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Ribonucleoproteins Package 700 Nucleotides of Pre-mRNA into a Repeating Array of Regular Particles

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An assay for the in vitro assembly of HeLa cell 40S nuclear ribonucleoprotein particles (hnRNP particles) has been developed. The substrates were single-stranded nucleic acid polymers of defined length and sequence prepared in vitro and the six major core particle proteins from isolated 40S hnRNP. The fidelity of in vitro assembly was evaluated on various physical parameters, including sedimentation, salt dissociation, polypeptide stoichiometry, UV-activated protein-RNA cross-linking, and overall morphology. Correct particle assembly depended on RNA length and on the input protein/RNA ratio but not on the concentration of the reactant mixture nor on the presence or absence of internal RNA processing signals, a 5′-cap structure, a 3′-poly(A) moiety, or ATP as energy source. RNA lengths between 685 and 726 nucleotides supported correct particle assembly. Dimers and oligomeric complexes that possessed the same polypeptide stoichiometry as native hnRNP assembled on RNA chains that were integral multiples of 700 nucleotides. Intermediate-length RNA supported the assembly of nonstoichiometric complexes lacking structural homogeneity. An analysis of these complexes indicates that proteins A1 and A2 may be the first proteins to bind RNA during particle assembly. We conclude that the major proteins of 40S hnRNP particles contain the necessary information for packaging nascent transcripts into a repeating "ribonucleosomal" structure possessing a defined RNA length and protein composition but do not themselves contain the information for modulating packaging that may be required for RNA splicing.

The biochemical events of pre-mRNA processing are known largely through studies on the RNA intermediates produced during splicing and studies on the nucleotide sequences required for RNA maturation (for reviews, see references 17 and 42). Nascent transcripts, however, are packaged by a specific subset of major nuclear proteins during transcription to form a ribonucleoprotein (RNP) complex in which the events of RNA splicing occur (see references 8 and 10 for reviews). Historically, these complexes were termed heterogeneous nuclear ribonucleoprotein particles (hnRNP) because the packaged moiety was heterogeneous nuclear RNA and not because the individual particles were known to be heterogeneous in composition and structure (10, 38, 41). Several observations indicate that nascent transcripts are in fact packaged into a regular repeating structure composed of a contiguous array of 40S hnRNP complexes. These observations suggest that the complexes may play only a passive role in RNA splicing. Other observations suggest that packaging may be transcript specific and thus imply that hnRNP may play a more direct role in the events of RNA processing.

Observations in support of a fundamental packaging function for hnRNP are the following. (i) The intranuclear concentration of the major hnRNP is high. For example, in actively growing mammalian cells there is 20 to 30% as much individual core particle hnRNP as individual core particle histone (24). (ii) The majority of nuclear pre-mRNA sequences are recovered from nuclear extracts as 40 to 500S complexes which yield 40S monoparticles upon endogenous or exogenous ribonuclease activity (31, 32, 37, 38, 41). (iii) The 40S hnRNP particles from actively growing HeLa cells sediment in sucrose density gradients as morphologically homogeneous structures which are primarily composed of multiple copies of six major proteins at the protein A1/A2/B1/B2/C1/C2 molar ratio of 3:3:1:1:3:1 (25). (iv) Isolated 40S particles possess the same ultrastructural morphology as those observed in gently spread transcriptive units (45). (v) Finally, previous in vitro reconstitution studies have shown that hnRNP particles form on substrates of single-stranded DNA (ssDNA), mRNA, and homoribopolymers, suggesting no sequence-specific requirement for particle assembly (12, 39, 49).

