Reconstitution of Nucleoprotein Complexes with Mammalian Heterogeneous Nuclear Ribonucleoprotein (hnRNP) Core Proteins

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ABSTRACT   Newly transcribed heterogeneous nuclear RNA (hnRNA) in the eucaryote cell nucleus is bound by proteins, giving rise to large ribonucleoprotein (RNP) fibrils with an inherent substructure consisting largely of relatively homogeneous ~20-nm 30S particles, which contain core polypeptides of 34,000–38,000 mol wt. To determine whether this group of proteins was sufficient for the assembly of the native beaded nucleoprotein structure, we dissociated 30S hnRNP purified from mouse ascites cells into their component proteins and RNA by treatment with the ionic detergent sodium deoxycholate and then reconstituted this complex by addition of Triton X-100 to sequester the deoxycholate. Dissociation and reassembly were assayed by sucrose gradient centrifugation, monitoring UV absorbance, protein composition, and radiolabeled nucleic acid, and by electron microscopy. Endogenous RNA was digested and reassembly of RNP complexes carried out with equivalent amounts of exogenous RNA or single-stranded DNA. These complexes are composed exclusively of groups of *n* 30S subunits, as determined by sucrose gradient and electron microscope analysis, where *n* is the length of the added nucleic acid divided by the length of nucleic acid bound by one native 30S complex (about 1,000 nucleotides). When the nucleic acid: protein stoichiometry in the reconstitution mixture was varied, only complexes composed of 30S subunits were formed; excess protein or nucleic acid remained unbound. These results strongly suggest that core proteins determine the basic structural properties of 30S subunits and hence of hnRNP. In vitro construction of RNP complexes using model nucleic acid molecules should prove useful to the further study of the processing of mRNA.

Ultrastructural and biochemical lines of investigation have independently shown that heterogeneous nuclear RNA (hnRNA) is complexed with proteins throughout its existence in the cell nucleus (reviewed in reference 20). In electron micrographs of transcriptional complexes spread from lysed nuclei, hnRNA-protein complexes (hnRNP) frequently appear as fibrils consisting of 20-nm beads connected by RNA strands (1, 2, 21, 23). The biochemical entity isolated from purified nuclei by either isotonic extraction or mechanical disruption, referred to by us as "30S RNP" since it usually sediments more slowly than the 40S ribosomal subunit, is 18–25 nm in diameter and contains 700–1,000 nucleotides of rapidly labeled RNA and 40–60 copies of a group of four to six proteins of 34,000–40,000 mol wt with similar amino acid compositions and isoelectric points (pI = 8.0–9.0) (3, 5, 8, 11, 17). These proteins are highly conserved throughout the ver-

tebrates and turn over slowly in contrast with many other proteins (17–19). Since they only partially protect hnRNA from nuclease digestion (18), it has been suggested that these proteins form a core around which the nucleic acid is wrapped (30), and hence their designation as "core proteins." This hypothesis is attractive in that it allows for new transcripts to be maintained in a compact yet untangled conformation, to be accessible to processing enzymes, and for mature mRNA to be removed and exported from the nucleus while potentially stable protein cores are recycled.

One difficulty with this model is that, when stripped of nucleic acid in vitro, core protein complexes dissociate unless previously cross-linked (18) or maintained at an extremely high concentration (13). Another problem is the variability in the ratios of the individual core proteins in different preparations (3), as well as the variability of 30S RNP particle size

and morphology within a single preparation (18). In contrast to the notion that a simple set of proteins is largely responsible for the primary folding of hnRNA is the contention, based on limited RNase digestion of large hnRNP, that certain high molecular weight proteins associate directly with the hnRNA moiety and give a conformational stability to polyparticulate RNP which is lost when 30S RNP subcomplexes are purified (32, 33).

In vitro reconstitution experiments with purified core proteins and defined nucleic acid sequences can not only explore the feasibility of a relatively simple model for RNP structure but might also lay the groundwork for biochemical studies of hnRNA processing by providing a way of making nativelike RNP substrates containing a single species of nucleic acid. A basic question is whether the core proteins contain sufficient information by themselves to faithfully assemble protein cores, 30S monomeric RNP and polyparticle complexes in vitro without generating aberrant structures. The products of in vitro assembly therefore should possess the relative stoichiometry of the core proteins, the original RNA:protein ratio and the native quaternary structure.

A useful model for testing this idea is that of Samarina et al. (30) which predicts that, since native 30S RNP contain 700–1,000 nucleotides of RNA per complex, an RNA molecule this size or smaller should assemble into a single 30S sized complex. A larger RNA, or possibly a single-stranded DNA molecule, would form a chain of 30S particles linked by short stretches of nucleic acid with little or no bound protein. When the native protein:nucleic acid ratio is preserved, the number of 30S particles per complex should approximately equal the length of added nucleic acid divided by the length bound per 30S RNP in the absence of cooperative binding. A lower protein:nucleic acid ratio should result in fewer 30S particles along the length of the nucleic acid molecule.

These experiments require a method for reversible dissociation of native RNP. Possible choices of reagents which dissociate hnRNP without denaturation include high concentrations of monovalent cations (13, 29), low concentrations of the ionic detergent sodium deoxycholate (DOC) (1, 7, 24), and extensive RNase treatment (32). We have tested these three reagents and have found that DOC dissociation is the easiest to reverse without causing protein denaturation. To accomplish this, we used Triton X-100, a nonionic detergent which forms mixed micelles with an ionic detergent, causing a rapid reduction in its effective concentration. In this paper we use in vitro reconstitution as a model for polyparticle assembly and examine the effects of varying the nucleic acid:core protein stoichiometry and the polynucleotide size and sequence on the structure of the reconstituted hnRNP.

MATERIALS AND METHODS

Purification of 30S RNP: 30S RNP were prepared from mouse taper liver tumor (TLT) cell nuclei as previously described (16, 17). Briefly, TLT cells were grown in ascites form in the peritoneal cavity of Swiss Webster mice for 5–7 d, harvested, and swollen by two cycles of low speed centrifugation and resuspension in water at 0°C. The cells were then pelleted at low speed, rapidly transferred to a Dounce homogenizer using approximately 10 times the pellet volume of 0.25 M sucrose and one-tenth TKM buffer (TKM buffer is 80 mM KCl, 50 mM Tris, 15 mM MgCl₂, pH 7.6), and the cells were immediately lysed by three to five strokes with a "B" pestle. The nuclei were pelleted at 2,000 rpm for 5 min, washed twice with one-tenth TKM buffer, once with STM7 (STM is 0.1 M NaCl, 10 mM Tris, 1 mM MgCl₂; buffered at pH 7, 8, or 9) buffer, and once with STM9 buffer. The nuclear pellet was then resuspended in a small volume of STM9 buffer and gently shaken in an ice water

bath for 3–4 h. The nuclear extract was separated from the nuclei by low speed centrifugation, and then layered on 10–30% sucrose gradients buffered with STM8 and centrifuged until the 30S RNP peak was one-half to two-thirds down the gradient, well separated from the slowly sedimenting material at the top of the gradient: 14 h at 24,000 rpm in an SW27 rotor; 3 h at 45,000 rpm in an SW50.1 rotor (all rotors of Beckman Instruments, Inc., Palo Alto, CA). The 30S RNP A₂₅₄ peak fraction was then either used directly or was pelleted for 10 h at 45,000 in a 60Ti rotor. The 30S RNP pellet was resuspended on ice over 1–2 h in a volume of STM8 buffer to yield a final concentration of 8–20 A₂₅₄ U/ml.

Preparation of Nucleic Acids: Tobacco mosaic virus (TMV) RNA was prepared from purified virus, grown on tobacco leaves using a stock provided by Dr. R. Haselkorn (University of Chicago), and isolated from infected leaves by the polyethylene glycol method of Gooding and Hebert (9). The ³²P-labeling of TMV RNA was accomplished by incubating a few leaves in a [³²P]phosphate solution for several days. The RNA was extracted from the virus with buffered chloroform/phenol (1:1). ϕX174 DNA was similarly extracted from purified virus (Miles Laboratories, Elkhart, IN). Globin mRNA was chloroform/phenol extracted from a reticulocyte lysate (Pel-Freez, Inc, Rogers, AR) and separated from ribosomal RNAs by oligo(dT)-cellulose column fractionation followed by preparative centrifugation on 5–20% sucrose gradients and isolation of the 7S O.D.₂₅₄ peak. Poly(U) was purchased from P. L. Biochemicals (Milwaukee, WI). Poly(A+) mRNA was prepared from TLT cells as previously described (22). All nucleic acids were assayed for size and purity by sucrose gradient centrifugation, and in some cases by gel electrophoresis, prior to use in experiments described in this paper.

