In Vitro Reconstitution of 35S Ribonucleoprotein Complexes†

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ABSTRACT: Ribonucleoprotein complexes (hnRNP) sedimenting at 30–40 S and containing fragments of heterogeneous nuclear RNA (hnRNA) have been extracted from HeLa cell nuclei. Besides hnRNA fragments (8–12 S), the complexes contain eight mostly basic core proteins of M_r 31 000–41 000 as shown by two-dimensional gel electrophoresis. Other proteins (mostly of higher molecular weight) seem to be peripherally associated since they are lost after pelleting and recentrifugation of the hnRNP complexes. The particle dissociates into its protein components after digestion of the endogenous hnRNA fragments by micrococcal nuclease. After inactivation of the nuclease and addition of a wide variety of exogenous RNAs [MS2 phage RNA, poly(U), poly(C), poly(A), and poly(A,U)], a RNP particle is re-formed which resembles the native nhRNP complex according to its sedimentation value (35 S), its appearance in the electron microscope, its density in metrizamide, and its protein composition. No particles are formed on double-stranded RNA [poly(A)·poly(U)] or native DNA whereas denatured DNA allows complex formation. On MS2 RNA (3569 nucleotides), the formation of tri- and tetrameric complexes is observed. This indicates the presence of 900–1200 nucleotides per particle. In vivo, 40S hnRNP particles are a unit component of larger RNP structures. Hence, we conclude from our results that the hnRNP core proteins have the intrinsic capability to associate with nascent single-stranded hnRNA regions to form these RNP complexes. Because of the lack of any sequence specificity, the complexes may function in packaging of the hnRNA and in connection with other nuclear components may provide a scaffold for subsequent processing reactions.

In eukaryotic cells, heterogeneous nuclear RNA (hnRNA)[1] is associated with proteins to form complex ribonucleoprotein structures (hnRNP). These structures can be visualized in the electron microscope as having a fibrous (7 nm thick) or beaded (20–25-nm diameter) appearance (Beyer et al., 1980). They form on nascent nhRNA during transcription, and it is generally assumed that they have a function during post-transcriptional hnRNA processing (van Venrooij & Janssen, 1978; Beyer et al., 1981). hnRNP structures can be isolated from cell nuclei in a wide range of sedimentation values (30–>200 S) (Samarina et al., 1968; Georgiev & Samarina, 1971; Pederson, 1973), depending on the method of preparation, probably due to different original lengths and varying degrees of degradation of the endogenous hnRNA. In all cases, however, hnRNP particles of 30–40 S can be isolated either as a direct result of the extraction procedure or by subjecting the larger RNP complexes to a mild ribonuclease treatment (Samarina et al., 1966, 1968; Martin & McCarthy, 1972; Stevenin & Jacob, 1974; Kinniburgh et al., 1976; Stevenin et al., 1977, 1979). These 30–40S hnRNP particles contain hnRNA fragments (8–10 S $\simeq$ 500–1000 nucleotides) and an assembly of proteins. More than 50% of the particle mass is made up by a group of mostly basic proteins (core proteins) with molecular weights between 30 000 and 40 000 (Beyer et al., 1977). The core proteins found in the 30–40S hnRNP particle and their stoichiometry are reproducible for a given cell in a given physiological state but may vary among different cells. However, it is always by members of the group of core proteins that the body of the 30–40S hnRNP particle is formed. Other proteins, mostly of higher molecular weight, appear associated with the core RNP complex, but their presence is not necessary to maintain the RNP particle structure. The core proteins themselves usually do not assemble into particles without a RNA template (Beyer et al., 1977). In analogy to chromatin structure, it has been suggested that the core proteins may serve in packaging the hnRNA chains whereas proteins functioning in enzymatic processing steps may belong to the associated group of proteins (Beyer et al., 1977; Billings & Martin, 1978). As a consequence of this assumption, the hnRNP core proteins should be unspecific with respect to RNA sequence when binding to the hnRNA but may have a specificity with respect to secondary structure. This can be tested by degrading the endogenous hnRNA fragments and replacing them with exogenously added RNA which can deliberately be chosen with respect to sequence and possible secondary structure. The formation of hnRNP complexes can then be monitored and, if formed, their features compared to the originally extracted native particles. In this paper, we demonstrate that 30–40S nhRNP particles from HeLa cells can be reconstituted on single-stranded ribonucleic acid as well as deoxyribonucleic acid but will not form on double-stranded polynucleotides. The reconstituted particles according to a number of parameters are identical with the native particles. The only proteins necessary for particle constitution are the group of about eight core proteins. Oligomeric complexes are formed on sufficiently long RNA chains. On MS2 RNA (3569 nucleotides), tri- and tetrameric structures can be observed which sediment at 50–55 S.

Materials and Methods

Buffers and Solutions. The following buffers were used: PBS, 150 mM NaCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4,

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[1] Abbreviations: hnRNA, heterogeneous nuclear RNA; hnRNP, ribonucleoprotein complex containing heterogeneous nuclear RNA; snRNA, small nuclear RNA; snRNP, ribonucleoprotein complex containing small nuclear RNA; PBS, phosphate-buffered saline; $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; poly(U), poly(A), and poly(C), polymers of uridylic, adenylic, and cytidylic acid, respectively; poly(A,U), statistical copolymer of adenylic and uridylic acid; poly(A)·poly(U), double-stranded complex of poly(uridylic acid) and poly(adenylic acid); RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; EDTA, ethylenediaminetetraacetic acid.

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pH 7.4; lysis buffer, 10 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris–HCl, pH 7.0; STM-A buffer, 100 mM NaCl, 1.5 mM $MgCl_2$, and 10 mM Tris-HCl, pH 7.0; STM-B buffer, STM-A adjusted to pH 8.0; magic buffer, 6.6% Tween 80 and 3.3% sodium deoxycholate in H_2O (w/v) (Penman, 1966).

Nucleic Acids. Bacteriophage MS2 RNA and all homopolymeric RNA were obtained from Boehringer (Mannheim, West Germany). The homopolymers were run on a sucrose gradient and shown to sediment between 8 and 12 S as indicated in Figure 5.

HeLa Cell Culture and Isotope Labeling. HeLa S3 cells were grown in MEM medium [Joklik's modification (Gibco)] with 5% newborn calf serum (Gibco). Cells were kept in logarithmic growth by sequential dilution every 24 h, keeping the cell concentration between 2.5×10^5 and 6×10^5 cells/mL. For labeling of hnRNA, the cells were concentrated to 5×10^7 cells/mL, and [^{3}H]uridine was added (50 μCi/mL) for 15 min. Proteins were labeled with L-[*methyl*-^{3}H]methionine (0.3 μCi/mL) at normal cell concentrations for 20 h.

