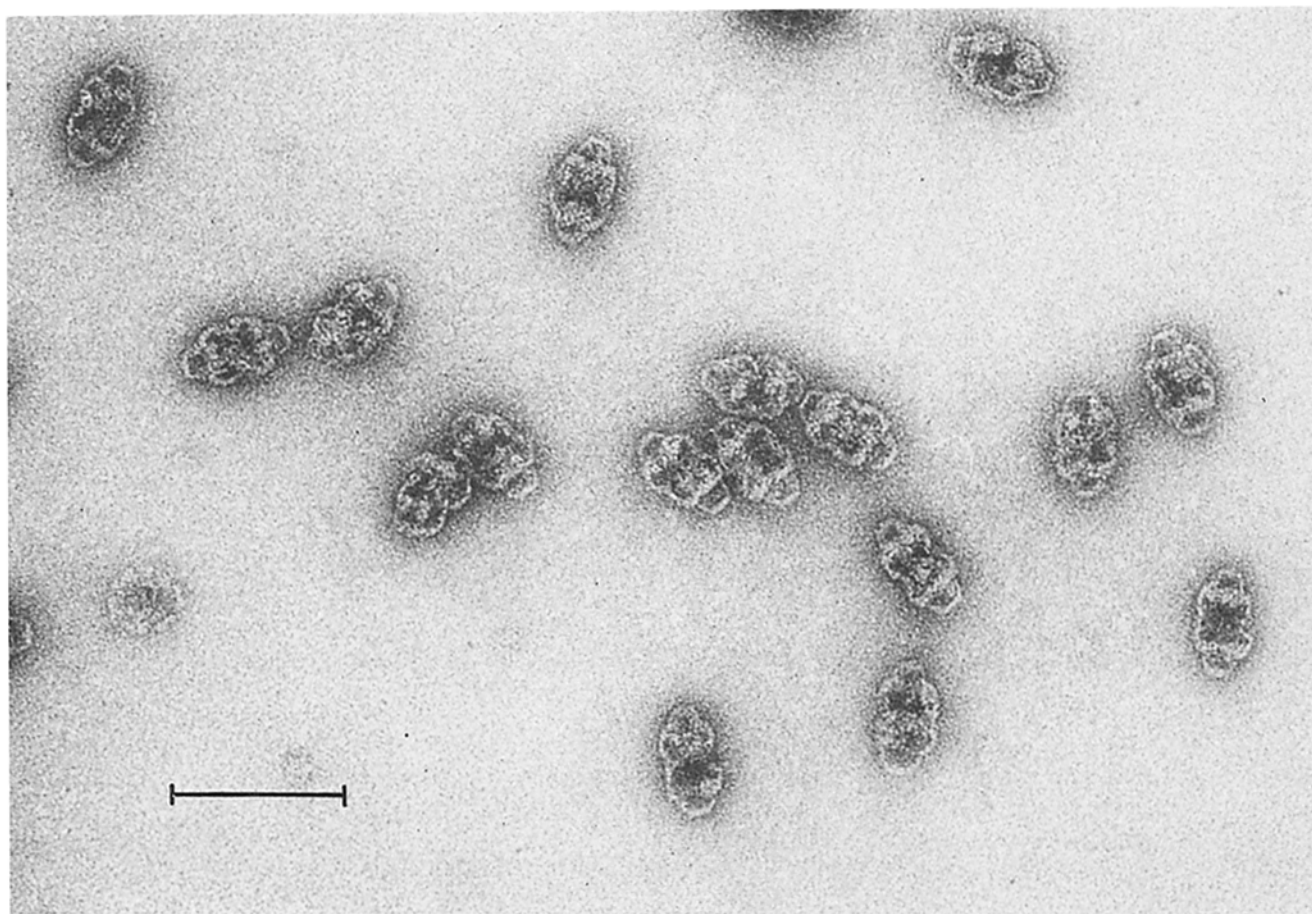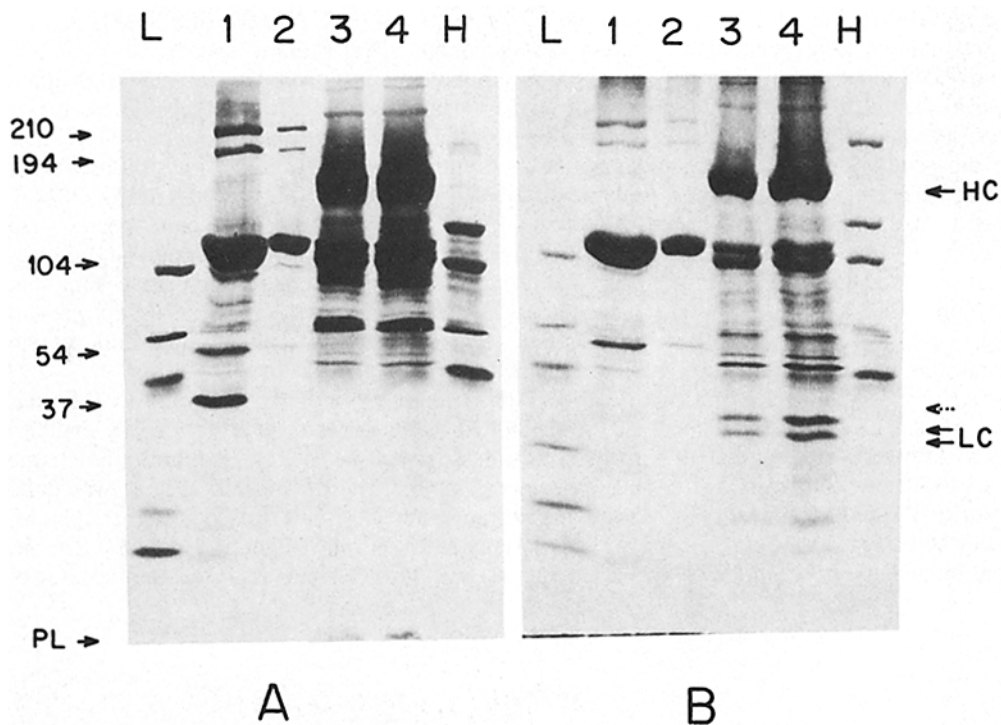

Figure 4. Electron micrograph of negatively stained vaults. Samples from fraction 9 in Fig. 3 B were negatively stained with uranyl acetate and viewed. Bar, 100 nm.

Figure 5. Protein composition of vaults and coated vesicles. Equal amounts of sample were applied to parallel lanes in the same SDS polyacrylamide gel, which was cut in half after the run. One side of the gel was stained with silver (*A*) and the other side with Coomassie Brilliant Blue (*B*). Lane *1*, 4 μg vaults; lane *2*, 1 μg vaults; lane *3*, 2 μg coated vesicles; and lane *4*, 6 μg coated vesicles. The major molecular species of the vaults are indicated in kD (*arrows*). The clathrin heavy chains (*HC*) and light chains (*LC*) are indicated. Note that the M_r 37,000 species stains prominently with silver but is completely unstained by Coomassie Brilliant Blue. Conversely, the clathrin light chains stain readily with Coomassie Brilliant Blue but poorly with silver. The migration position of the phospholipids is visualized using silver stain (*A*) and is indicated (*PL*). Lanes *L* and *H* contain, respectively, Bio-Rad standard low and high molecular weight proteins.

similar size and charge. Once again, the M_r 37,000 species behaved anomalously: it was present as a continuous streak between the pI 4.5 and 6.0 regions of the gel.

Treatment of Structures with Proteinase K and RNase A