Radiolabeling of hnRNA in 30S RNP: 5- to 6-d-old TLT cells were harvested, washed by low speed centrifugation twice with ice-cold Earle's balanced salt solution (Gibco Laboratories, Grand Island, NY), and preincubated at 37°C for 5 min in 5 to 10 times the original ascites fluid volume of minimal essential medium with Earle's salts (Gibco Laboratories), supplemented with L-glutamine and total amino acids. A volume of [5-³H]uridine (specific activity 25 Ci/mmol) (ICN Radiochemicals, Irvine, CA) was added to make a final concentration of 50–100 μCi/ml and the incubation continued at 37°C for 15–25 min. The incubation flask was then plunged into an ice water bath briefly, and the cells were washed several times with Earle's balanced salt solution before proceeding to the 30S RNP extraction procedure.

Analytical Sucrose Gradient Ultracentrifugation and Fractionation: Samples containing 0.16 O.D.₂₆₀ U of 30S RNP were layered on 5 ml of STM8 buffered sucrose gradients, usually 10–30% wt/vol. Occasionally, phosphate buffer was used instead of Tris-HCl with no change in sedimentation profiles of either glutaraldehyde-fixed or unfixed specimens. Gradients were centrifuged at 45,000 rpm in Beckamn SW50.1 rotors for times generally ranging from 1 to 3 h. Gradients were fractionated with an ISCO Model D gradient pump through a 1-cm path length UV flow cell and the O.D.₂₅₄ was continuously recorded as a function of distance along the gradient. For analyzing protein composition or counting radioactivity, gradients were collected in 16 fractions after being pumped through the UV flow cell. Fractions were mixed with 1 vol of 10% (vol/vol) trichloroacetic acid (TCA), precipitated overnight at −0°C, collected, and washed with 5% TCA on Whatman glass filter disks (Whatman Laboratory Products Inc., Clifton, NJ), dried, and counted in Liquifluor-toluene. When total counts were desired, samples were mixed directly with 10 ml of Triton X-100 Liquifluor and counted.

Protein Gels.: A standard 10% polyacrylamide, 0.1% SDS slab gel (15) was used to analyze protein composition. Samples were denatured with 2 vol of ethanol without predigestion of nucleic acid, precipitated overnight at −20°C, pelleted at low speed, and resuspended in standard denaturation buffer (50 mM Tris, pH 7.0, 2% SDS, 5% 2-mercaptoethanol). Protein bands in gels were stained either with Coomassie Blue or with silver (27).

Electron microscopy: All samples were fixed in solution with 0.01 M phosphate-buffered 1% glutaraldehyde prior to deposition on carbon-coated formvar grids rendered hydrophilic by glow discharge. Dilution of amine-containing buffers (e.g., Tris) with phosphate buffer was sufficient to prevent artifacts from Schiff base cross-linking with glutaraldehyde. Specimens on the grid were rinsed several times with double-distilled water and stained with 1% uranyl acetate. A Siemens 101 electron microscope operated at 80 kV with a 100-μm objective aperture was used to examine and photograph specimens.

RESULTS

Dissociation and Reconstitution of 30S RNP

All the experiments described here use 30S RNP purified from a nuclear extract of TLT ascites cells as described in Materials and Methods. The O.D.₂₅₄ profile of a sucrose

gradient analysis of purified 30S RNP shows a discrete peak centered at about 35S, nearly symmetric but with a slowly sedimenting tail (Fig. 1A). Acid-precipitable [³H]uridine counts (Fig. 1A) and SDS PAGE analysis of gradient fractions (Fig. 1E) show that the rapidly labeled RNA co-sediments with this peak, as do most of the proteins, which are seen to fall almost exclusively in the molecular weight range corresponding to core proteins, and comprise six species with molecular weights between 34,000 and 40,000. A small proportion of the 34,000- and 35,000-dalton proteins are sometimes preferentially lost from the RNP complex, as seen here.
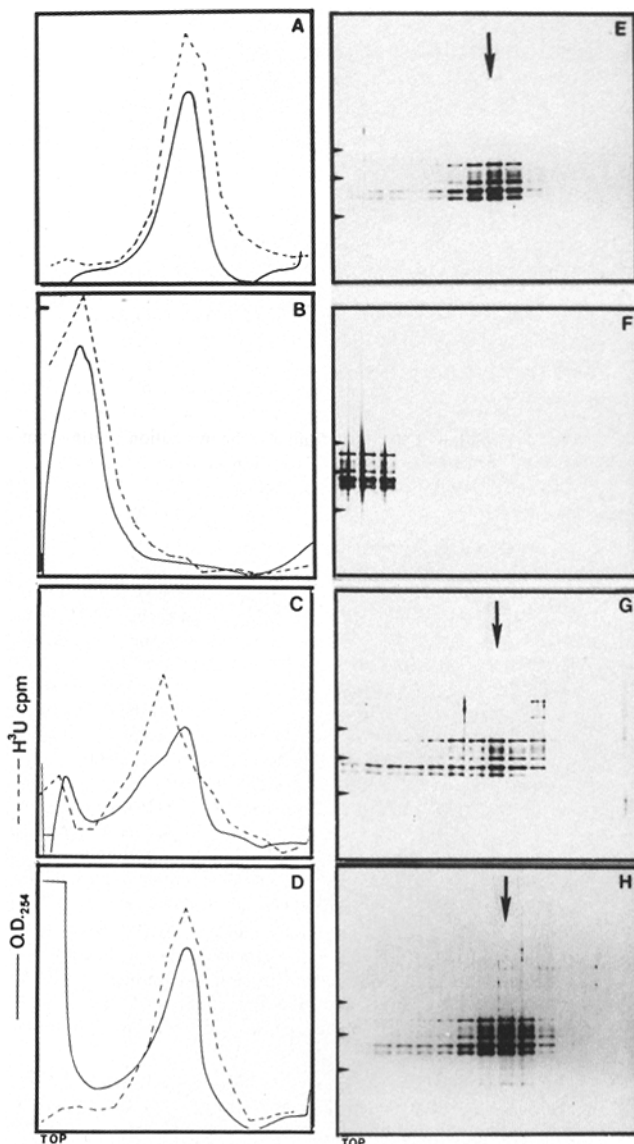

FIGURE 1   Sucrose gradient analysis of deoxycholate dissociation and Triton X-100 reconstitution of 30S RNP. (A–D) O.D.$_{254}$ profiles, 0.2 O.D. full scale, and acid precipitable ³H counts, 10,000 cpm full scale, of 16 fractions across the gradient. (E–G) SDS PAGE analysis of the same fractions shown to the right. Arrows indicate relative positions of the corresponding O.D.$_{254}$ peaks. Arrows indicate molecular weight markers: carbonic anhydrase (29,500), glyceraldehyde 3-phosphate dehydrogenase (36,000), ovalbumin (45, 000). (A and E) 30S RNP control (B and F) 30S RNP + 0.5% DOC, 0.2% DOC in gradient buffer (C and G) 30S RNP + 0.5% DOC, no DOC in gradient buffer (D and H) 30S RNP + 0.5% DOC + 1.0% Triton X-100.

If 0.5% DOC (Sigma Chemical Co., St. Louis, MO) is included in the gradient buffer, the O.D.$_{254}$ peak shifts to the top of the gradient along with the RNA and proteins (Fig. 1, b and f). Inclusion of 0.5% DOC in the sample layered on top of the gradient, but not in the gradient itself results in a partial shift of both protein and RNA peaks towards the top of the gradient (Fig. 1, C and G). This suggests that 30S RNP undergo dissociation into slowly sedimenting species in the presence of DOC, an effect that can be partially reversed by centrifugation of DOC-treated 30S RNP into a gradient effectively lacking the detergent. Again, it is the 34,000- and 35,000-dalton proteins that re-bind least tightly to the complex. Dissociation can be completely reversed by the addition of Triton X-100 to a final concentration equivalent to that of the DOC. Such a reconstitution results in the formation of an RNP complex identical to the native complex in its sedimentation profile, content of rapidly labeled RNA, and protein composition (Fig. 1, D and H). The Triton X-100 most likely partitions the DOC into mixed micelles, which sediment slowly and remain near the top of the gradient (where the absorbance peak of the Triton X-100 can be seen).

Reconstitution from high salt has several disadvantages in comparison to the DOC/Triton X-100 method. Lowering the ionic strength by dialysis takes many hours, during which time core proteins may be denatured or cross-linked (13). To circumvent this problem, we performed reconstitution from high salt by rapid dilution or by sedimentation into a low-salt containing sucrose gradient (data not shown). However, sample dilution makes it difficult to layer an adequate amount of reconstituted material on a sucrose gradient for further study, while performing reconstitution on the gradient makes analysis by techniques other than sedimentation difficult. Therefore, in consideration of its rapidity, convenience, and versatility, we performed most of the reconstitutions described in this paper using the DOC/Triton X-100 method.