Preparation of Nuclear Extracts and Isolation of hnRNP Complexes. If not indicated otherwise, all steps were performed in the cold. The cells were collected by centrifugation (10 min at 350g), and the pellet was washed twice with cold PBS. After resuspension in lysis buffer at a concentration of 2×10^7 cells/mL, the solution was adjusted to 0.5% (v/v) of the nonionic detergent NP-40 and left on ice for 10 min. The cells were then broken by six strokes in a glass homogenizer with a loosely fitting pestle. Nuclei were pelleted at 600*g* for 10 min. The nuclei were resuspended in lysis buffer and freed of cytoplasm by three further strokes (loose pestle) in the presence of 1% (v/v) magic detergent mixture. Nuclei were pelleted again as above, washed twice in STM-A buffer and once in STM-B buffer, and finally resuspended in STM-B at about $(3–5) \times 10^8$ nuclei/mL. The extraction was done by magnetic stirring for two periods of 30 min at 20 °C. The combined extracts were cleared at 16000*g* for 10 min and the supernatants layered on 15–30% sucrose gradients in STM-A. Centrifugation was at 87000*g* for 16 h (Beckman SW40 rotor) at 2 °C. Gradients were collected in 0.8-mL fractions. Absorption at 260 nm was continuously monitored by using a Gilford photometer. Radioactivity was determined as trichloroacetic acid precipitable counts per minute on glass/fiber filters (Schleicher & Schuell) in a toluene-based cocktail in a Beckman 9000 scintillation counter.

The fractions of the gradient containing the hnRNP complexes were combined and pelleted at 100000*g* for 16 h (Beckman Ti75 rotor) at 2 °C. The pellet was resuspended in STM-A containing 15% (w/v) sucrose to a final concentration of 1–2 mg/mL.

Electrophoretic Techniques. Proteins were analyzed on 10% polyacrylamide slab gels containing 0.1% $NaDodSO_4$ (Laemmli, 1970) or on two-dimensional gels under nonequilibrium isoelectric focusing conditions in the first dimension (1800 V·h) (O'Farrell et al., 1977).

Metrizamide Gradient Centrifugation. RNP-containing fractions were layered on metrizamide step gradients (Nyegaard, Oslo) [40% and 60% (w/v) metrizamide in H_2O, 25% and 50% in D_2O] and spun at 130000*g* for 60 h (Beckman SW41 rotor) at 5 °C. Gradients were collected in 0.3-mL fractions, and the refractive index was measured. The corresponding densities were calculated from the formulas $\rho_{5\,^\circ C}^{H_2O} = 3.453\eta^{20\,^\circ C} - 3.601$ and $\rho_{5\,^\circ C}^{D_2O} = 3.0917\eta^{20\,^\circ C} - 2.9984$ as given by the manufacturer.

Reconstitution of RNP Complexes. Nuclear extracts, RNP-containing gradient fractions, or resuspended pelleted RNP particles (0.2–1 mg of protein) were treated with 150 units/mL micrococcal nuclease (Boehringer or P-L Biochemicals) in the presence of 1 mM $CaCl_2$ for 30 min at 37 °C to degrade the endogenous nucleic acids. The reaction was stopped and the enzyme inhibited by adding EGTA up to a final concentration of 4 mM. Various RNAs were then added, maintaining a protein:RNA ratio of 4:1 (8:1 in the case of nuclear extracts), 2.9984 by incubation at 25 °C for 1 h under occasional agitation. The mixtures were then analyzed on sucrose gradients as described above. Note: most commercial samples of micrococcal nuclease contain a trace contamination of another nuclease which cannot be inhibited by EGTA. This nuclease degrades MS2 RNA (28 S) within 30 min at 25 °C to fragments of 15–20 S but does not attack intact RNP particles (preparations obtained from P-L Biochemicals showed the lowest content of this contaminant). To minimize possible degradative effects, the reconstitution of oligomeric complexes was done after digestion with 10–30 units of micrococcal nuclease under otherwise unchanged conditions.

Results

Isolation of Native 40S hnRNP Particles. hnRNP complexes in the form of 40S core complexes were isolated essentially according to the classical procedure devised by Samarina (Samarina et al., 1966, 1968). This procedure uses a shift from pH 7 to 8 at temperatures of 18–22 °C to activate an endogenous endoribonuclease which liberates the core particles from larger hnRNP structures. Our protocol was optimized with respect to maximum yield of hnRNP complexes. Washing the nuclei with the detergents NP-40 and sodium deoxycholate/Tween 80 (magic buffer) before extraction reduced the ribosomal contamination without changing the yield or properties of the extractable hnRNP particles. These "native" hnRNP particles sedimented at slightly less than 40 S on sucrose gradients (Figure 1A). From 10^8 cells, we routinely obtained 0.15–0.2 mg of hnRNP protein. When desired, the particles were pelleted. After resuspension in STM-A buffer containing 15% sucrose, they could be stored at −20 °C for months without a noticeable change in properties.

Characterization of Native 40S hnRNP Complexes. Since they had to be used as standards in comparison to the reconstituted particles, the native 40S hnRNP particles from the gradients were characterized with respect to their RNA and protein components to ascertain their hnRNP nature and define their properties.

(*A*) *RNA.* HeLa cells were pulse labeled for 10 min with [^{3}H]uridine, more than 85% of the nuclear RNA label was found in the nuclear extracts, and more than 80% of this radioactivity was associated with the 40S peak. These labeling kinetics and this location are expected for the fragmented hnRNA remaining in the native 40S complexes. The length of the RNA was found to be heterogeneous, ranging between 100 and about 1000 nucleotides with a maximum between 500 and 800 nucleotides as determined from migration on polyacrylamide gels (not shown). It should be mentioned that after long labeling periods (16 h) small nuclear RNAs (mainly species U1 and U2) are found in association with native 40S complexes. These RNAs, probably together with its snRNP protein components, are lost at salt concentrations above 100 mM NaCl (data not shown) (Zieve & Penman, 1981).

(*B*) *Proteins.* One-dimensional gels of the gradient fractions show a protein pattern across the 40S peak which is typical for hnRNP complexes from HeLa cells (Beyer et al., 1977) and similar to the pattern from other mammalian cells (Billings & Martin, 1978; Karn et al., 1977) (Figure 1A). A group