Most of the native vault polypeptides were largely resistant to several proteases, among them trypsin, chymotrypsin, and *S. aureus* V-8 protease (Fig. 7). As these mild proteases are frequently used as structural probes, it appeared likely that the resistance of these polypeptides to the proteases was due to their complex tertiary structure. Alternatively, the presence of an underlying membrane could explain the protease resistance of many of the polypeptides. When purified vault structures were treated with proteinase K, a highly nonspecific protease with the capacity to degrade protease-resistant proteins such as RNase A (31), all of the vault species were degraded (Fig. 7, lane *9*) with the exception of the M_r 37,000 species whose mobility on SDS PAGE was unaltered. This species remained resistant to proteinase K even in the presence of 1% Triton X-100. Examination of proteinase K–treated vaults by electron microscopy revealed no trace of underlying membrane or vesicle, nor any other visible species that might sequester the M_r 37,000 species and thereby account for its resistance to enzymatic proteolysis. Its anomalous staining and focusing properties (see above), in addition to its immunity to proteolysis, suggested that it might not be protein in nature. Although its mobility on SDS PAGE was unaffected by DNase I (not shown), it was completely destroyed by treatment with RNase A (Fig. 7, lane *8*). In addition, this species stained with ethidium bromide and could be [^{32}P]labeled using both T4 ligase and T4 kinase (not shown).