To show that dissociation and reconstitution are complete in the absence of centrifugal forces, and that these processes are almost instantaneous upon addition of DOC or Triton X-100, we used electron microscopy to confirm and supplement the results of centrifugation experiments. Furthermore, it can be shown that Triton X-100 reconstituted RNP retain the distinctive appearance of the native complex with respect to average dimensions and the range of observed morphological forms. Glutaraldehyde fixed, uranyl acetate stained 30S RNP from a standard preparation (similar to that in Fig. 1A) are ~22 × 25 nm and range in shape from ovoid to rectilinear (Fig. 2A), with the latter forms (indicated by arrows) being the most striking and characteristic of nuclear RNP (18).

Treatment of a sample of the same 30S RNP preparation with 0.5% DOC prior to fixation and staining results in the disappearance of most 25-nm particles (Fig. 2B). Instead the appearance is suggestive of a layer of small, poorly contrasting objects near the limit of resolution of the negative staining technique, ~2–4 nm. Dilution of the DOC-treated sample with DOC-containing buffer confirms this impression since discrete particles 3–8 nm in diameter are now visible (Fig. 2C). These probably correspond to material in the slowly sedimenting peaks in dissociating gradients (Fig. 1B), and represent single proteins or small protein aggregates. The effect of DOC on particle adsorption to the electron microscope (EM) substrate film and on negative staining is negligible, as is shown by the presence of a few remaining intact particles (Fig. 2B), and by the result of reversing the order of
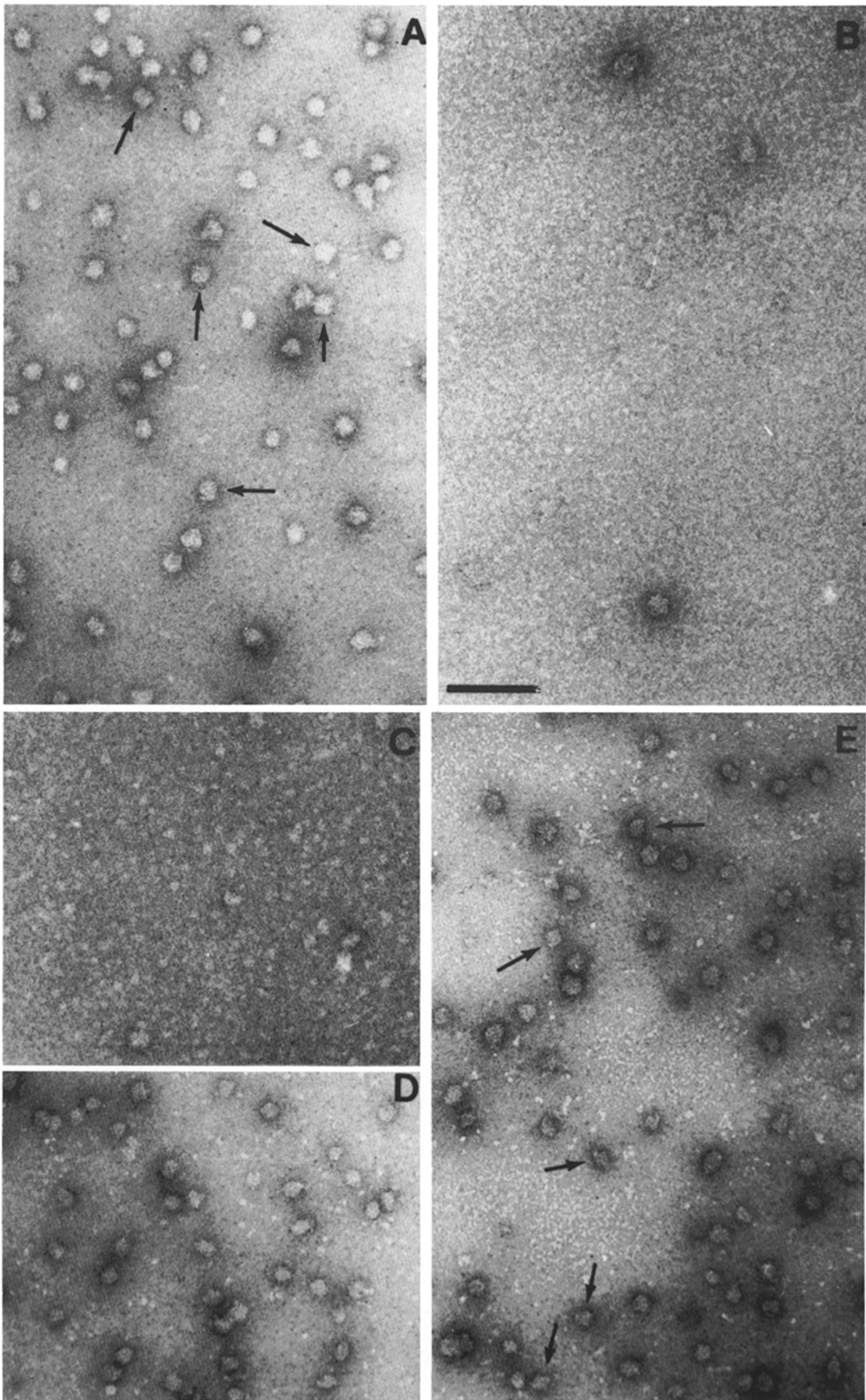
fixation and DOC addition (Fig. 2d). Addition of Triton X-100 to DOC dissociated 30S RNP results in the reappearance of 25-nm particles (Fig. 2e) which are similar in their morphology and distribution on the EM substrate to native 30S RNP. The subset of rectilinear forms (some indicated by arrows) has also reappeared in approximately the same frequency as before dissociation.

Digestion of Endogenous hnRNA

Single-stranded nucleic acids can replace native hnRNA in 30S RNP complexes reconstituted by procedures similar to those just described. However, in addition to dissociation, it is desirable to remove endogenous hnRNA prior to reconstitution. This is most readily accomplished by nuclease treatment of 30S RNP, either preceded or followed by DOC dissociation. Nuclease treatment by itself causes a variable degree of dissociation (33) (see also section on protein composition below and Fig. 8A).

However, without high salt or DOC to stabilize them in the dissociated state by charge neutralization, we find that core proteins sometimes denature; whether they remain dissociated or reaggregate nonspecifically and irreversibly may then depend on uncontrollable factors such as trace contaminants. We, therefore, used DOC in conjunction with nuclease to insure complete dissociation without denaturation.

The choice of nuclease used is important to avoid digesting the exogenous nucleic acid that will be added to the dissociated protein. For the bulk of our studies, which use various exogenous RNAs, micrococcal nuclease (Boeringer-Mannheim, Indianapolis, IN) at a final concentration of 300 U/ml in the presence of 1 mM $CaCl_2$ will digest 80–90% of the endogenous RNA in 20 min at 0°C and can be inactivated by the addition of EGTA (10 mM). We also performed some experiments with the circular single-stranded DNA from phage ϕ174. This DNA behaves no different from RNA in reconstitution experiments but is not degraded by the "endogenous" ribonuclease activity often found as a contaminant in 30S preparations. For some of these experiments involving DNA, pancreatic ribonuclease (Sigma Chemical Co.) at a concentration of 100 μg/ml is used instead of micrococcal nuclease.

Reconstitution of Nucleoprotein Complexes with Defined Species of Nucleic Acids

The DOC/Triton X-100 reconstitutions with nuclease pre-digestion have been performed as described above using a variety of single stranded nucleic acids, including TMV RNA (6,400 nucleotides), a mixture of rabbit α and β globin mRNAs (580 and 620 nucleotides), and ϕX174 DNA (5,500 nucleotides). In addition we have used poly(U) in the size range 5S–12S. To avoid degradation by endogenous nuclease, we sometimes fixed the complexes with glutaraldehyde prior to running on gradients. To reconstitute these species as single size classes of mono- or polyparticles, we maintained the native RNA:protein ratio of approximately 1,000 nucleotides per 30S RNP protein core by adding an amount of exogenous

RNA or DNA equimolar in total nucleotides to the endogenous hnRNA it is replacing. The latter number has been calculated from the $O.D._{254}$ of the 30S RNP preparation prior to nuclease digestion. The protein component contributes very little to the absorption at this wavelength, as is shown by the 80% reduction in the height of the 30S RNP absorbance peak in a gradient profile where the 30S RNP were first cross-linked and then RNase digested (data not shown). Under these stoichiometric conditions, TMV RNP should give an average of 6.4 30S-sized particles per complex, ϕ174 DNP (DNA protein complex) 5.5 particles per complex, globin RNP 1 particle per complex, and poly(U)NP one particle per complex. These predictions have been tested by electron microscopy and sucrose gradient analysis.