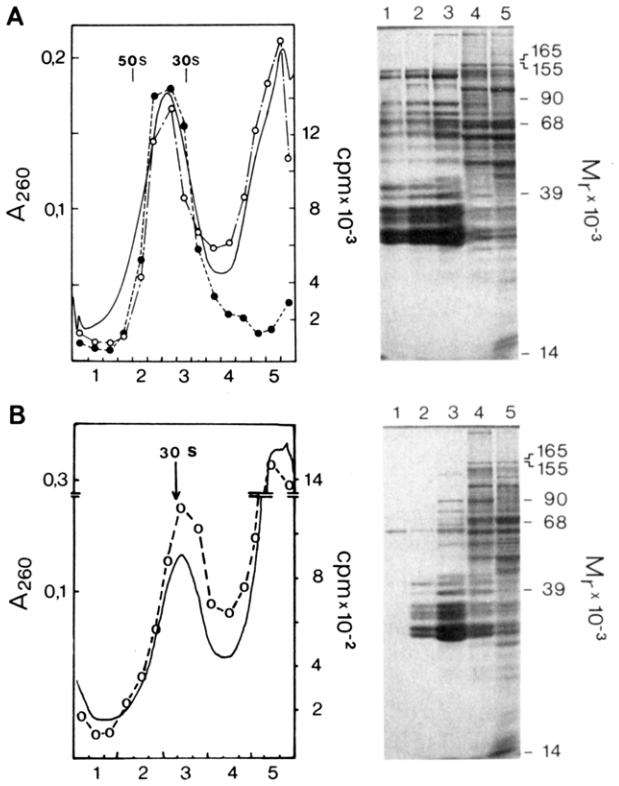

FIGURE 1: Characterization of native and reconstituted RNP complexes by sucrose gradient centrifugation and electrophoretic gel analysis. Nature nuclear extract (1 mL) (A) or a reconstituted sample from 800 μg of nuclear extract protein and 100 μg of MS2 phage RNA (B) was sedimented through a 15–30% sucrose gradient in STM-A buffer at 26 000 rpm for 16 h. Every three fractions were pooled to give corresponding lane 1–5 on 10% polyacrylamide slab gels (Laemmli, 1970). In (A), the closed circles represent acid-insoluble [^{3}H]uridine-labeled RNA (15-min label, 50 μCi/mL), and the open circles represent L-[*methyl*-^{3}H]methionine-labeled proteins, respectively. In (B), acid-insoluble radioactivity from L-[*methyl*-^{3}H]methionine-labeled proteins is represented as open circles. Absorbance was monitored at 260 nm (—) in (A) and (B). *s* values were determined by cocentrifugation of EDTA-treated *Escherichia coli* ribosomes.

of six proteins migrating in three doublet bands corresponding to the A1, 2, B1, 2, and C1, 2 proteins (Beyer et al., 1977) with molecular weight values between 30 000 and 40 000 constitutes 65% of the native particle protein mass (see Figure 3B).

These are the core proteins. Two-dimensional gel analysis resolves about eight members in this group (Figure 2B). The individual spots have been operationally defined as core proteins on the basis of (i) their presence in 40S native RNP particles from HeLa and other mammalian cells, (ii) their constant ratios with respect to the other core proteins, (iii) an unchanged stoichiometry in response to various handling procedures (e.g., recentrifugation or pelleting and resuspension), and (iv) their occurrence in reconstituted RNP complexes, again exhibiting a stoichiometry equal to native particles (Figures 1B and 3D). Some very minor proteins have not been included although they may fulfill these conditions. There is some heterogeneity among the B and C group proteins complicating the simple B1/B2 and C1/C2 pattern (Figure 2B). Assignments have been made in Figure 2B on the basis of electrophoretic mobility, but they should be regarded as preliminary, until possible interrelationships (like precursor/product or unmodified/modified protein) have been investigated. The A proteins, most B proteins, and the C1 core proteins are basic with apparent isoelectric points between pH 8 and 9 whereas the B1 and C2 proteins are found in the acidic region around pH 6.5. The streaking of C proteins is probably caused by residual oligonucleotides as discussed below. This pattern of the hnRNP core proteins is similar for RNP particles from a number of different cells isolated in different laboratories (Beyer et al., 1977; Peters & Comings, 1980; Suria & Liew, 1979). It should be pointed out, however, that only two-dimensional techniques have sufficient resolving power to permit reasonable comparisons. We found, in addition, that a mixture of equal amounts of hnRNP proteins from HeLa cells and bovine lymphocytes produces an unchanged, i.e., superimposable, pattern of spots including the noncore proteins. This indicates, on one hand, the conserved nature of this group of proteins and, on the other hand, the reproducibility of the native 40S hnRNP preparations. The occurrence of charge isomers is also typical for the hnRNP proteins. We consistently find that the degree of charge isomerization is much lower (mainly one or two isomer spots) in HeLa cells than in lymphocytes (mainly four to five charge isomers) or in rat liver cells (Peters & Comings, 1980; Suria & Liew, 1979). In vivo and in vitro labeling experiments with ^{32}P have shown that the majority of these isomers seem to arise by some kind of phosphorylation (I. Gabaldõn de Koch, H.-E. Wilk, and K. P. Schäfer, unpublished results).

The noncore proteins are relatively loosely associated with the hnRNP core complexes since they are more or less lost after pelleting and resuspension of the RNP complexes. In addition, their amount is drastically reduced in reconstituted particles (Figure 3A–D). The loss of peripheral noncore proteins leads to a reduced *s* value (35 S) of the pelleted and recentrifuged native RNP complex with an unchanged stoichiometry of the core proteins.

Other Methods of Characterization. The density of native 40S or pelleted and recentrifuged 35S hnRNP particles in metrizamide gradients was found to be 1.28 g/cm^3 in water and 1.33 g/cm^3 in D_2O. The particles gave a single band in the density gradient which contained all the core proteins in the usual amounts (Figure 2A). These values are similar to results from other laboratories (Karn et al., 1977). Various preparations of the RNP complexes were negatively stained and analyzed in the electron microscope. The population of monodisperse roughly spherical particles (Figure 2C) shows a diameter range from 15 to 25 nm which again is comparable to published data (Beyer et al., 1977; Karn et al., 1977; Martin et al., 1978; Stevenin et al., 1979).

In Vitro Reconstitution of hnRNP Complexes. Our experimental rationale was as follows: the amount of the core proteins within the nucleus, their ubiquity among eukaryotic cells of widely varying origin, and their conservative nature have led to the above-mentioned view that they have a structural role in packaging and transport of hnRNA and in providing a scaffold for processing reactions, which in some cases must have stringent topological requirements. However, if this is in fact the role of these proteins, we would not expect them to have any RNA sequence specificity in their binding to hnRNA, but they may well be able to recognize secondary or tertiary structures. In addition, the core proteins should have the capacity to self-assemble into the 35–40S hnRNP complexes, once a RNA template is provided. Therefore, an in vitro system could be set up in which the endogenous hnRNA fragments are degraded by a reversibly activated nuclease and are replaced by exogenous RNA. This should allow us to study the self-assembly process of the RNP complexes, to determine the number and nature of all proteins necessary for this process, and to produce a homogeneous