Phenol Extraction and Urea PAGE of the Vault RNA

The purified structures were extracted with phenol, phenol/chloroform, and chloroform as described in Materials and Methods. The material remaining in the aqueous phase was precipitated by the addition of 2 vol of ethanol, incubated at −20°C overnight, and pelleted at 12,000 *g* for 30 min. When this material was reapplied to SDS PAGE, it migrated to exactly the same position as the original M_r 37,000 species (not shown). As SDS does not denature RNA, SDS PAGE cannot be used to accurately determine the size of RNA species. The extracted material was therefore treated with 8.3 M urea at 60°C and run in a urea PAGE system commonly used in nucleic acid size determination (see Materials and Methods). A single major species was observed, which exhibited a mobility between the 5S and 5.8S ribosomal RNA standards (Fig. 8). Using DNA standards obtained from restriction enzyme digests of pBR322, we estimated its size to be ∼140 bases. Its mobility in urea PAGE under denaturing conditions appears to closely resemble that of U8 RNA (see Fig. 1 in reference 22), which also contains 140 bases. The nucleoside composition of the vault RNA was determined by high performance liquid chromatography (Table I). The unusually low molar ratio of adenosine (12 %) indicates that the vault RNA is probably unrelated to either U8, 5S, or 5.8S rat RNAs.
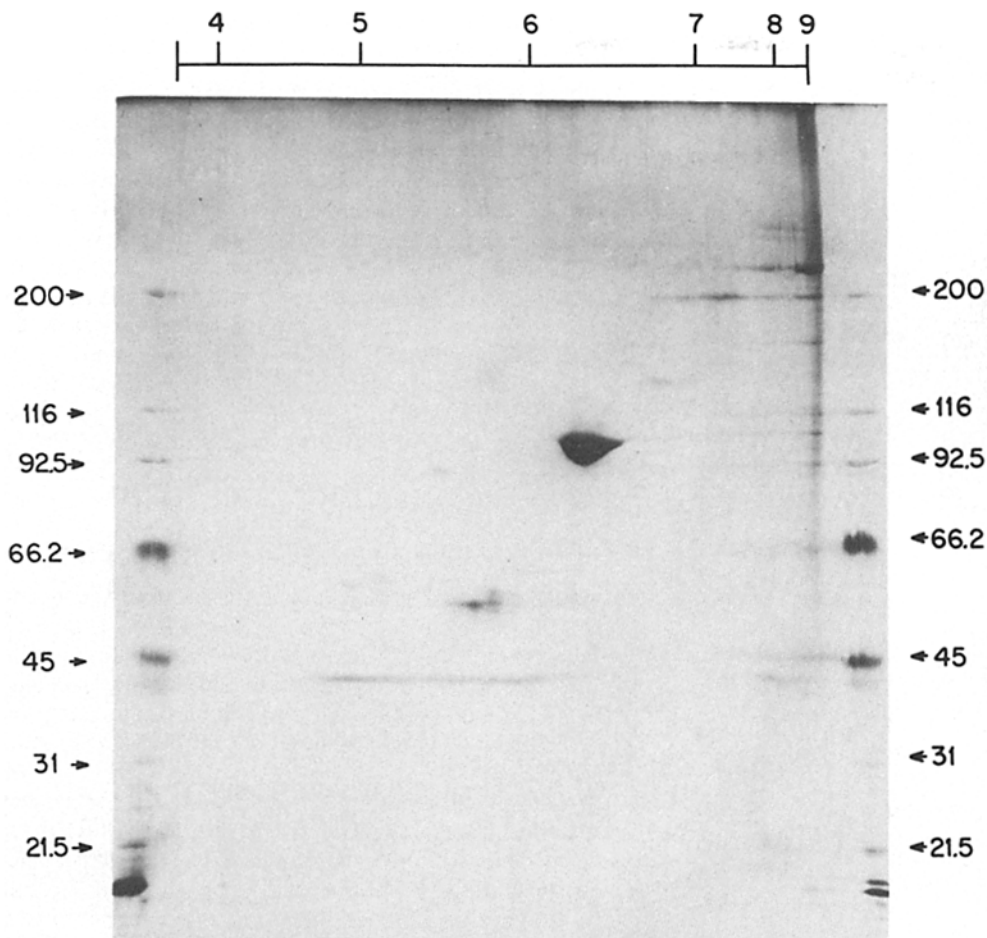