Electron microscopy of total reaction mixtures prior to running on gradients as well as of gradient fractions demonstrate the polyparticle nature of TMV RNP and ϕ174 DNP (Fig. 3,a and b). The micrographs show that the subunits comprising both types of polyparticle are grossly similar to native 30S RNP in their morphology and overall size range, although there are more subunits near the low end of this size range (20 nm) than the high end (25 nm). Reconstituted globin RNP and poly(U)NP are also quite similar to the native complex and, as predicted, are largely monomers (Fig. 4). For all reconstituted complexes, rectilinear forms (indicated by arrows in Figs. 3 and 4) are at least as prominent as they are in micrographs of native 30S RNP. The boundaries of 30S subunits within polyparticles are sometimes obscured by the tendency of the subunits to abut, overlap or even fuse with their neighbors, and complexes occasionally have a fibrillar appearance. Similar close packing is observed in native hnRNP spread from lysed nuclei (1, 2, 21) and probably reflects the saturation or near saturation of the nucleic acid strand with protein. Linking strands of nucleic acid are rarely visible in the 90% of the complexes in the micrographs in which subunits can be distinguished. The average number of particles per complex is calculated as 5.5 ± 1.3 for TMV RNP and 4.8 ± 1.3 for ϕX174 DNP. These numbers compare reasonably well with the predicted values. Also, histograms of the measured size distributions (Fig. 3, C and D) show that polyparticles are not random aggregates.

Sucrose gradient profiles (Fig. 5A, B, E, and F) show that all species reconstitute as single gaussian peaks identical in half-width to the native 30S RNP peak (Fig. 5, D and G), and corroborate the EM data. The purified nucleic acids all sediment more slowly than the 30S peak (data not shown). The approximate number of particles per complex can be calculated from the positions of the reconstituted peaks relative to the native 30S peak using the formula $S = kM^{2/3}$, where S is the sedimentation coefficient, M is the molecular weight and k is a proportionality constant. The equation should apply to comparison of poly and monoparticles if there is no significant difference in their frictional coefficients, an assumption shown to be valid when it has been applied to polyribosomes (14). Since $M_p = nM_o$, where the subscript p refers to a polyparticle composed of n 30S monomers and the subscript o refers to

Figure 2 Electron micrographs of 30S RNP. Negative staining with 1% uranyl acetate. All samples were fixed with 0.5% glutaraldehyde buffered with 10 mM phosphate, pH 7.5, prior to deposition on the grid and staining. (A) Native 30S RNP; (B) 30S RNP + 0.5% DOC; (C) 30S RNP + 0.5% DOC, tenfold diluted; (D) 30S RNP pre-fixed with 0.5% glutaraldehyde + 0.5% DOC; (E) 30S RNP + 0.5% DOC + 1.0% Triton X-100. Bar, 100 nm. × 160,000.
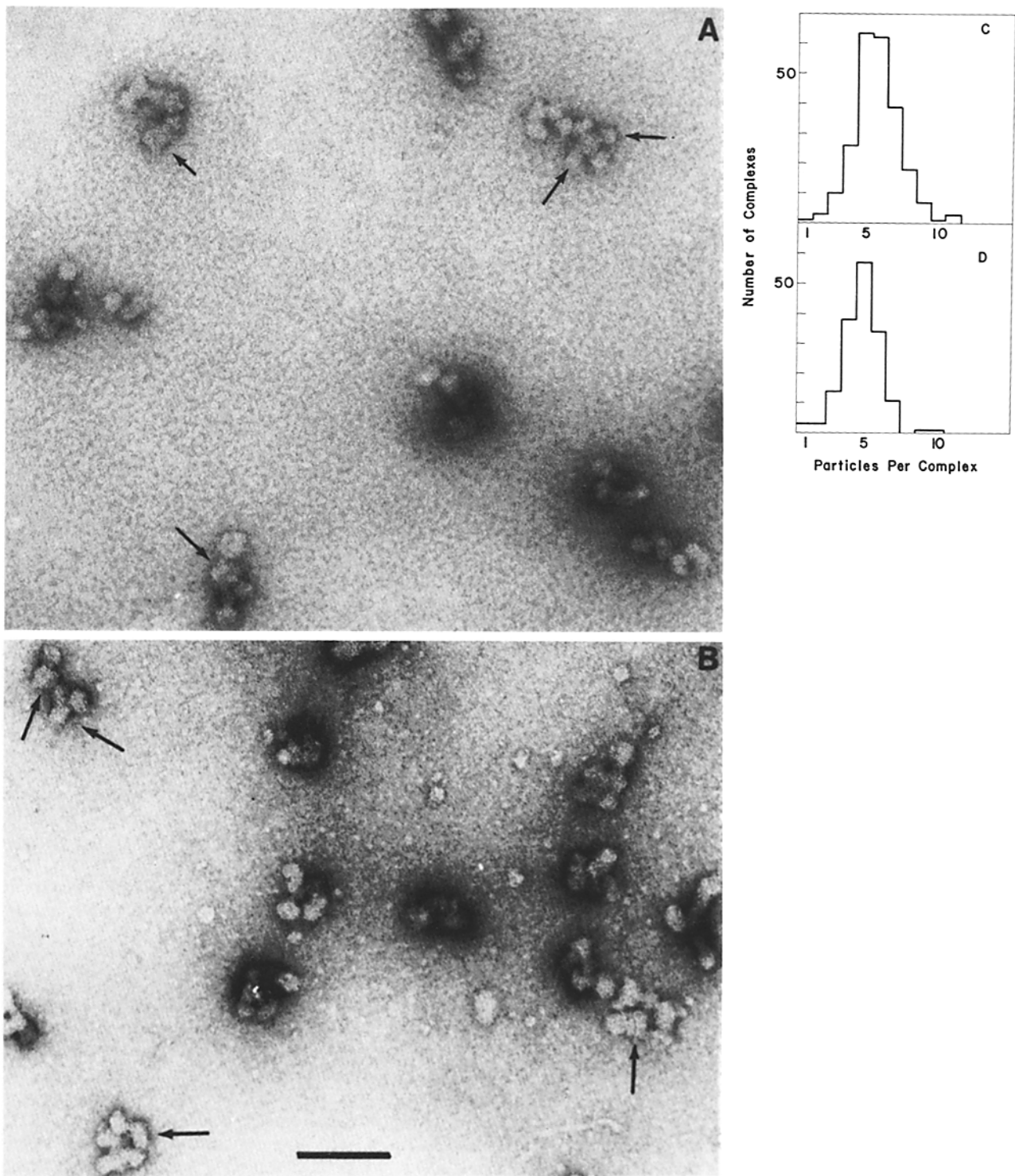
FIGURE 3 Electron micrographs of (A) TMV RNP and (B) φX174 ssDNP nucleoprotein complexes, with histograms of the number of 30S subunits per (C) TMV complex and (D) φX174 complex. Polyparticle samples taken from the peak fractions of gradients similar to those in Fig. 5,a and b were glutaraldehyde fixed and negatively stained with uranyl acetate. Arrows point to rectilinear forms. All complexes in which subunits could be distinguished were counted (~95% of the total from five fields of view for the φX174 ssDNP and 15 fields of view for the TMV RNP). Bar, 100 nm. × 160,000.

the 30S monomer, then it follows that $n = (S_p/S_o)^{3/2}$. Assuming that the 10–30% sucrose gradients used in the SW50.1 rotor are approximately isokinetic, then S_p/S_o is the ratio of the relative distances of migration of the two peaks. This gives values of n as 5.6 for TMV RNP, 4.9 for φX174 DNP, and 1.0 for globin RNP and poly(U)NP. That the peak observed

with the φX174 reconstitution does contain polyparticles with 30S sized subunits is shown by a shift of this peaks to the 30S position upon limited nuclease digestion (Fig. 5C). A similar result is obtained with TMV RNA reconstitution (results not shown).