Among the observations interpreted as evidence of a direct function of hnRNP in splicing are electron micrographs of negatively stained transcriptive units showing the sequence-specific distribution of 25-nm RNP complexes on nascent transcripts and the aggregation of these structures to form RNA loops which are excised during transcription (1, 2, 4). More recently it has been possible to identify the *Drosophila* chorion genes s36-1 and s38-1 in spreads of follicle cell chromatin (35). Transcripts of these genes reveal 25-nm RNP complexes at the 5′ and 3′ splice junctions of the single intron (36). However, these findings are often misinterpreted because the stable 25-nm structures are not likely to be 40S hnRNP particles. For example, the procedure used to visualize the 25-nm complexes in spread transcriptive units (ionic detergent and alkaline pH) are known to dissociate the six major proteins from the pre-mRNA fragments present in isolated 40S hnRNP (24, 25). In support of this point is the finding that when less-dispersive conditions are used to spread the same transcripts, a contiguous array of 22.5-nm particles (the size of negatively stained 40S particles) are observed along the length of the RNA molecule (27, 28, 36, 45). As discussed by Osheim et al. (36), this finding indicates that the stable 25-nm structures located at splice junctions are not 40S hnRNP particles but are probably

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complexes of small nuclear ribonucleoprotein (snRNP), perhaps with remnants of an association of these structures with 40S hnRNP complexes.

To better characterize the ribonucleoprotein structure of nascent transcripts and to define the length of RNA packaged in 40S monomers, we have developed an in vitro assay for the assembly of HeLa cell hnRNP on RNA of defined length and sequence transcribed in vitro. Without the complication of heterogeneous-length RNA, the composition and physical properties of reconstituted hnRNP complexes have been monitored as a function of RNA length, protein/RNA ratio, reactant concentration, and sequence requirements. These studies demonstrate that the six major ribonucleoproteins of 40S particles function to package RNA, through a length-dependent mechanism, into a regular repeating particulate structure possessing a defined protein stoichiometry and containing approximately 700 RNA nucleotides per particle. Lengths of RNA which were not integral multiples of 700 nucleotides supported the assembly of nonstoichiometric structures which did not sediment as structurally homogeneous entities. Correct in vitro assembly of mono-, di-, and oligomeric hnRNP did not depend on reactant concentration, the presence of eucaryotic processing signals, a 5'-cap structure, a 3'-poly(A) moiety, or ATP as energy source. Correct assembly depended on RNA length and the protein/RNA ratio in the reaction mixture. While these finding argue that hnRNP functions to package nascent transcripts into a fundamental repeating structure, they do not demonstrate the absence of a role for hnRNP in splicing. The finding that monoclonal antibodies against the C proteins (7) and polyclonal antibodies against the core proteins of 40S hnRNP (43) block in vitro splicing support a role in splicing. The ability of hnRNP to package nascent transcripts in vitro into a regular repeating structure regardless of sequence suggests that *trans*-acting elements such as snRNP (not present in the in vitro reconstitution assay used here) may function in vivo to exclude hnRNP from reactive sites in the nascent transcripts or to phase particle assembly so as to accommodate RNA splicing.

MATERIALS AND METHODS

Plasmid constructions. Plasmids were constructed by using standard cloning techniques (29). The plasmid pMBG2020 was constructed in two steps: first by insertion of a *Sau*3A-*Bam*HI fragment of mouse β-globin (containing the first exon, first intron, and the majority of the second exon) into the *Bam*HI site of the polylinker in the vector pGEM1 and then by insertion of a 1,537-nucleotide *Bam*HI-*Xba*I fragment from λ-gt WES-MBG2 (containing the remainder of the globin gene; kindly provided by Philip Leder) behind the previous insert. The clone pAd2MLT-1967 was constructed by inserting the *Xho*I-*Bam*HI fragment of pAd2-BalIE (containing the first and second leaders and the first intron of the adenovirus type 2 major late transcript region) into the *Sal*I-*Bam*HI sites in the polylinker of pGEM1. This clone was then used in the construction of pAd2MLT-1487/IVS which contains a shortened form of the intron. The clone pAd2MLT-1967 was digested with *Bss*HII, which removed 491 nucleotides of intron sequence, and ligated to yield pAd2MLT-1487/IVS. The clone pRSP1 was a gift from P. Grabowsky and P. Sharp and has been described previously (15, 19). The plasmid pGL737b was provided by G. Lin and contains the gene 22/23 region of bacteriophage T4. Templates for RNA synthesis were linearized with restriction enzymes (see Fig. 2).