Figure 6. Two-dimensional PAGE of vaults. Vaults were treated as indicated in the text and subjected to two-dimensional gel electrophoresis according to the method of O'Farrell (19). Molecular weights of the standards are indicated ×10⁻³. The pH of the first dimension is indicated at the top of the figure.
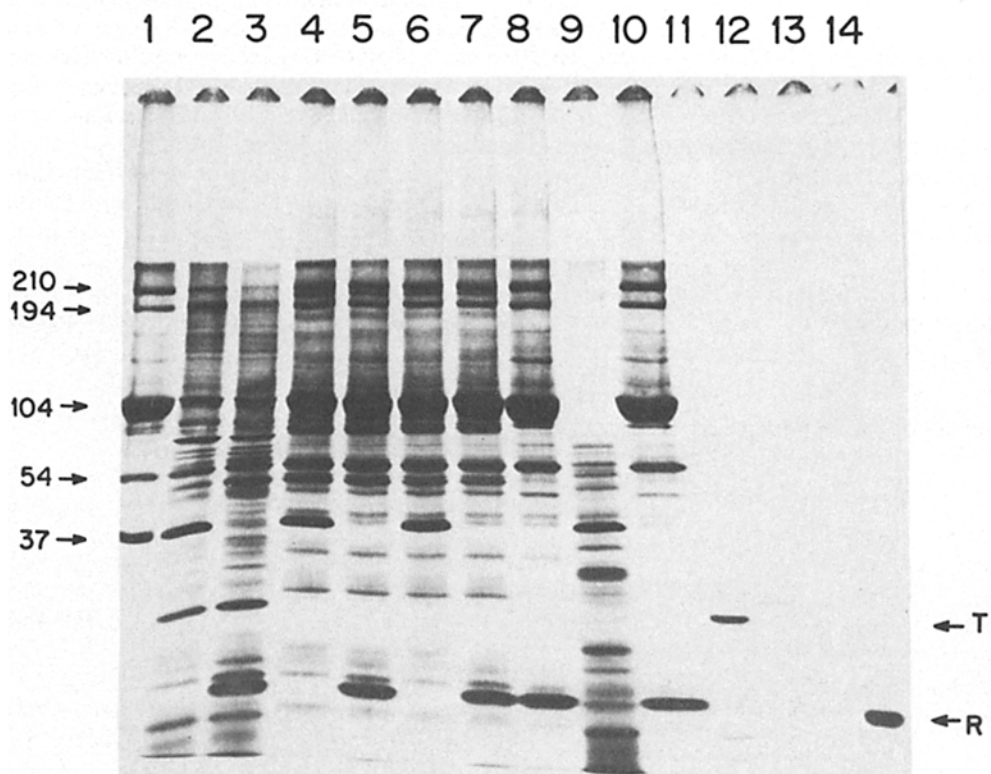
Figure 7. Digestion of vaults with proteases and RNase A. 3-μg aliquots of either RNase A–pretreated (100 ng, 1 h) or untreated native vaults were digested with 200 ng of one of the following: trypsin, V-8 protease, α-chymotrypsin, or proteinase K, for 150 min at 25°C. Samples were then treated with SDS and subjected to SDS PAGE. The gel was stained with silver. Samples in lanes *3, 5, 7, 8, 10* were treated with RNase. Lane *1,* control; lanes *2* and *3,* trypsin digests; lanes *4* and *5,* V-8 protease digests; lanes *6* and *7,* α-chymotrypsin digests; lane *9,* protease K digests. Lanes *11–13* show the elution positions of the proteases trypsin, V-8 protease, α-chymotrypsin, protease K, respectively. Lane *14* contains RNase. The molecular masses of the major vault polypeptides are indicated ×10⁻³; the migration positions of trypsin (*T*) and RNase A (*R*) are indicated by arrows.
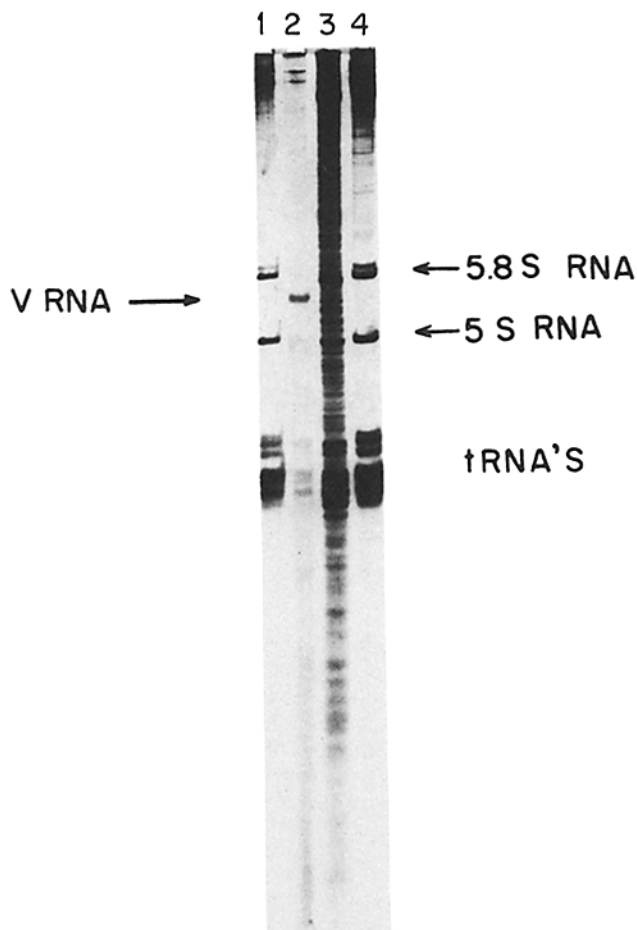
Figure 8. Urea gel of RNA showing size relative to known standards. Lanes *1* and *4* are poly A⁻ RNA standards; lane *2*, phenol-extracted vaults; lane *3*, phenol-extracted crude rat microsomes.