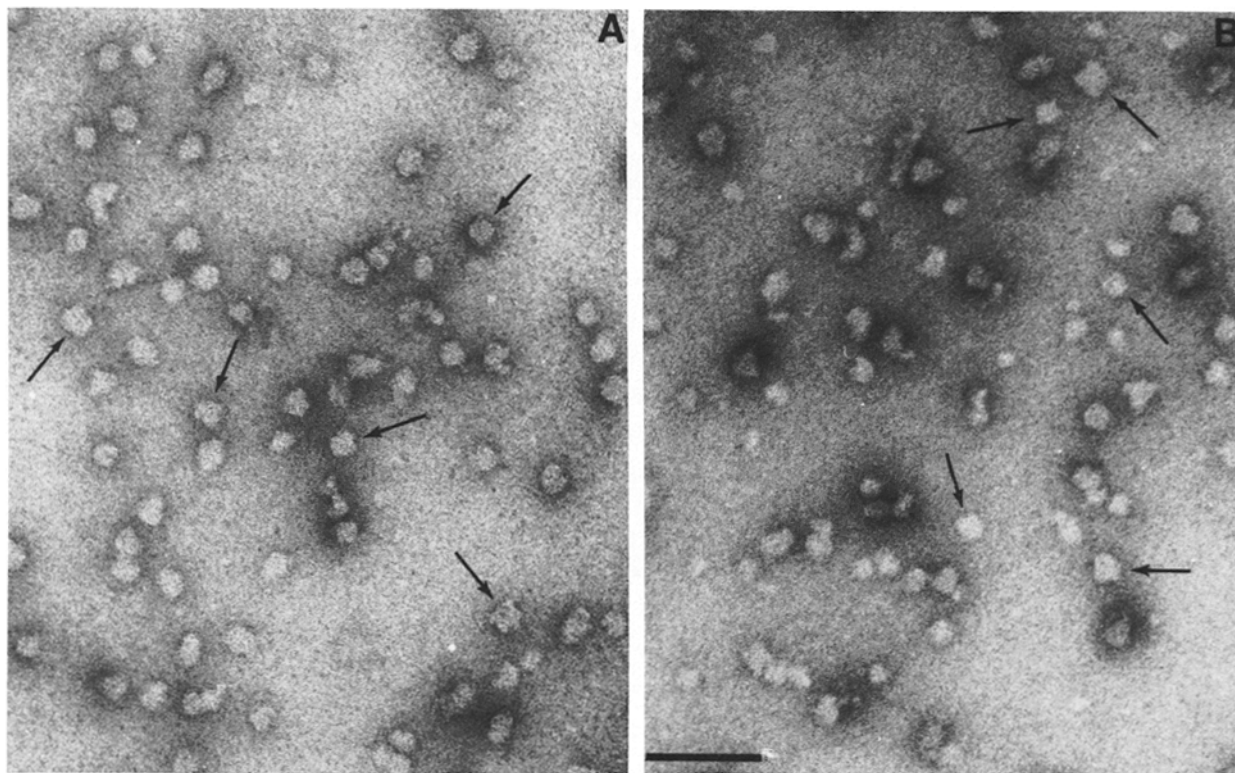The average number of nucleotides per 30S complex cal-

FIGURE 4 Electron micrographs of globin (A) and polyU (B) RNP complexes. Samples from the gradient peaks in Fig. 5, e and f were glutaraldehyde fixed and negatively stained with 1% uranyl acetate. Arrows indicate rectilinear forms. Bar, 100 nm. × 160,000.

culated from either the sucrose gradient or the EM data is 1,150 for TMV RNA complexes and 1,140 for ϕX174 DNA complexes. That essentially the same result is obtained from reconstitutions using two types of nucleic acid attests to the reproducibility of the method used to calculate the amount of exogenous nucleic acid to be added, as well as to the overall fidelity of reconstitution. The value of 1,150 bases per 30S complex is slightly higher than previous estimates of the average number of nucleotides of hnRNA bound to one 30S complex, which are in the range of 700–1,000 (20), and does allow for the possibility of some RNA linker regions between particles and the presence of folded sequences normally lost from purified 30S RNP (12).

Reconstitution with Total Poly(A⁺)mRNA

Poly(A⁺)mRNA isolated from TLT cytoplasm contains a mixed population of RNA molecules, as shown by the sucrose gradient profile (Fig. 6A). The size of the molecules ranges continuously from 4S to 28S, or about 100–5,000 nucleotides. A similar result is obtained with denaturing (methyl mercury) agarose gels (22). Reconstitution with this RNA performed as described in the previous section yields a heterogenous set of RNP complexes. The sedimentation profile of such a reconstitution resembles that of the purified RNA in its general shape, with the exception of a prominent peak at the position of the native 30S RNP (Fig. 6B). This is most likely due to the fact that mRNA molecules less than 1,000 nucleotides long should reconstitute as 30S complexes and not form any smaller structures. No such quantization is seen with the larger complexes, and the inability to resolve discrete poly-particle peaks is probably due to the limitations of sucrose gradient centrifugation, since limited micrococcal nuclease

digestion of the poly(A⁺)RNP shifts the sedimentation coefficients of all material to the position of native 30S RNP (Fig. 6C), indicating that the poly(A⁺)RNP are composed entirely of 30S subunits. Electron microscopy of gradient fractions (indicated by arrows in Fig. 6B) show that the number of 30S particles per complex increases with the distance of sedimentation as expected (Fig. 7).

The Protein Composition of Complexes Reconstituted with Exogenous Nucleic Acids

The sedimentation and EM experiments described in the previous several sections indicate that reconstituted complexes resemble native 30S RNP in that they are composed of 30S subunits; we found that core proteins are present in the same stoichiometry relative to one another as in native 30S RNP. Fig. 8 shows an O.D.$_{254}$ sedimentation profile and an SDS PAGE analysis of proteins across a sucrose gradient of unfixed ϕX174 DNP from an experiment similar to those described above. The major core proteins migrate with the reconstituted ϕX174 DNP peak and are present in the same ratio as seen in native 30S RNP (Fig. 8D). A similar result is found for the protein composition of TMV RNP (results not shown). As with the native RNP, it is primarily the 34,000 and 35,000-dalton proteins which are most readily lost from the complex and which sediment more slowly than the reconstituted peak. Fig. 8, a and c, is a control reconstitution performed at 0°C with Triton X-100 but without any nucleic acid added after nuclease digestion of native hnRNA, showing that the sedimentation of the proteins in Fig. 8d is dependent on the presence of nucleic acid.
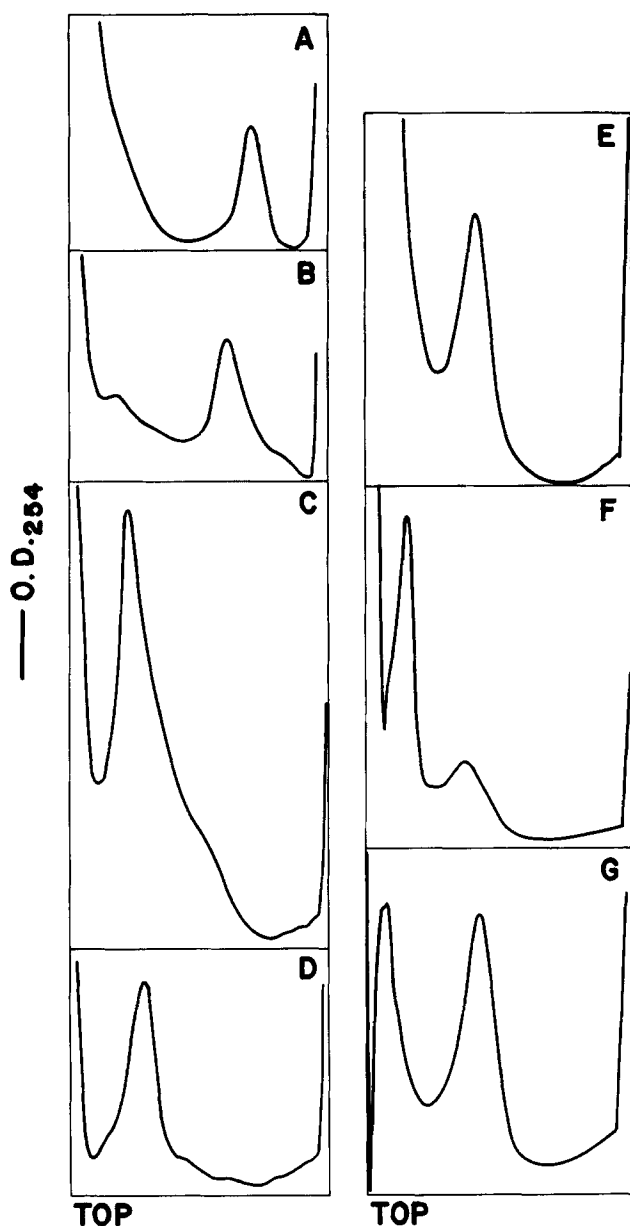
FIGURE 6 Sucrose gradient analysis of RNP complexes reconstituted with TLT cytoplasmic poly(A^+)RNA. (A) Sucrose gradient profile of poly(A^+) RNA, full scale 0.5 O.D.$_{254}$. 0.3 O.D.$_{254}$ U of RNA prepared as described in the Materials and Methods section were layered on 5–20% sucrose Tris, pH 7.5, gradients and centrifuged for 2.5 h at 48,000 rpm on a SW 50.1 rotor. In B and C, samples were prepared from an equimolar amount of poly(A^+)RNA reconstituted with core proteins as described in the text and centrifuged on sucrose gradients for 2 h at 45,000 rpm. (B) poly(A^+)RNA reconstitution, pre-fixed with glutaraldehyde. C is the same as B, but treated with 100 U/ml of micrococcal nuclease for 5 min at 0°C after reconstitution and prior to fixation. 0.2 O.D.$_{254}$ U full scale. Arrows refer to fractions from which samples were taken for the electron micrographs in Fig. 7, a and b.