In vitro transcriptions. In vitro transcription with SP6 polymerase was performed in a 400-μl reaction under the following conditions: 40 mM Tris hydrochloride (pH 7.5), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 800 U of RNase (Promega Biotec), 90 μg of DNA template per ml, and 30 U of SP6 polymerase (New England Nuclear Corp.) at 40°C for 1 h. Transcriptions carried out with T7 polymerase were performed in a 400-μl reaction as described above with the following modifications: 50 mM NaCl, 8 mM $MgCl_2$, 40 mM Tris hydrochloride (pH 8.0), and 50 U T7 polymerase (Stratagene) at 37°C for 1 h. Following transcription, the template was removed by adding 320 additional units of RNase and DNase to a final concentration of 20 μg/ml and incubated at 37°C for 10 min. RNA was then extracted with phenol-chloroform-isoamyl alcohol (50:50:1), and unincorporated nucleotides were removed by spin-column chromatography on Sephadex G-50 (29) equilibrated in TE buffer (10 mM Tris hydrochloride [pH 8.0], 1 mM EDTA). Capped-RNA synthesis was performed with ^{7}mGpppG or GpppG (Pharmacia Fine Chemicals) as described previously (20). RNA was determined by optical density at 260 nm, and the optical density at 260 nm/optical density at 280 nm ratio was calculated as a measure of RNA purity. Prior to particle reconstitution, all transcription products were examined for correct length and absolute homogeneity by electrophoresis of 1 μg of RNA in 8 M urea sequencing-type gels, followed by staining in ethidium bromide and visualization with UV light (29). Typical 400-μl reactions with SP6 polymerase yielded 20 to 30 μg of RNA. A 400-μl reaction with T7 polymerase yielded 150 to 200 μg of RNA.

hnRNP reconstitution and particle analysis. HeLa cell culture and the isolation of monomer 40S hnRNP particles by the sonic disruption method were performed as described elsewhere (23, 25, 47). All experimental procedures were performed at 4°C unless stated otherwise. Pooled fractions from sucrose gradients containing 40S hnRNP were dialyzed against STM (90 mM NaCl, Tris hydrochloride [pH 8.0], 1 mM $MgCl_2$) to remove sucrose. The particles were then adjusted to 1 optical density at 260 nm per ml with STM and to 1 mM dithiothreitol and 1 mM $CaCl_2$. To remove endogenous RNA, micrococcal nuclease (Pharmacia) was added to a final concentration of 300 U/ml, and the particles were incubated for 40 min at 25°C. After incubation, the nuclease was inactivated by the addition of EDTA to 10 mM. The particle proteins were then placed on ice, synthetic RNA was added to the desired protein/RNA ratio and reactant concentration, and reconstitution was allowed to proceed for 1 h. Protein concentrations were assayed by the Bradford (5), Lowry (26), or micro-biuret (18) methods. Following reconstitution, samples were analyzed by sedimentation in sucrose density gradients. Reconstitutions performed with 36-, 73-, 454-, 512-, 685-, 726-, 736-, 801-, 962-, 1,119-, 1,287-, and 1,452-base RNAs were analyzed on 10 to 25% sucrose gradients spun for 16 h at 25,000 rpm in an SW28 rotor. Structures reconstituted on 1,509-base RNA were analyzed on 15 to 30% gradients, while reconstitutions on 1,999-base RNA was analyzed on 15 to 40% gradients; both were spun as described above. Reconstitutions on φX174 DNA were analyzed on 15 to 40% gradients spun as above but only for 10 h. Following centrifugation, fractions were collected from the gradients, and proteins were analyzed by electrophoresis as described previously (22). Sedimentation values were assigned by centrifugation of *Escherichia coli* ribosomes in parallel gradients.