In addition to the major RNA species, trace amounts of species with mobilities similar to the tRNAs were observed in phenol-extracted vaults. These minor bands were consistently observed in several preparations. Although large amounts of tRNAs might be expected to contaminate any species purified from a postnuclear extract, the purification procedure used here includes four pelleting steps, one 16-h isopycnic ultracentrifugation, and agarose gel electrophoresis. It is possible that these minor RNA species interact with the native vaults and are thereby co-purified.

Western Blot Analysis Reveals That Vaults Are Unrelated to CVs

Antibodies initially raised against purified vaults (B3 antisera) appeared to react largely with the major vault polypeptide (M_r 104,000) as shown by Western blot analysis (Fig. 9 *C*, lane *2*), however, after 5 mo of repeated booster injections of vaults, an antisera was obtained (B7) which showed some reactivity to all of the vault polypeptides with the exception of the M_r 210,000 species (Fig. 9 *A*, lane *1*). Although mobility of the M_r 104,000 polypeptide appeared similar to that of the larger of the coated vesicle–associated $M_r \sim 100,000$ polypeptides in SDS PAGE (Fig. 5 and Fig. 9), no cross-reactivity between the two polypeptides was observed (Fig. 9 *C*, lanes *4* and *6*) indicating that the major vault protein is not identical to the CV peptide of similar mobility. Neither antibody showed reactivity with the M_r 37,000 vault RNA species.

Anti–vault Antibodies Precipitate Vault 5.6S RNA

Although no direct antibody reactivity was observed against the 5.6S RNA by Western blot analysis (Fig. 9 *A*, lane *1*), immunoprecipitation of Ficoll-purified microsomes revealed that the 5.6S RNA was immunoprecipitated by the vault antibody in addition to the vault polypeptides, indicating that the 5.6S RNA is tightly associated with the vault proteins (Fig. 10).

To determine if the vault RNA was degraded during vault purification, we examined the size of labeled vault RNA immunoprecipitated from cultured cells. H4 cells were labeled with [³²P]phosphate for 16 h, and homogenized in ice cold MES buffer in the presence of RNase inhibitor to prevent any potential degradation of the vault RNA during the subsequent procedures. The homogenate was centrifuged to remove nuclei, mitochondria, and lysosomes, and the supernatant was immunoprecipitated and phenol extracted as described (see Materials and Methods). When subjected to urea PAGE, a single major ³²P-labeled species was detected which co-migrated with the 5.6S vault RNA standard (Fig. 11). The presence of a small amount of 5.8S RNA may represent ribosomal RNA contamination; whether this is due to a possible vault–ribosome interaction in vivo is not clear.

A minor species of RNA which co-migrates with the immunoprecipitated vault RNA is apparent in both the initial cell supernatant (Fig. 11, lane *1μs*) and the parallel aliquot of cell supernatant which was incubated for the duration of the immunoprecipitation (Fig. 11, lane *2μs*). No appreciable

Table I. Nucleoside Composition of Vault RNA (Molar Ratios)

	Adenosine	Guanosine	Uridine	Cytidine	Reference
Vault RNA (rat)	12.0	29.7	30.9	27.4	This study
U8 RNA (rat)	24.3	22.9	31.4	21.4	21
5S RNA (rat)	18.3	30.3	29.0	22.3	1
5.8S RNA (rat)	21.2	29.5	20.5	28.8	26
Control rRNA (50S subunit, *E. coli*)	22.6	31.3	24.2	21.9	This study
rRNA (50S subunit, *E. coli*)	26.1	31.8	20.1	22.1	4, 8*