FIGURE 5 Sucrose gradient analysis of complexes reconstituted with exogenous nucleic acids equimolar in amount to the endogenous hnRNA being replaced. Reconstitution was carried out with (A) TMV RNA; (B) ϕX174 DNA; (C) ϕX174 DNA treated after reconstitution with 1U/ml micrococcal nuclease for 5 min at 0°C; (E) globin mRNA, and (F) poly(U); D and G are native 30S RNP controls. Samples centrifuged on sucrose gradients for either 1 h (A–D) or 2 h (E–G) at 45,000 rpm in an SW50.1 rotor. Full scale on D is 0.2 O.D.$_{254}$ U; other profiles on the same scale.

Variation of the Nucleic Acid-Protein Stoichiometry in Reconstitution with Exogenous Nucleic Acids

In the experiments described above, polyparticles of a defined size could be formed when using TMV RNA or ϕX174 DNA in quantities equivalent to the endogenous RNA they were replacing. If the amount of either of these nucleic acids is decreased (or if the amount of nucleic acid-free core protein is increased) the reconstituted polyparticle does not change either its sedimentation coefficient or the number of particles it contains. The protein-nucleic acid stoichiometry of the
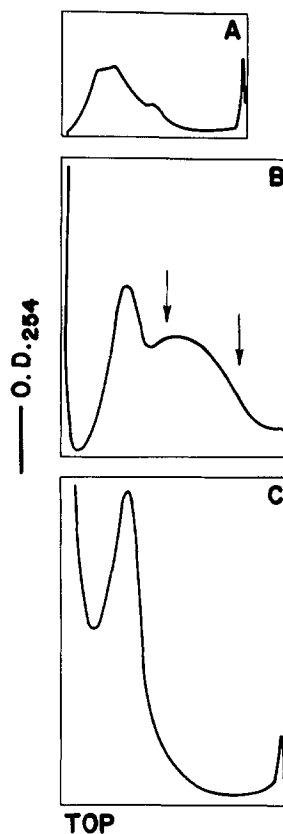
polyparticle does not change, and the excess protein appears at the top of the gradient since it has no nucleic acid to bind. This is illustrated for the case of reconstitution with one-half the equimolar amount of ϕX174 DNA. The ϕX174 DNP peak appears at the same relative position on the sucrose gradient profile (Fig. 9A) as the peak in the equimolar reconstitution (Fig. 8B). Most of the 30S core proteins have not complexed with the ϕX174 DNA and remain at the top of the gradient. Again, it is the lower molecular weight components which are preferentially lost from the reconstituted complex, while the larger polypeptides are included, giving a slightly altered protein stoichiometry relative to equimolar reconstitutions. The total digestion of endogenous hnRNA (as shown by the flat [^{3}H]uridine profile) shows that the core proteins have not reformed 30S RNP complexes. Native complexes must therefore be near saturation with respect to nucleic acid binding, and the average number of nucleotides bound per complex in the ϕX174 DNA or TMV RNA complexes as determined above, 1,140 and 1,150, must be close to the saturation number.

If instead the exogenous TMV RNA or ϕX174 DNA is increased, complexes form which sediment more slowly but still as discrete peaks. This is illustrated for a reconstitution with twice the equimolar amount of ϕX174 DNA (Fig. 9B). The reconstituted peak has sedimented about one and one-half times as far as native 30S RNP. The [^{3}H]uridine profile shows that all endogenous hnRNA has been digested and therefore makes no contribution as a 30S RNP complex to the O.D.$_{254}$ peak.

A similar study with TMV RNA shows that the sedimentation coefficient of the reconstituted complex decreases with the addition of larger amounts of nucleic acid until it approaches the value for native 30S RNP. Reconstitution with
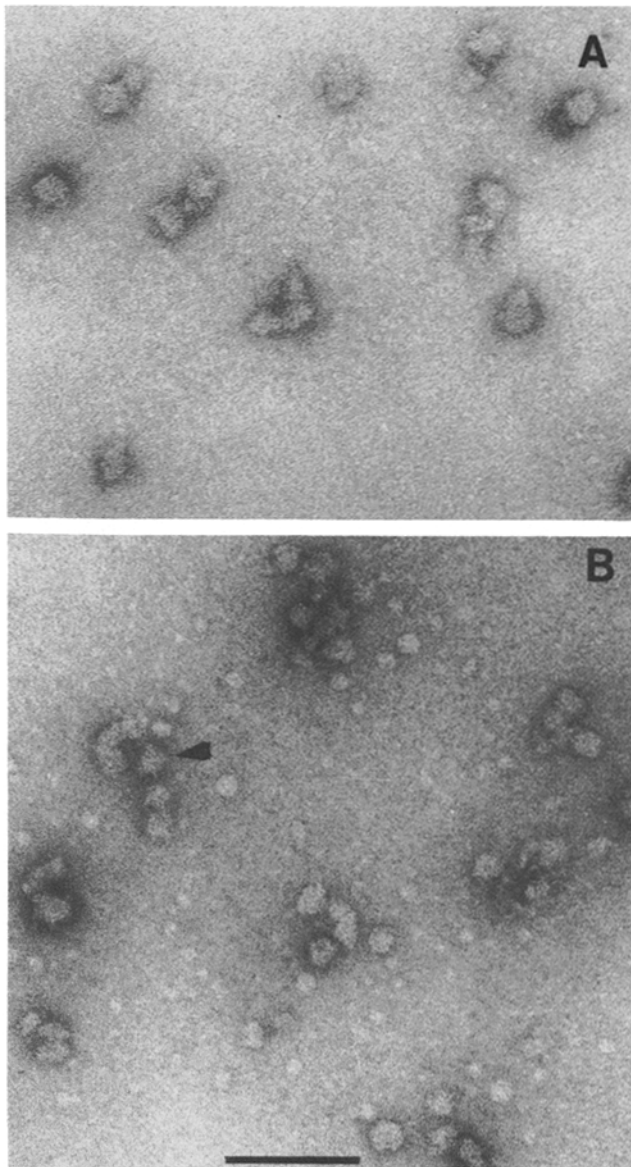
FIGURE 7 Electron micrographs of two different sizes of poly(A⁺)RNP reconstitutions. Samples were taken from the gradient fractions indicated by arrows in A and negatively stained with 1% uranyl acetate. (A) Polyparticles averaging two subunits per complex, with monomer, dimer, and trimer complexes visible. (B) complexes averaging four 30S subunits per complex . The arrowhead points to a complex that is particularly well separated from its neighbors and appears to have a single RNA strand emerging from opposite sides of the particle. Bar, 100 nm. × 180,000.

one, two, three, and four times the equimolar amount of TMV RNA results in complexes sedimenting 3.1 times, 2.5 times, 1.6 times, and 1.3 times as fast respectively as native 30S RNP (Fig. 10, A–D). The ^{32}P-labeled TMV RNA has been used in this experiment to verify the presence of exogenous nucleic acid in the more slowly sedimenting reconstituted complexes. It can be seen that all label migrates with the putative reconstitution peak, and the use of total rather than acid-precipitable counts shows that degradation of TMV RNA is minimal since there are few counts at the top of the gradient.

Electron microscopy of gradient fractions from reconstitutions where single-stranded nucleic acid is in excess shows that O.D.$_{254}$ peaks contain 30S subunits in polyparticle form.

The number of 30S particles per complex decreases as the nucleic acid to core protein ratio is raised and the position of the corresponding gradient peak shifts towards the top of the gradient. Fig. 11 shows two examples of negatively stained TMV polyparticles from peaks sedimenting 1.4 times and 1.6 times as far as native 30S RNP. The number of particles per complex as calculated from the sedimentation velocity is 2.4 and 3.3, respectively, which is approximately equal to the mean number calculated from several micrographs of these two specimens.

Based on these data, it seems that undersaturation of a large nucleic acid with core proteins results in fewer 30S complexes forming along its length, rather than the protein distributing in some other fashion. This suggests that core proteins cannot assemble into any form other than a 30S complex, and will not for example, evenly coat a nucleic acid molecule with protein regardless of the relative stoichiometry as has been observed with nonspecific protein binding to nucleic acids (10). There is little evidence of cooperativity between particles in the assembly of nucleoprotein complexes.

DISCUSSION

In the experiments described above, we used a novel method of dissociation and reconstitution to show than hnRNP core proteins by themselves contain sufficient information to self-assemble with single stranded RNA or DNA into 30S nucleoprotein complexes or polyparticles composed of 30S subunits. This method, which uses DOC to dissociate and Triton X-100 to reconstitute, differs significantly from those previously published in that it does not involve dialysis, large volume changes (such as would be required to rapidly dilute high salt or urea concentrations). Since strong denaturants are not involved the proteins are not subject to irreversible alteration and many enzymes can be used to modify the RNA or protein in the dissociated state.