UV cross-linking. Sucrose gradient fractions containing

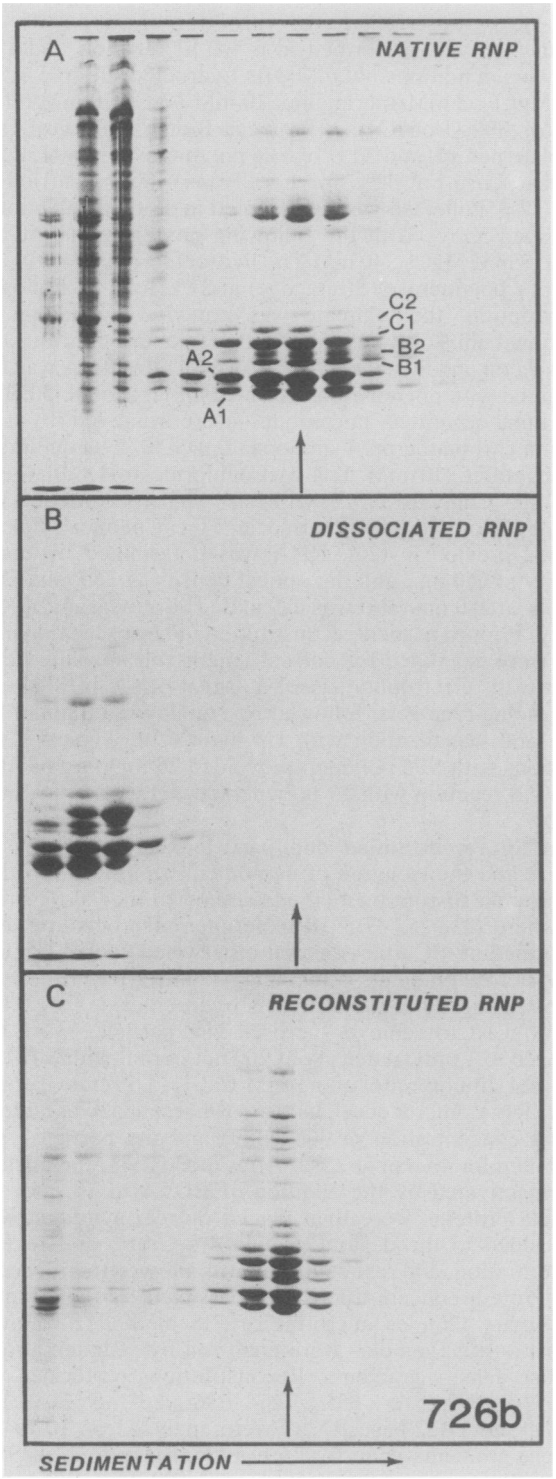

FIG. 1. Sucrose gradient analysis of intact, dissociated, and reconstituted 40S hnRNP particles. (A) Distribution and protein composition of intact 40S hnRNP in a 15 to 30% sucrose density gradient. The top of the gradient is at the left. The gradients were fractionated into 1.0-ml portions, and the protein present in each fraction was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8.75% polyacrylamide). The six major core proteins are labeled (A1, A2, etc.). The 40S position was determined by sedimentation of *E. coli* ribosomal subunits in separate gradients and is indicated by the vertical arrow. (B) Digestion with micrococcal nuclease of particles collected from 40S peak in panel A to dissociate the particle proteins. In this case, all protein and degraded RNA remained at the top of the gradient (left). (C) Inactivation of nuclease with EDTA and addition of 726-nucleotide RNA (726b) as the assembly substrate.

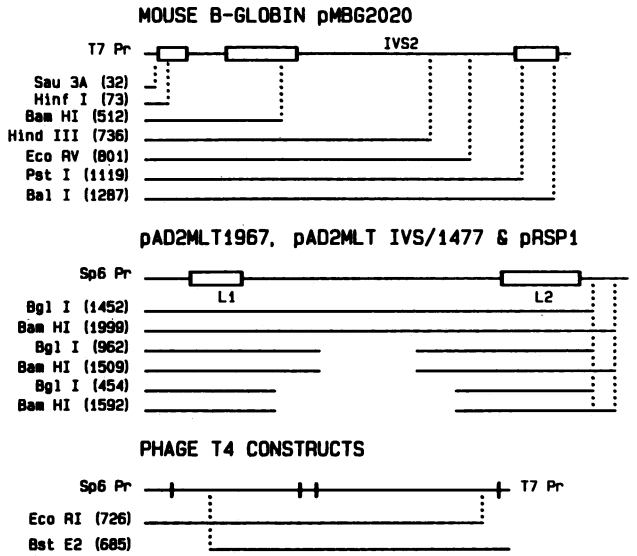

FIG. 2. DNA templates used for in vitro RNA synthesis. The RNA substrates used for in vitro hnRNP assembly were transcribed from the templates shown with SP6 or T7 polymerase. Transcript lengths and the restriction endonucleases used to truncate the templates are indicated at the left. Exons are noted by boxes. See Materials and Methods for details.