Agarose gel–purified vaults were treated with 0.3 M KOH for 12 h at 37°C, and the protein precipitated by the addition of HClO₄ to obtain a final concentration of 4%. After centrifugation at 20,000 *g* for 30 min, the supernatant was treated with alkaline phosphatase (5) and analyzed by high performance liquid chromatography using a Supelco C18 reverse-phase column and a nonlinear gradient of acetonitrile in 0.25 M ammonium acetate (pH 6) at room temperature essentially as described by Buck et al. (5). A sample of *E. coli* ribosomal 50S subunit was hydrolyzed in parallel to serve as a positive control.
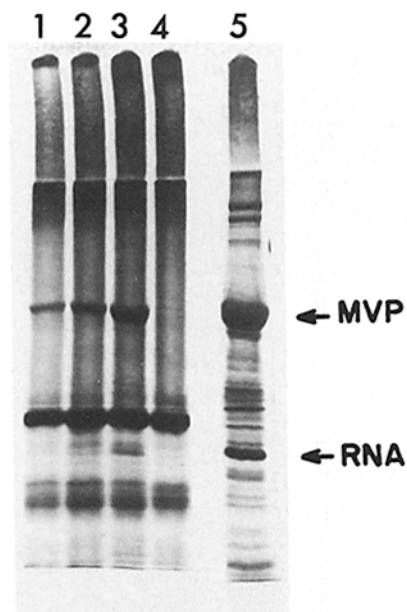* Calculated from the published sequences of 23S and 5S *E. coli* RNA.
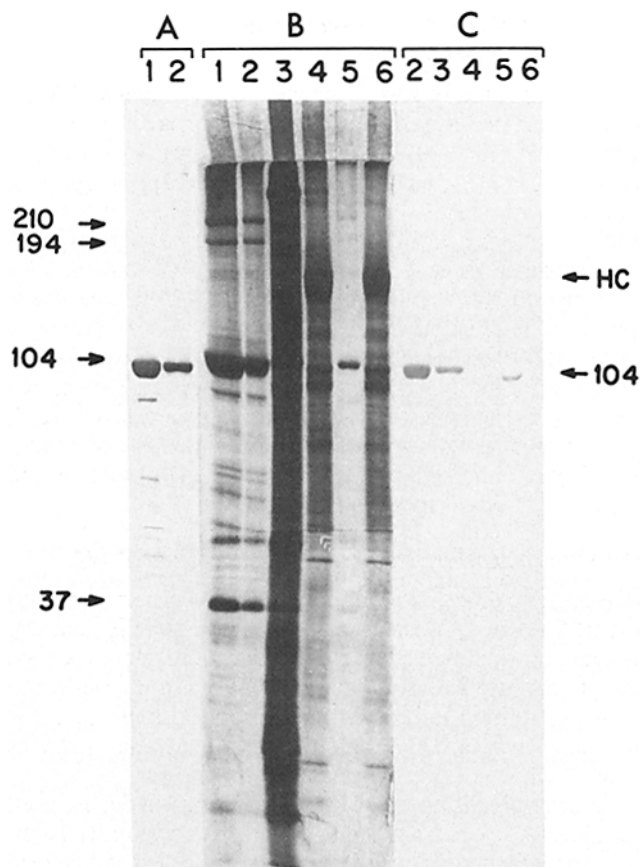
Figure 9. Western blot analysis using anti-vault antibodies. (*A*) Western blot analysis using anti-vault B7 antisera. Lane *1*, 4 μg purified vaults; lane *2*, 1 μg purified vaults. (*B*) Silver-stained SDS PAGE of same gel shown in other panels. Lane *1*, 4 μg purified vaults; lane *2*, 1 μg purified vaults; lane *3*, Ficoll-purified microsomes; lanes *4* and *6*, 4 μg coated vesicles; lane *5*, 200 ng purified vaults. (*C*) Western blot of identical samples shown in *B*, stained with anti-vault antisera B3.

Figure 10. SDS PAGE of vault immunoprecipitates. Ficoll-purified microsomes (see text) were incubated with varying amounts of anti-vault serum (B7) or non-immune serum and immunoprecipitated as described in text. Lane *1*, 5 μl anti-vault serum immunoprecipitate; lane *2*, 10 μl anti-vault serum immunoprecipitate; lane *3*, 20 μl anti-vault serum immunoprecipitate; lane *4*, 20 μl non-immune serum immunoprecipitate; lane *5*, vault standard.