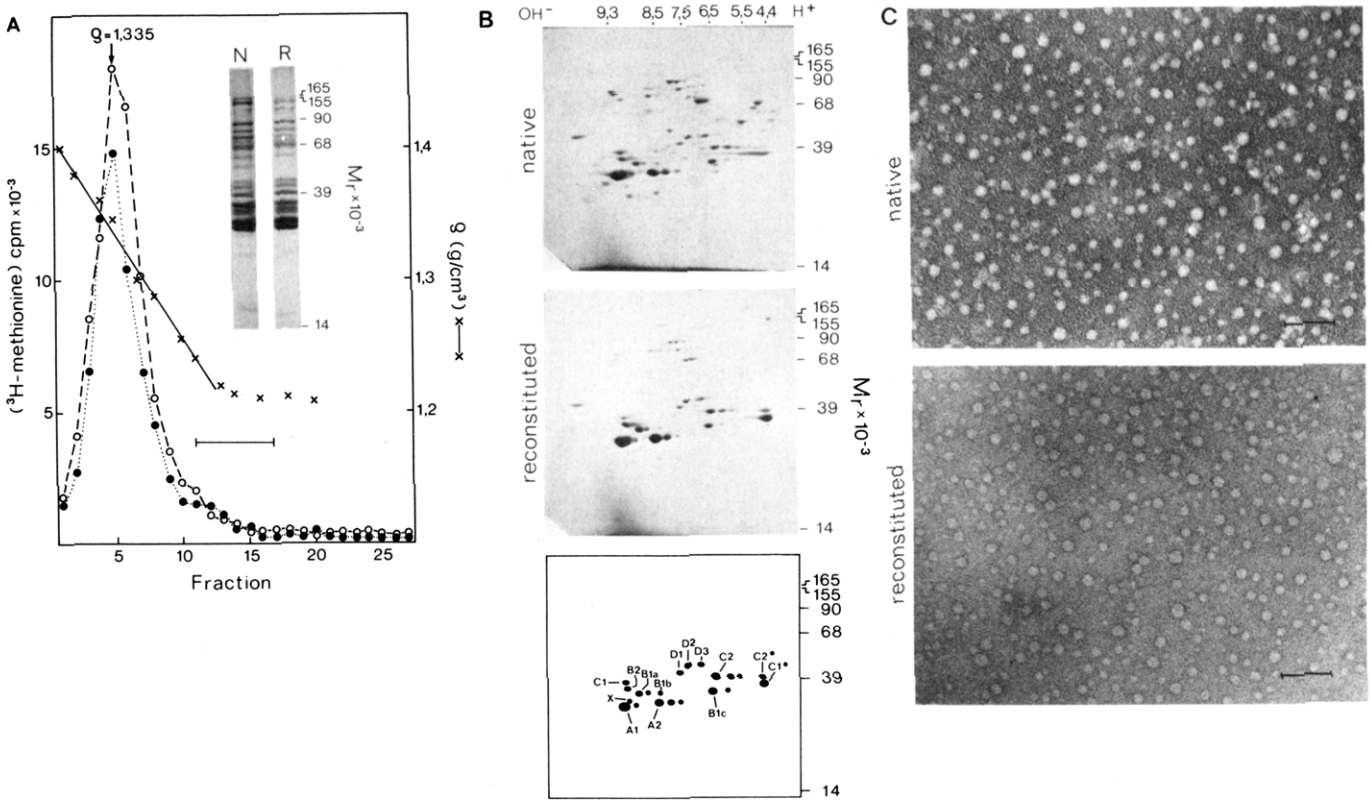

FIGURE 2: Comparison of native and reconstituted RNP particles. (A) Metrizamide density gradient centrifugation: native and reconstituted RNP complexes were taken from the 30–40S region of a sucrose gradient and layered on top of a metrizamide step gradient as described. The distribution of acid-insoluble radioactivity from L-[*methyl*-^{3}H]methionine-labeled proteins in native hnRNP particles (○) and reconstituted complexes (●) is shown. The gel lanes show the protein composition of the peak fractions from the metrizamide gradients of native (N) and reconstituted (R) particles. Control gradients showed that free core proteins after micrococcal nuclease digestion will band from 1.18 to 1.22 g/cm^3. The bar indicates the density range within which 80% of the ^{3}H radioactivity of free proteins is found. (B) Two-dimensional gel analysis: proteins of native and reconstituted particles were analyzed on two-dimensional polyacrylamide gels under nonequilibrium conditions (O'Farrell et al., 1977). Before electrophoresis, RNA was digested by 150 units/mg of particle protein of micrococcal nuclease and 10 μg/mg of RNase A for 60 min at 37 °C in the presence of 1 mM $CaCl_2$. The pH gradient formed during the first dimension run is indicated on top of the figure. The drawing gives the nomenclature of the core proteins as proposed by Beyer et al. (1977) and as given in the text. (C) Electron microscopy: RNP particles were isolated from the 30–40S region of sucrose gradients, diluted in PBS, and negatively stained by treatment with phosphotungstic acid. Pictures were taken at 24000× magnification. The bar corresponds to 0.1 μm.

population of RNP structures by using a defined RNA template.

Conditions of in Vitro RNP Reconstitution. We have used the calcium-dependent micrococcal nuclease to degrade the endogenous hnRNA fragments within the RNP complexes. The nuclease was then inhibited by the addition of EGTA and the reconstitution started by the addition of RNA. Reconstitution was done in most cases from purified 35S hnRNP complexes (after sucrose gradient separation and subsequent pelleting and resuspension) but could also be achieved with identical results directly from crude nuclear pH 8 extracts (see Figures 1 and 6C). At the end of the RNA digestion (30 min at 37 °C), the solution containing the RNP's becomes turbid. The free proteins obviously tend to aggregate when their concentration exceeds a certain limit. The precipitate can be pelleted and contains the core proteins as well as the high molecular weight proteins. After addition of RNA, the turbidity slowly decreases. We have therefore routinely allowed 60 min at 25 °C for RNP reconstitution.

RNP Complex Formation on Phage MS2 RNA. Phage MS2 RNA with a length of 3569 nucleotides (28 S) (Fiers et al., 1976) was chosen as exogenous RNA because it lacks all posttranscriptional modifications of eukaryotic hnFNA [5′ cap, 3′-poly(A), internal m^{6}A methylations] and does not contain introns. It has, however, complex secondary and tertiary structures, thereby resembling hnRNA and/or eukaryotic mRNA. After digestion of the endogenous hnRNA, the hnRNP complexes disintegrate, and in sufficiently dilute solutions (100 μg/mL), all proteins are recovered from the top of the gradient (Figure 4B). When increasing amounts of MS2 RNA are added, the formation of RNP complexes sedimenting at 35 S is observed (Figure 4C–F). It can be seen that the C group proteins together with protein A2 bind to the RNA with high affinity even at low RNA:protein ratios (Figure 4C). In this respect, it is interesting to note that the C proteins also show the tightest binding to RNA when the hnRNP complexes are dissociated by high ionic strength (Beyer et al., 1977). At RNA:protein ratios of about 1:4 (Figure 4E), the particle formation seems to be complete since practically all core proteins are associated with the 35S complexes. A RNA:protein ratio of 1:4 has been reported for the native 40S complexes (Beyer et al., 1977). When the length of MS2 RNA is considered, the formation of oligomeric RNP complexes should be possible but has not been observed from nuclear extracts. The reason becomes clear when the MS2 RNA is analyzed after reconstitution: the RNA now runs as a broad band of fragments from 400 to 800 nucleotides (data not shown) which still allow monomer RNP complex formation but do not permit dimeric structures. This RNA breakdown probably is caused by an endogenous RNase activity known to be present in RNP preparations (Niessing & Sekeris, 1970). For the purpose of studying the assembly of the monomeric core RNP particle, this nucleolytic activity can be neglected (it even increases the yield of monomeric 35S complexes), but

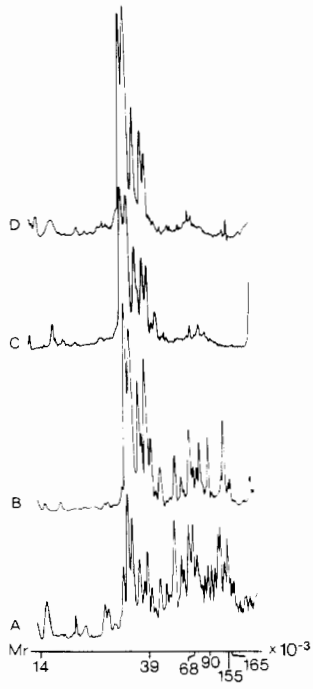

FIGURE 3: Core protein distribution in various RNP fractions. Samples were analyzed on 10% polyacrylamide slab gels (Laemmli, 1970) and stained with Coomassie Blue, and the respective lanes were scanned at 578 nm in a Gilford photometer. (A) Top fraction of a sucrose gradient of native 40S RNP particles representing a soluble nonparticular protein pool; (B) native 40S RNP particle (peak fraction); (C) native particles after pelleting; (D) reconstituted particle (35S peak).

it has to be controlled when the formation of oligomeric RNP structures is to be studied.