DOC probably stabilizes core proteins by binding to positively charged amino acid residues that interact with nucleic acid phosphate groups. The hydrophobic interactions of DOC with core proteins are of equal importance since they allow DOC to be an effective dissociating agent at a concentration of 8 mM, far less than the minimum concentration of monovalent salt (0.8 M) that is required for complete dissociation (4).

Reversing the effects of DOC is particularly easy since it dissociates from the proteins in the presence of Triton X-100, with which it forms mixed micelles. As would be expected, 0.5% SDS also dissociates 30S RNP, but its effect cannot readily be reversed by Triton X-100 (results not shown), even though these two detergents do form mixed micelles (28). This suggests that DOC does not bind tightly to core proteins, and only minimally denatures them. The fact that 30S RNP complexes reconstituted from DOC with Triton X-100 regain the sedimentation properties and the EM appearance of native complexes supports this conclusion. By comparison, urea is a reagent which denatures 30S RNP proteins and will dissociate 30S RNP complexes. It can be removed by dialysis, but the resulting complexes sediment differently on sucrose gradients than the starting material (Billings, P., and T. Martin, unpublished results).

Our reconstitution experiments demonstrate several interesting properties of nuclear RNP assembly. First, no high molecular weight proteins are present in the gradient purified 30S RNP that are used as starting material, and the five or
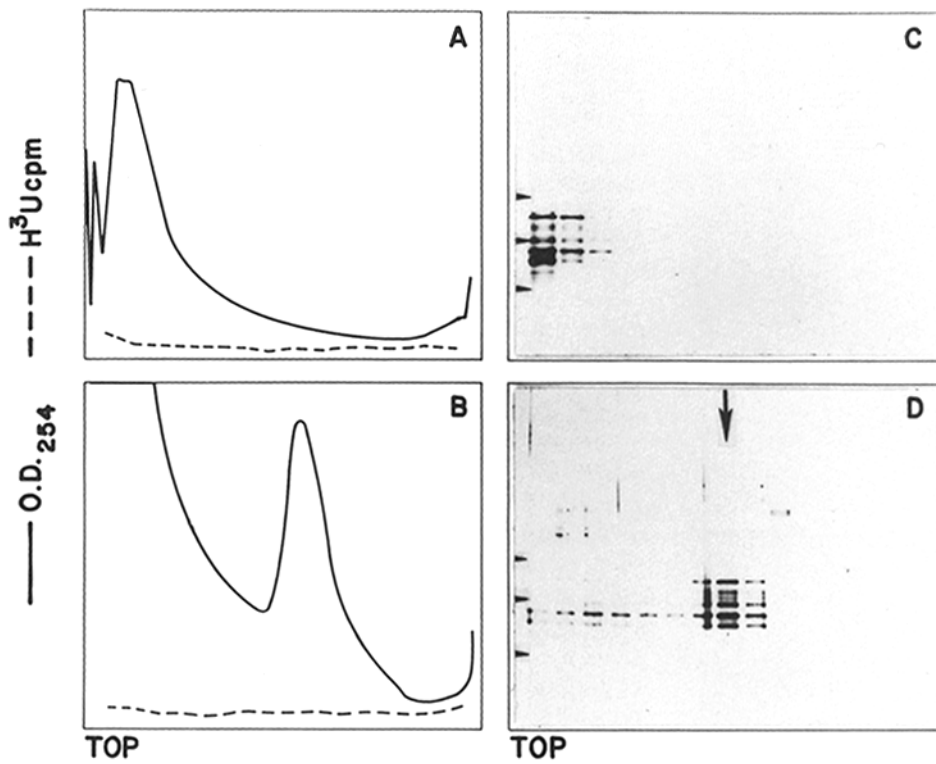
FIGURE 8 Sucrose gradient analysis of the reconstituted φX174 complex to show protein composition. Reconstitution and centrifugation as described for Fig. 5, A and B. 0.2 O.D.₂₅₄U, and 10,000 cpm full scale. Endogenous [³H]uridine-labeled hnRNA and protein analyses are as described in Fig. 2. (A and C) control reconstitution with no added exogenous nucleic acid after nuclease predigestion. (B and D) reconstituted φX174 complexes. Arrow in D indicates relative position of the corresponding O.D.₂₅₄ peak in B.
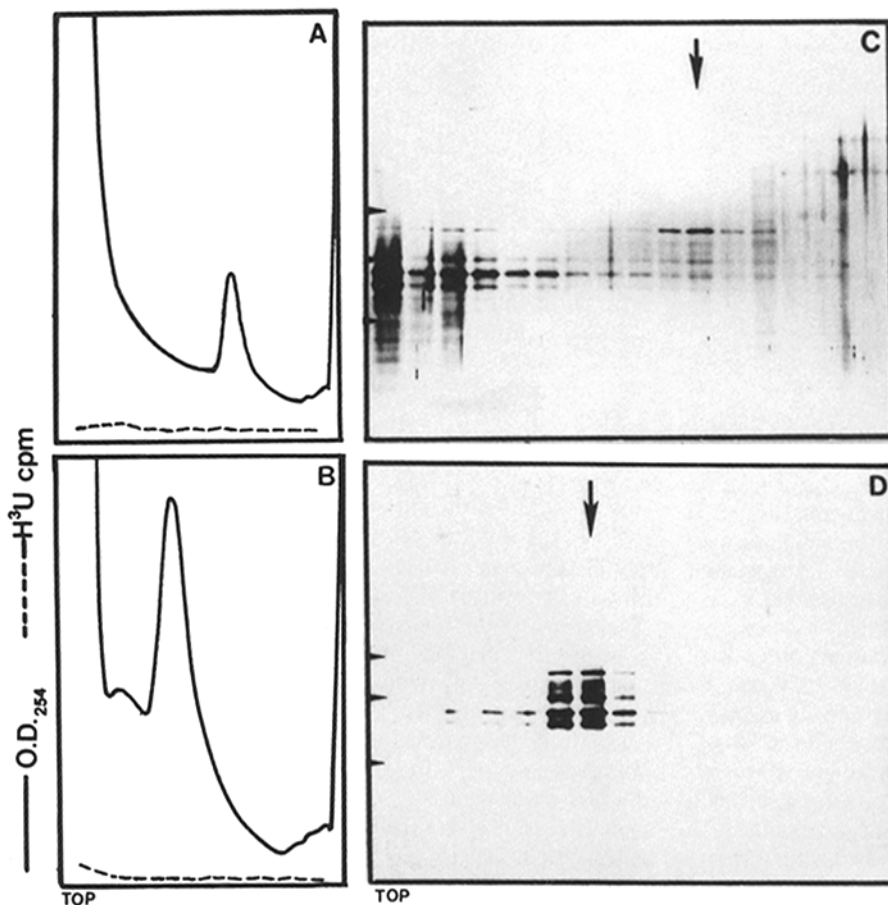
FIGURE 9 Reconstitution of φX174 ssDNP using one half (A and C) and twice (B and D) the molar equivalent amount of DNA. Reconstituted complexes were prepared as described in the text and centrifuged for 1 h at 45,000 rpm. The endogenous [³H]uridine-labeled hnRNA, and SDS PAGE analyses of proteins are as described in Fig. 1; full scale is 0.2 O.D.₂₅₄ and 10,000 cpm. Arrows in C and D indicate relative positions of the corresponding O.D.₂₅₄ peaks.

six core proteins retain the same relative stoichiometry after reconstitution as they have in the originally isolated state. Quaternary structure is retained as well, to the extent shown by similarities in redissociability, EM morphology, and sedi-

mentation properties. Thus, our studies support the fundamental hypothesis of Samarina et. al. (30) on the basic subunit structure of hnRNP and the existence of a small set of core proteins which alone are capable of maintenance of particle
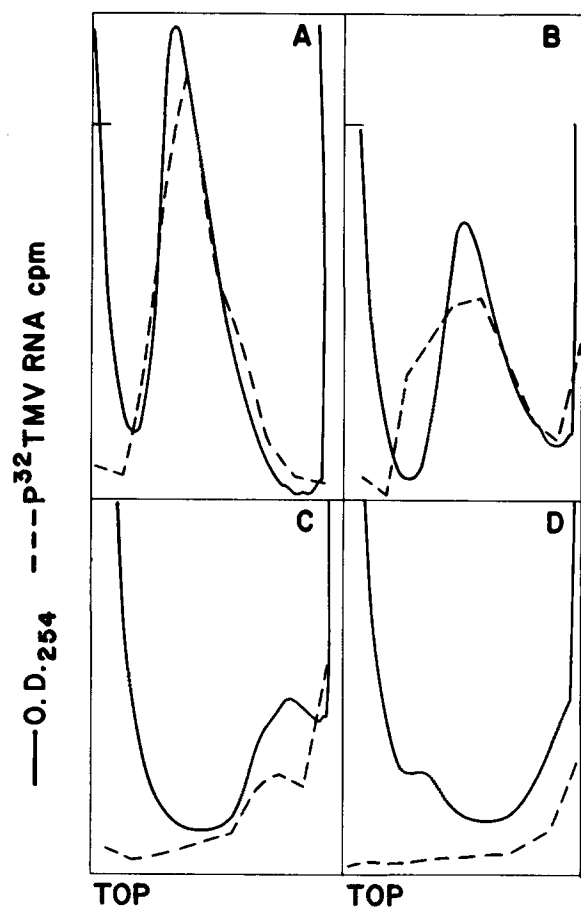
FIGURE 10 Reconstitution using a constant amount of core proteins and varying the amount of TMV RNA added. ^{32}P-labeled TMV RNA was added at (A) four times, (B) three times, (C) two times, and (D) one times the endogenous RNA content of the starting material. Samples were fixed in glutaraldehyde and centrifuged for 1.5 h at 45,000. 0.2 $O.D._{254}$ U and 5,400 cpm full scale in C and D: to left-hand notch marks in A and B.