differences are seen between the *1μs* and *2μs* samples, indicating that no degradation of RNA occurs during the 3-h immunoprecipitation procedure. This suggests that the 5.6S vault RNA is initially present as a minor cytoplasmic species and is not generated from a larger species during the purification procedure. Furthermore, the vault RNA appears to be tightly bound to the vault proteins in the cell extract.
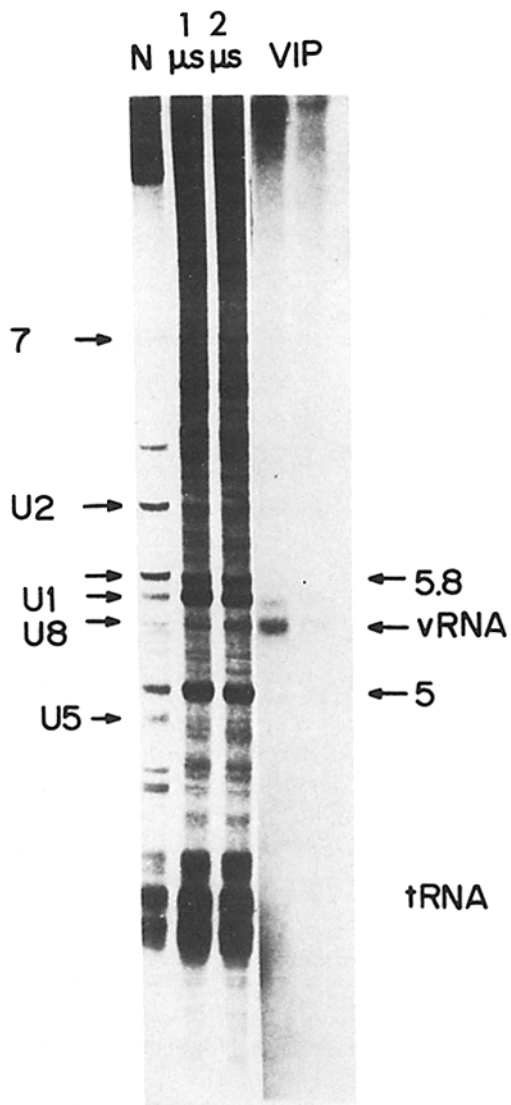
Discussion

The studies presented here describe the purification of a novel subcellular structure of unknown function. These particles, which we refer to as vaults, have a complex morphology resembling the multiple arches which form cathedral vaults. They have been purified from cytoplasmic extracts of rat liver and exhibit a number of unusual properties: their morphology is unique; they contain a single species of 5.6S RNA and no detectable membrane, therefore they constitute a ribonucleoprotein particle of unprecedented size; they possess a small RNA of unusual base composition; and their protein composition is greatly dominated by one species of polypeptide despite their relatively large size. Furthermore, we have found that these structures are present in partially

purified extracts from all cell types examined to date, including such diverse species as rat glia and hepatocytes, human fibroblasts, rabbit alveolar macrophages, as well as murine 3T3 and KNIH cells.

Unlike the protein composition of other ribonucleoprotein particles, the composition of the vaults is dominated by a single polypeptide species (M_r 104,000). This is also unusual considering the relatively large size (35 × 65 nm) of the native vaults. The *Escherichia coli* ribosome, best characterized of the ribonucleoprotein structures, contains three species of RNA in addition to 52 proteins (18); of the protein species, only two are present in multiple copies of four per ribosome (13). Although we have not determined the absolute stoichiometry of the vault proteins, densitometry values obtained from scanning the Coomassie-stained SDS polyacrylamide gel (shown in Fig. 5) allow a molar ratio of 3:1:55:7 to be determined for four major polypeptides of M_r 210,000, 192,000, 104,000, and 54,000, respectively. The M_r 54,000 species is resolved into at least four components by two-dimensional PAGE (Fig. 6). Using these values, and assuming that at least one molecule of the M_r 192,000 species is present per vault, the minimum molecular mass of the native particle (minus the RNA) can be estimated to be 8 × 10⁶ D. The apparent bilateral symmetry of the native vault particle suggests that the actual molecular mass may be twice this value. The extremely high molar frequency of the M_r 104,000 species relative to all other protein constituents is unlike the composition of the ribosome or any other ribonucleoprotein particle. It is, however, strongly reminiscent of coated vesicle composition, where clathrin has been estimated to account for 43% (32) to over 70% (20) of the total protein.

Table II. Stoichiometry between Protein and RNA Components in Vaults

	RNA	Protein
Assay	10.76 μg	221 μg
Moles	234×10^{-12}*	27×10^{-12}‡
Molar ratio	8.7 molecules/vault	6.4 MVPs/RNA

Vaults were treated with KOH and $HClO_4$ as described in the legend to Table I. The RNA content was calculated from the absorbance at 260 nm of the supernatant fraction using an average nucleotide molar extinction coefficient of 10^4 at 260 nm, assuming an average nucleotide molecular mass of 330. Protein content of the vault sample was determined by the method of Lowry et al. (15) using bovine serum albumin as a standard.