reconstituted monomers formed on 736-nucleotide globin RNA were irradiated for 10 min with 254-nm UV light at a dose of 2,000 mW/cm^2. Following irradiation, the NaCl concentration was adjusted to 0.7 M and the samples were sedimented on 5 to 25% sucrose gradients containing 0.7 M NaCl, 10 mM Tris hydrochloride (pH 8.0), and 1 mM EDTA. Fractions from the gradient were then digested with 50 μg of RNase A at 37°C for 1 h and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8.75% polyacrylamide).

Electron microscopy. Samples for electron microscopy were prepared as described in detail elsewhere (50). Reconstituted or native RNP in sucrose gradient fractions were fixed on 0.1% glutaraldehyde (electron microscopy grade; Polyscience) for 10 min. Samples were then absorbed to polylysine-coated grids, stained with 5% uranyl acetate, and platinum shadowed.

RESULTS

Conditions for in vitro assembly of hnRNP. In these experiments, sucrose gradient-purified 40S hnRNP particles from isolated HeLa cell nuclei were dissociated by complete digestion of the endogenous pre-mRNA moiety with micrococcal nuclease. The enzyme was then inactivated with EDTA before exogenous RNA was added. To demonstrate complete RNA digestion and subsequent particle dissociation, the RNA component of isolated 40S monoparticles was labeled in vivo with [^{3}H]uridine. No labeled nucleotides or protein were observed to enter gradients, with the conditions