Formation of Oligomeric Complexes. The high level of this endogenous nuclease activity could be the reason for our failure to detect the formation of oligomeric complexes when the reconstitution on MS2 RNA was done from nuclear extracts. We therefore used pelleted and resuspended 35S material for further reconstitution experiments. As an additional precaution, the concentration of the micrococcal nuclease was lowered to 10–30 units/mL since practically all commercial samples of this enzyme were contaminated by varying amounts of another nuclease which could not be inhibited by withdrawal of calcium. Control experiments showed that even at this low level of micrococcal nuclease the native 35S peak completely disappeared and all RNP proteins were found on top of the sucrose gradients. We have not tested what the minimum concentration of nuclease for 35S hnRNP complex degradation would be.

When pelleted 35S particles were treated in this way, the addition of MS2 RNA resulted in the appearance of a peak at about 55 S (Figure 5A) which contained all the core proteins in the usual stoichiometry (Figure 5B). On inspection in the electron microscope, a high proportion of tri- and tetrameric complexes could be observed (Figure 5C). The diameter of the subunit particles is about 20 nm but shows a certain variation like the monomeric particles taken from the 40S region (Figure 2C). The RNA extracted from the 55S complexes gave a size spectrum from about 400 nucleotides up to intact MS2 RNA [main peak around 800 nucleotides (not shown)]. If we rule out any effect of the micrococcal nuclease contaminant, this would indicate that the endogenous nuclease is still present and active although at reduced levels as compared to complete nuclear extracts. Dimeric or monomeric particles in the 55S peak (Figure 5C) may arise by continuing action of the enzyme. Since we do not observe structures with more than four monomer particles and if we assume that the tetramer fully covers the complete MS2 RNA, we may infer a length of 900 nucleotides per monomeric core particle. The reported length of the hnRNA fragments in native 40S particles—between 400 and 900 nucleotides (Georgiev & Samarina, 1971; Beyer et al., 1977)—results from degradation during isolation. We have not systematically tested what minimum length is required for monomeric complex formation in the reconstitution assay, but the successful use of denatured DNA from dimeric nucleosomes (length about 400 nucleotides), as described below, indicates that even such relatively short chains still allow formation of a core complex. Maintenance of the particle structure seems to be possible even on RNA chains down to 200 nucleotides, however (W. M. LeStourgeon, personal communication).

Reassociation of RNP Complexes with Various Polynucleotides. The reconstitution assay can even be further simplified with respect to the RNA component by using homopolymers like poly(A) or poly(U). Furthermore, topological requirements of particle formation can be probed by using single-stranded poly(A) or poly(U), partly double-stranded poly(A,U), or the fully double-stranded polynucleotide poly(A)·poly(U). All polynucleotides were obtained commercially and screened for their length. Material of 8–12 S (about 600–1200 nucleotides) was used for in vitro reconstitution to assure proper 35S RNP complex formation. We routinely used a RNA:protein ratio of 1:4 in all assays since optimum yields had been obtained under these conditions with MS2 RNA (see Figure 4).

It turned out that all homopolymers tested were equally efficient in 35S RNP formation. No preference in affinity of the core proteins to associate with various polynucleotides was observed. (Figure 6). Poly(U), poly(C) (data now shown), and poly(A) gave identical results. Again, it was of no importance whether the reconstitution was done directly from nuclear extracts or from purified 35S complexes (see Figures 1 and 6C). These results support our assumption that the core proteins have no RNA sequence specificity for their binding and particle formation. The single-stranded homopolymers have rather little secondary structure. The importance of the secondary structure of RNA was probed with poly(A,U), a statistical copolymer of AMP and UMP which has a high degree of internal self-complementarity and hence a secondary structure like double-stranded stems and loops, or by using the double-stranded complex poly(A)·poly(U). The formation of 35S RNP complexes can only be observed on the poly(A,U) copolymer which actually may not be so different from MS2 RNA in its secondary structure. Double-stranded poly(A)·poly(U) was ineffective in complex formation. The small amount of proteins entering the gradient may be bound to single-stranded regions or ends which are certainly present in the commercial poly(A)·poly(U) (Figure 6B). The exclusion of double-stranded regions from hnRNP complexes has also been observed in vivo (Calvet & Pederson, 1978) and hence may be of biological significance.

The lack of any sequence specificity in the association of the core proteins with RNA prompted us to extend our reassociation experiments to DNA. We used DNA fragments from di- and trinucleosome peaks after micrococcal nuclease digestion of mouse cell nuclei. These fragments are about 400–600 nucleotides long. In their native form, the DNA fragments do not allow 35S RNP particle formation although some binding of the core proteins, especially the A group, is observed (Figure 6F). After denaturation of the DNA, how-