* Moles of RNA were calculated based upon our size estimations of vault RNA (140 nucleotides).

‡ Moles of vault particle protein were calculated using densitometry which indicated that 71% of the native vault mass was contributed by the M_r 104,000 protein (MVP) present at a minimum frequency of 55 copies per vault structure (see text).

Figure 11. Urea PAGE autoradiograph of ^{32}P-labeled H4 cell extracts and immunoprecipitated vaults. H4 cells were labeled and homogenized as described in the text. The homogenate was fractionated into nuclear and postnuclear fractions, each of which was immediately phenol extracted and ethanol precipitated. An aliquot of the supernatant was immunoprecipitated with anti–vault antibodies, while another aliquot was incubated in parallel to serve as an indicator of endogenous RNase activities. Lane N, nuclear extract; lane 1 μs, supernatant immediately phenol extracted; lane 2 μs, supernatant phenol extracted after incubation for 3 h at 4°C; VIP lanes, vault immunoprecipitates (two concentrations). vRNA denotes the position of RNA extracted from bulk-purified vaults, which co-migrated with the ^{32}P species obtained from the immunoprecipitate and was visualized by silver stain. The VIP lanes were visualized using a longer exposure (2 d) than the other lanes (2 h).

The relative protein to RNA ratio was determined essentially as described (30) and calculated to be 4.6% by weight (Table II). Using the above molar ratios and the minimum vault mass (8 × 10⁶ D), and assuming a molecular size of 140 bases for the vault RNA, we calculate that there are ∼9 RNA molecules per vault. This corresponds to a molar ratio of 6.4 of the M_r 104,000 peptides per vault RNA molecule.

Signal recognition particle is a well-characterized ribonu-cleoprotein structure which contains six polypeptides present in equimolar amounts (29) in addition to a 7S RNA (30). It is considerably smaller than ribosomes with a sedimentation coefficient of 11S (29) and it appears unlikely that it is related to vaults; although they both contain a species of M_r 54,000, the other proteins are dissimilar in molecular mass and their RNA moieties differ in size. An additional small cytoplasmic ribonucleoprotein, which has been reported to inhibit cell-free protein synthesis (25), also appears unrelated to vaults in terms of both protein and RNA composition.

Vaults were initially observed as contaminants of coated vesicle preparations and were also observed to survive treatment with 2 M urea under conditions which completely remove the clathrin coats from the underlying vesicle. A family of polypeptides with molecular sizes similar to that of the major vault polypeptide have been reported to mediate the interaction between clathrin triskelions and the underlying vesicle (28), thus it seemed possible that the vaults might constitute a coated vesicle nucleation particle. This hypothesis was tested several ways: liver coated vesicles were further purified by S-1000 chromatography (23), which separated the bulk of the coated vesicles from the vaults. The vault-free coated vesicles were treated with 2 M urea and reapplied to the S-1000 column equilibrated with 2 M urea. No species of M_r 104,000 were found to elute from the column at the position expected for vault particles. Numerous attempts were made to detect an underlying membrane within the vaults. After proteinase K treatment, no vesicle was observed by electron microscopy using conditions which clearly showed the vesicular components of proteinase K-treated coated vesicles. No phospholipids were detected in vaults using silver stain and SDS PAGE, although they were readily detected in coated vesicles. Finally, the results presented in Fig. 11 demonstrated that the M_r 104,000 vault protein is immunochemically distinct from coated vesicle polypeptides of similar mass. We must, therefore, conclude that the vaults are unrelated to coated vesicles, as indeed their ribonucleoprotein nature suggests.

These studies raise a number of immediate questions. How could such relatively large structures escape morphological detection to date? Given their superficial resemblance to coated vesicles by electron microscopy using negative stain, thin section views of vaults might resemble tangential (mem-

braneless) views of coated vesicles. Small structures such as ribosomes are easily detected in the electron microscope due to their great numbers and to the ordered nature of their distribution along the rough endoplasmic reticulum; were they present in much lower frequency and more randomly distributed, they would not be as readily apparent. Another important question is the function of vaults in vivo. Their large size suggests complex function, and their RNA content suggests interaction with other nucleic acids. To address these questions, we are using the antibodies we have raised against the vault proteins to investigate their subcellular localization. Immunological and structural studies on these novel particles are presently in progress.

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