integrity. If there are other proteins present in large, polyparticulate hnRNP prepared by sonication that are lost, perhaps due to RNase action during extraction procedures (33), our experiments show that they are not required for polyparticle stabilization. Second, reconstitution of the 30S RNP subcomplex appears to be a highly cooperative process, and no products of DOC/Triton X-100 reconstitution smaller than the full sized 30S complex can be produced. Furthermore, addition of deoxycholate or high salt to reconstituted complexes causes their redissociation (results not shown), indicating that protein-protein and protein-RNA interactions are reversible as well as cooperative. Kulguskin et al. (13) reported a high degree of cooperativity in the reversible transition between 30S protein cores and dissociated proteins at high ionic strength in the absence of RNA. However, upon adding RNA and lowering the ionic strength they found that only those RNP reconstituted from 30S cores and not RNP reconstituted from the protein subunits could be redissociated. Our results indicate that 30S RNP can be completely and reversibly dissociated into RNA and core polypeptide monomers (or possibly dimers), and that all the reassociated particles have properties similar to those of the starting material. The discrepancy in results may arise from the the tendency of core proteins to cross-link or denature.
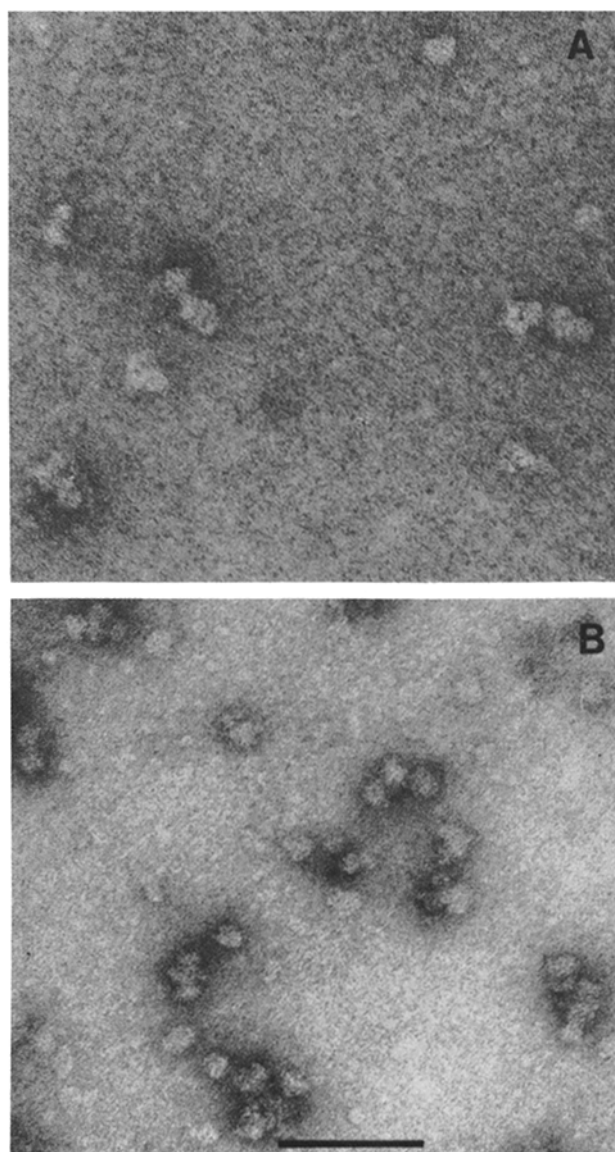

FIGURE 11 Electron micrographs of undersaturated TMV RNP negatively stained with uranyl acetate. Particles were reconstituted with a (A) 2- or (B) 1.5-fold excess of TMV RNA and isolated from the O.D. peaks of sucrose gradients similar to those shown in figure 10. Bar, 100 nm. × 180,000.

Formation of 30S subcomplexes on larger exogenous nucleic acids is also a cooperative and reversible process. Regardless of the total core protein:nucleic acid stoichiometry chosen, the proteins appear to organize only as 30S particles, without forming intermediate or aberrant assembly products. Variation of the protein:nucleic acid ratio changes only the average number of 30S particles per complex, and there are no apparent cooperative interactions between 30S subunits. Cooperativity in the assembly of two or more 30S subunits would be detected in a reconstitution with core proteins where a large nucleic acid is present in molar excess over the amount of hnRNA it is replacing. The result would be an unequal distribution of 30S cores over the nucleic acid molecules, so that complexes with numbers of subunits deviating widely from the mean would be formed. Since the standard deviation of the average number of 30S subunits per complex, as measured by the $O.D._{254}$ peak half-width and by direct counting on electron micrographs, does not change with changing

stoichiometry or nucleic acid length, we conclude that there is little or no cooperativity between 30S subunits. More direct evidence is desirable, however, and it is still possible that there is some interaction between 30S subunits, since we have little information on the actual spacing between 30S particles in undersaturated complexes.

Although quite similar to the original 30S RNP in their sedimentation properties, size distribution, characteristic morphology, and number of nucleotides bound to the protein core, the 30S subunits formed in reconstitutions with exogenous nucleic acids differ in a way relevant to the cooperativity of assembly. This difference is that the average size of the subunit is ~20% smaller than that of 30S RNP as isolated from cells. An explanation for this size difference is that the RNA in 30S RNP prepared by nuclear extraction is in the form of fragments, and the continuous strand of an exogenous nucleic acid might maintain the complex in a tighter conformation.

By using various defined single stranded nucleic acids in reconstitutions, we have been able to study the influence of nucleotide sequence on 30S RNP structure and on the arrangement of 30S subunits within polyparticles. As regards morphology, our results can be summarized quite simply: there is no obvious correlation with nucleotide sequence. Different phasings of the nucleic acid in the polyparticle might obscure such a pattern, but the results with poly(U) eliminate this as a possible explanation. An alternative determinant of particle morphology might be protein composition, for, although the stoichiometry of core proteins relative to one another is maintained during reconstitution, they might be non-uniformly distributed in 30S subunits. Stevenin et al. (31, 32) proposed a similar hypothesis to account for shape heterogeneity in native polyparticles from rat brain; however, the protein composition they reported was far more complex and included many proteins outside the 34,000–40,000-mol wt range. We expect that the possibility can be tested with monoclonal antibodies currently being characterized in our laboratory.

That all reconstituted nuclear RNP have the same beaded structure, independent of nucleotide sequence, suggests that core proteins may denature some regions of RNA or ssDNA secondary structure. The completeness of denaturation as well as the pattern of denaturation may vary. It has been also suggested on the basis of low double-stranded RNA content of hnRNP (5, 12) and the low molar ellipticity of hnRNA in RNP (11, 25, 34) that hnRNA is largely denatured in hnRNP. A protein of 40,000 mol wt (HD40) purified from brine shrimp and present in 30S complexes from this organism has been shown to denature nucleic acids by following variation in the circular dichroism spectrum with the relative amount of HD40 in a reconstitution with φX174DNA. However, HD40 differs significantly from 30S hnRNP core proteins in its reconstitution properties. At low protein to nucleic acid ratios, it will uniformly coat a nucleic acid molecule rather than form a globular subunit. At a high protein to nucleic acid ratio, HD40 will form polyparticles containing globular complexes of smaller diameter than 30S hnRNP and considerably more complexes appear to form on one φX174 molecule (26, 34), indicating that the maximum number of nucleotides bound in one HD40 complex is much less than the 1,000 we have found for the vertebrate hnRNP particles.

The role of individual hnRNP core polypeptides in the maintenance of the protein core and the secondary and ter-

tiary structure of the hnRNA associated with them remains to be determined. However, the application of in vitro reconstitution using model nucleic acids with various defined secondary structures or containing identifiable sequences thought to be important to RNA processing would appear to be a valuable approach to the unravelling of nuclear RNA metabolism.

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