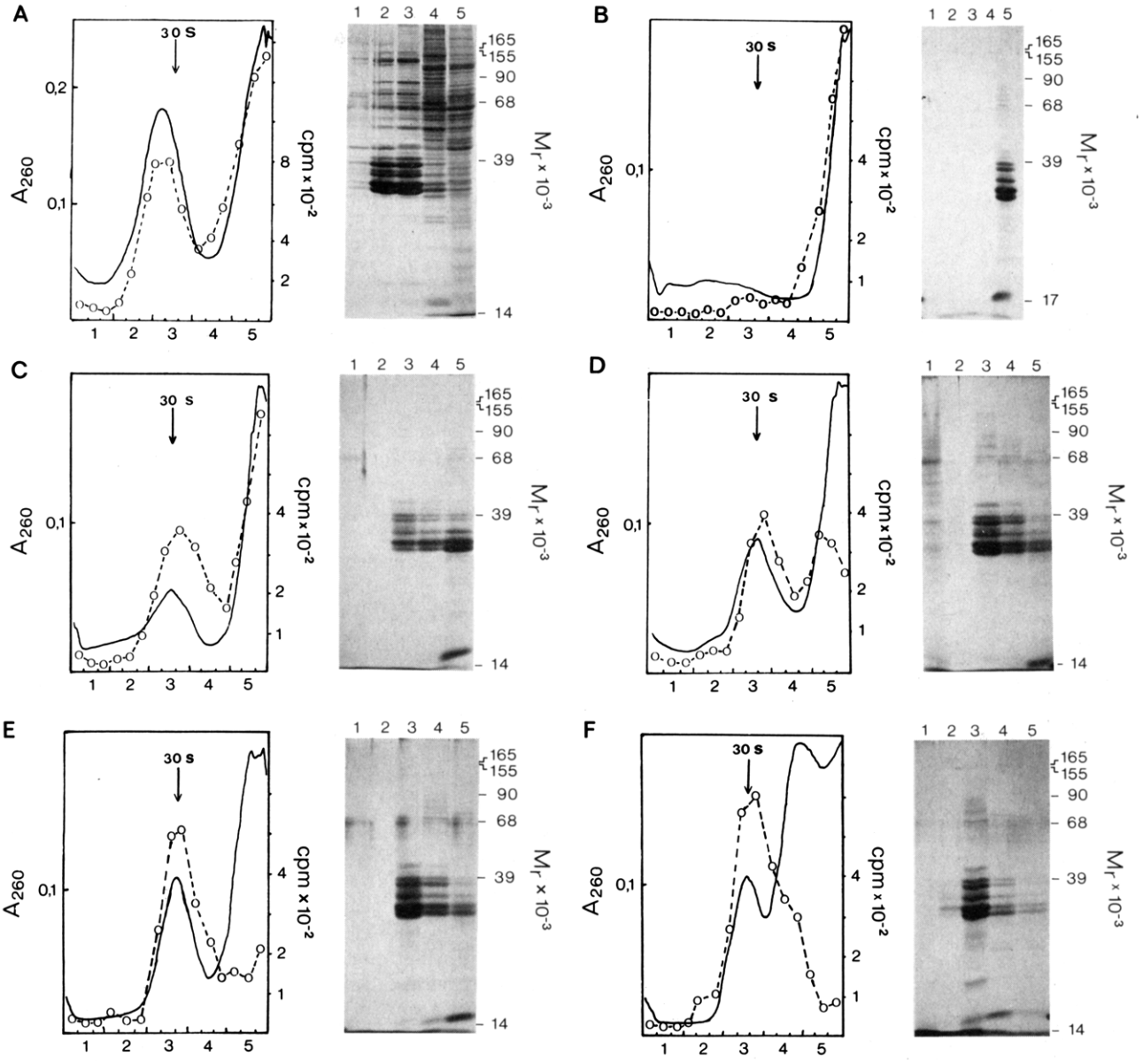

FIGURE 4: Reassociation of core proteins and MS2 phage RNA. Native 40S hnRNP complexes were isolated from sucrose gradient fractions (A), and the endogenous RNA was digested with 100 units/mg of particle protein of micrococcal nuclease for 30 min at 37 °C in the presence of 1 mM $CaCl_2$. Recentrifugation shows the total lack of intact RNP particles (B). After inactivation of the nuclease by 4 mM EGTA, reconstitution was achieved by addition of MS2 phage RNA. Samples of 200 μg of core proteins were incubated with 10 (C), 25 (D), 50 (E), and 100 μg (F) of MS2 RNA for 60 min at 20 °C. The reconstituted particles were analyzed on sucrose gradients as before. Absorption at 260 nm was monitored (solid line) and protein distribution determined by acid-insoluble radioactivity [tracer labeling of the cells before extraction by L-[*methyl*-^{3}H]methionine (open circles)]. Pooled fractions of the gradients were analyzed on 10% polyacrylamide slab gels.

ever, all core proteins are found in a distinct peak around 35 S with a stoichiometry indistinguishable from that of other RNP complexes (Figure 6E). Apparently, the core proteins will associate with any polynucleotide offering single-stranded regions of sufficient length.

Comparison of Native and Reconstituted RNP Complexes. For routine purposes, we have used the sedimentation behavior of the RNP particles and one-dimensional gel electrophoresis to characterize the proteins of the RNP complexes. To show the identity of native and reconstituted RNP complexes, we have analyzed the proteins on two-dimensional gels (Figure 2B) under nonequilibrium conditions for a better resolution of the basic components (O'Farrell et al., 1977). The native particles show a complex pattern of proteins. However, it is evident that the core proteins make up more than two-thirds of the particle protein mass. After reconstitution (on MS2 RNA) and recentrifugation, the core proteins still exhibit the same stoichiometry among each other whereas practically all other components are reduced to traces (Figure 2B). We have not included the group of proteins labeled D (M_r 40 000–43 000) amont the core proteins although they are frequently found in RNP preparations, but their distribution on a sucrose gradient differs from that for the A to C group proteins. The D proteins have not been included among the core proteins since they appear enriched in RNP fractions heavier than 35S and may be preferentially bound to larger complexes or oligomeric particles (Figure 1A,B and Figure 5B). Pelleting of the native RNP particles and recentrifugation of the redissolved pellet yield 35S complexes which are considerably depleted of the higher molecular weight proteins (M_r above 40 000) (Figure 3C,D) and resemble reconstituted particles in this respect. The density of the reconstituted complexes in metrizamide is identical with that of the native RNP, and there is no difference in the appearance in the electron microscope (Figure 2A,C). All these data support the assumption that native and reconstituted particles are identical.

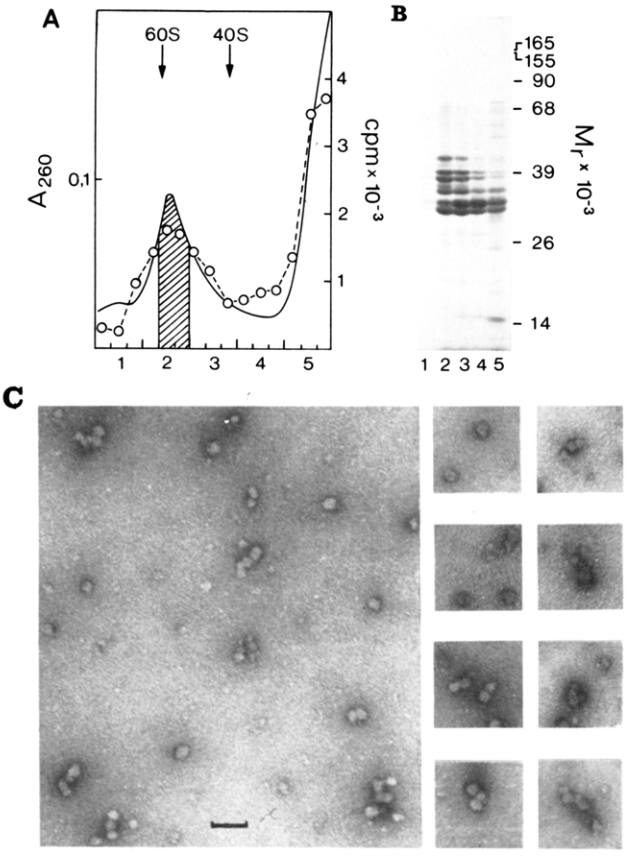

FIGURE 5: Reconstitution of oligomeric RNP complexes with MS2 RNA. Conditions of reconstitution and analysis were as before with the only exception being that the micrococcal nuclease concentration was lowered to 25 units/mg of RNP in the initial digestion; 100 μg of MS2 RNA was added for reconstitution starting with 500 μg of RNP. (A) Sedimentation profile of the reconstituted complexes. (B) Protein analysis of gradient fractions (Laemmli, 1970). (C) Electron microscopy: RNP complexes were taken from the 55S peak, fixed with glutaraldehyde, and negatively stained with uranyl acetate. Pictures were taken at 48000× magnification. The bar corresponds to 0.1 μm.

Discussion

In the same way as the study of the nucleosome has provided many insights into general chromatin structure, the 30–40S hnRNP complexes may be helpful in understanding the processing apparatus for hnRNA. In addition, any in vitro system aiming at a reconstitution of the processing machinery will require an understanding of hnRNP complex formation. The first step is an analysis of the protein components of these particles. although it was known for some time using one-dimensional polyacrylamide gels that more than 50% of the particle mass was represented by a few proteins in the molecular weight range between 30000 and 40000 (the core proteins), some discrepancies about the number of proteins persisted mainly because some components were not resolved into separate bands. The use of two-dimensional gels has revealed a strikingly common pattern for different mammalian cells [see Figure 2B and Beyer et al. (1977), Peters & Comings (1980), Suria & Liew (1979), and Brunel & Lelay (1979)]. When a proposed nomenclature (Beyer et al., 1977) is adopted, the basic core proteins A1 and A2 are present in equal amounts in our preparations of native 40S RNP complexes from Hela cells and represent about 50% of the core particles mass. The B group proteins split into several subcomponents with apparent isoelectric points from slightly basic into the neutral range. We have tentatively named them B1a, B1b, B1c, and B2. They are present in native particles at A1:A2:B1a–c:B2 approximate ratios of 6:6:2:1. The ratio of A2:B1 of about 3:1 seems to be a rather reliable and constant figure. The C proteins C1 and C2 are peculiar inasmuch as their strong affinity for nucleic acids seems to be responsible for the streaking observed on most two-dimensional gels. The streaking probably is caused by residual oligonucleotides which survive the nuclease treatment before electrophoresis. Whether the basic spot marked C1 is the free protein and the streak or the acidic spot (Figure 2B) represents a C1–RNA complex (marked C1*) or whether two different protein species are involved still has to be shown, e.g., by peptide mapping. The same argument may be valid for the slightly acidic protein C2 and its C2* counterpart. The compression of the streak into a distinct spot in reconstituted RNP particles could be due to the presence of an oligonucleotide of definite length since a homogeneous RNA has been used for reconstitution. The amount of total C protein is usually close to the amount of A1 and A2. All other proteins are not considered to be elements of the core RNP complexes. We may expect proteins and enzymes to be involved in the processing of hnRNA among this group [e.g., endonuclease(s) (Niessing & Sekeris, 1970) and ligase(s) involved in splicing, 5′ cap binding proteins(s), 3′-poly(A) polymerase, methylase(s), and protein kinase(s) (Blanchard et al., 1977) as well as phosphatase(s)].

After digestion of the 35S complexes with micrococcal nuclease (150–300 units/mg of RNP), the 35S peak has disappeared completely from the gradients. Under our conditions, we have never observed material heavier than 5–6 S (i.e., at the top of the gradient) after nuclease treatment. Nuclease-resistant protein–RNA complexes (sedimenting at 2 S) (Augenlicht et al., 1976) do not seem to interfere with any reconstitution experiments, if such material is present in our digested RNP preparations at all, since Augenlicht et al. used larger hnRNP structures for their experiments (up to 200 S) which contain many additional proteins. They found mainly proteins of M_r 40000 and 66000 associated with the nuclease-resistant oligonucleotides. Although the M_r 40000 protein could be related to the C group proteins, the presence of short oligonucleotides (as indicated by the smear in the gel of native particles in Figure 2B) obviously has no effect on the stoichiometry of the core proteins in the reconstituted particles (Figure 2B). Therefore, structures sedimenting at 10 S or above would have only been expected if the core proteins themselves would be able to associate in a particular form as has been proposed in the informofer model (Samarina et al., 1968; Georgiev & Samarina, 1971; Kulguskin et al., 1980). The nonexistence of protein particles, however, has been inferred by several other groups on the basis of the decay of the hnRNP complexes after RNAse or high salt treatment (Stevenin & Jacob, 1974; Stevenin et al., 1977, 1979; Beyer et al., 1977). In addition, Kulguskin et al. obtained free protein complexes only at protein concentrations above 6 mg/mL. These particles contained two major components as resolved on one-dimensional gels (Kulguskin et al., 1980). A comparison with the two-dimensional analyses of Suria & Liew (1979) and Peters & Comings (1980) for hnRNP complexes (also isolated from rat liver) makes it likely that these two components correspond to a mixture of the two core protein species A2 and B1a/B1b. In a model of 40S hnRNP complexes proposed by LeStourgeon and colleagues (LeStourgeon et al., 1981), these proteins form the inner rim of a thoroidal disk, thereby creating the overall form of the complex. Whatever the detailed inner structure of the 40S complex may be, however, the point can be made that protein–protein interactions are important for the maintenance of the particle

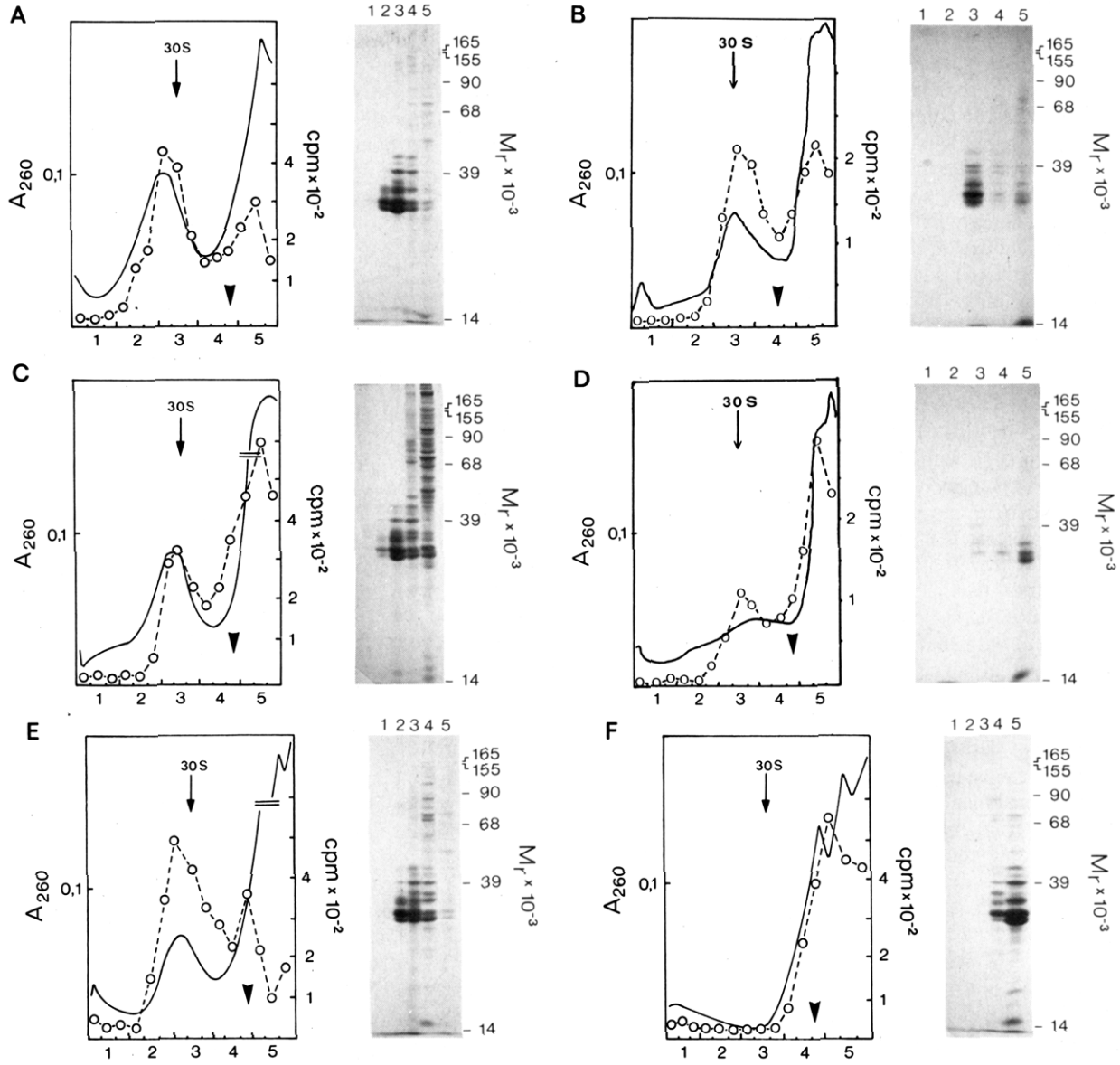

FIGURE 6: Reconstitution of RNP complexes with various nucleic acids. Conditions of reconstitution and analysis were as before. In the gradients, the absorption at 260 nm (solid line) and the acid-insoluble radioactivity incorporated into proteins (open circles) are given. After nuclease digestion of native particles, the following mixtures were used: (A) 400 μg of 40S hnRNP with 100 μg of poly(U); (B) 200 μg of 40S RNP with 50 μg of the statistical copolymer poly(A,U); (C) 800 μg of total protein of a nuclear extract with 100 μg of poly(A); (D) 200 μg of 40S RNP with 50 μg of double-stranded poly(A)·poly(U); (E) 400 μg of 40S RNP with 100 μg of denatured mouse DNA (400–600 nucleotides); (F) 400 μg of 40S RNP with 100 μg of native mouse DNA (400–600 base pairs). All experiments were at least repeated twice with identical results. The sedimentation positions of the free nucleic acids are given by arrowheads.

structures. It is therefore possible that at rather high protein concentrations complexes are formed due to such interactions. These complexes seen to be prone to stress-induced dissociation (e.g., during centrifugation), and their association with RNA leads to structures in which the RNA is protected against nuclease attack (Kulguskin et al., 1980). The last point especially together with the fact that all two-dimensional analyses reveal certainly more than two core protein constituents of hnRNP complexes makes it likely, in our opinion, that the formation of the protein complexes or their association with RNA as observed by Kulguskin et al. (1980) does not lead to structures identical with the original RNP particles. It seems important, therefore, as seen in Figure 2B, to show that the reconstituted particles do contain all core proteins present in native complexes. The specificity of their RNA association is underlined by the fact that the reconstitution can be done from crude nuclear extracts as well as from purified 35S complexes (Figure 1B and Figure 4D–F). It should also be pointed out that the C proteins, which have a high affinity for RNA (Beyer et al., 1977; Van Eekelen & Van Venrooij, 1981) and which are clearly the first to associate with MS2 RNA in our experiments (Figure 4C), seem to be missing from or are strongly reduced in many 40S hnRNP preparations (Kulguskin et al., 1980; Suria & Liew, 1979; Peters & Comings, 1980), although the "nativity" of the RNP complexes, i.e., the specificity of protein–RNA interactions, might crucially depend on their presence.

Another important point is the heterogeneity in size and shape of the 40S hnRNP complexes. Our samples were taken from the pooled 30–45S range of sucrose gradients. A variation of the particle diameter between 15 and 25 nm is clearly visible in both preparations (Figure 2C) and has also been observed by other groups [e.g., see Karn et al. (1977)]. Samples taken separately from the 30S, 40S, and 50S regions, respectively, have been shown to display a corresponding gradient of particle size (Stevenin et al., 1979). Higher res-

olution studies have revealed a considerable diversity of shapes and sizes for 30S complexes (Martin et al., 1978). The recurrence of this size variation in particles prepared under different conditions together with the data on the spontaneous reconstitution of the complexes as presented here makes it likely that we are not looking at an artifact but rather see the result of an intrinsic flexibility in the formation of the 30–40S RNP complexes. We have to keep in mind that hnRNP complexes necessarily have to be dynamic structures which can respond smoothly to the changes of their hnRNA templates during transcription and processing (Stevenin et al., 1979). The seemingly contradictory facts of constant core protein stoichiometry vs. variable particle size can be reconciled on the basis of subcore units (in which the core proteins are present in the known ratios) building up the 30–40S core complexes. Adding or detracting such subcore units from the complexes will lead to the variable size as observed in the electron microscope with isolated particles as well as with larger nascent structures in vivo (Beyer et al., 1980). A similar diversity of particle size is seen in the in vitro reconstituted oligomeric complexes, which, on MS2 RNA, either maximally contain three relatively large particles of similar size or accommodate four particles including at least one smaller member (Figure 5).

In agreement with their proposed structural role, our experiments show that the core proteins are able to assemble into 35S RNP complexes on any ribonucleic acid provided that the polynucleotide strand is at least partly single stranded. There is no restriction with respect to nucleotide sequence since homopolymers can be used as templates for reconstitution. No posttranscriptional modifications usually found in hnRNA like the 5′ cap, internal methylations, or a 3′-poly(A) tail are necessary for RNP complex assembly. Consequently, denatured, i.e., single-stranded, DNA also allows the formation of a 35S DNP complex which indicates that the affinity of the core proteins is generally directed against single-stranded polynucleotides (Martin et al., 1978). The inability of double-stranded RNA to form RNP complexes in vitro is in agreement with the finding of double-stranded hnRNA regions also being excluded from hnRNP structures in vivo (Calvet & Pederson, 1978).

The next step in the application of the in vitro reconstitution will be the use of a 5′-capped and 3′-polyadenylated but otherwise unprocessed hnRNA containing a defined gene sequence including at least one intron. This will allow one to probe the association of the small nuclear RNP complexes (snRNP's) with the hnRNP structures (Lerner et al., 1980; Zieve & Penman, 1981) in order to determine whether this binding is dependent on the presence of intron sequences.

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Registry No. Poly(U), 27416-86-0; poly(C), 30811-80-4; poly(A), 24937-83-5; poly(A,U), 25249-19-8.

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