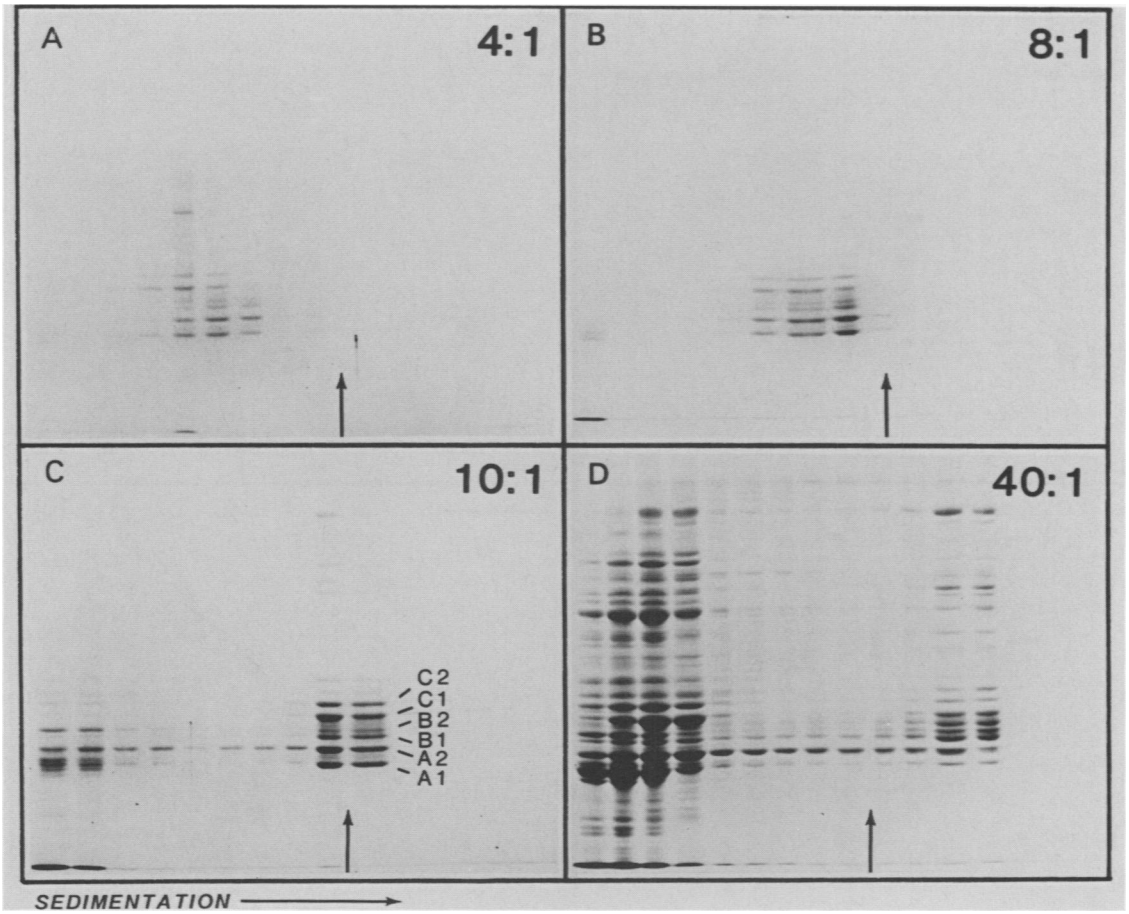

FIG. 3. Effect of protein/RNA ratio on 40S hnRNP assembly. (A to D) Protein compositions and sedimentation properties in 15 to 30% sucrose density gradients of hnRNP complexes formed at several different ratios of protein and RNA. Ratios are indicated in the upper-right corner of each panel. In each case, the top of the gradients are to the left. The vertical arrows indicate the 40S position. The RNA substrate was the 736-nucleotide transcript of mouse β-globin; 22 μg of RNA was used for each experiment. The six major core proteins are labeled in panel C.

used in the experiments (Fig. 1). The RNA substrates used for particle assembly were synthesized in vitro by using various gene constructs linked either to SP6 or to T7 promoters (34). Through the selective use of restriction endonucleases, the DNA templates were terminated at appropriate sites to yield 15 transcripts which varied in sequence and length from 32 to 1,999 nucleotides (Fig. 2).

To determine the optimum protein/RNA ratio for in vitro particle assembly, transcripts of 685, 726, or 736 nucleotides were used. The choice of these transcript lengths was based on our previous studies showing that the RNA fragments recovered from sucrose gradient-isolated 40S monoparticles ranged from 500 to 800 nucleotides, with most fragments being about 700 nucleotides in length (3, 23, 47). All three transcript lengths gave the same results (Fig. 3).

At a low protein/RNA ratio (4:1), proteins associated with all of the added RNA, forming two classes of structures: a slower sedimenting structure composed mostly of proteins of the A and C groups and a faster sedimenting structure enriched in proteins of the A and B groups (Fig. 3A). At an 8:1 ratio (Fig. 3B), a complex was formed which sedimented as a broad peak slightly less than 40S but which possessed nearly correct protein stoichiometry. At ratios from 10:1 to 20:1 (Fig. 3C), a homogeneous 40S structure was formed which possessed a protein composition and stoichiometry identical to those of isolated monoparticles. Under these conditions, protein was in a slight to moderate excess, so that unbound protein can be seen at the top of the gradient. An important point to note here is that when the soluble RNP proteins were not initially present in a perfect 3:3:1:1:3:1 ratio (because of overdigestion of the starting monoparticle preparation in the course of its isolation), the final protein stoichiometry of the reconstituted structure at the 10:1 ratio was correct. When reconstitution was performed under a large protein excess (40:1), a 45S complex highly enriched in the B proteins was formed and most of the major particle proteins (A1, A2, and C1) remained dissociated at the top of the gradients. All reconstitutions described below were performed at input protein/RNA ratios between 10:1 to 20:1.

Although this range of protein/RNA ratios is optimal for particle reconstitution, it does not imply that the protein/RNA ratio of the product is the same as the starting ratio in the reconstitution mixture. Analysis of the protein/RNA ratio of RNP formed on the 736-nucleotide RNA indicates a 6:1 ratio (data not shown). Some minor high-molecular-weight proteins which were not seen in the reconstituted product remained at the top of gradients. These proteins may be components of snRNPs or other contaminating structures or they may be true hnRNP-associated proteins that did not

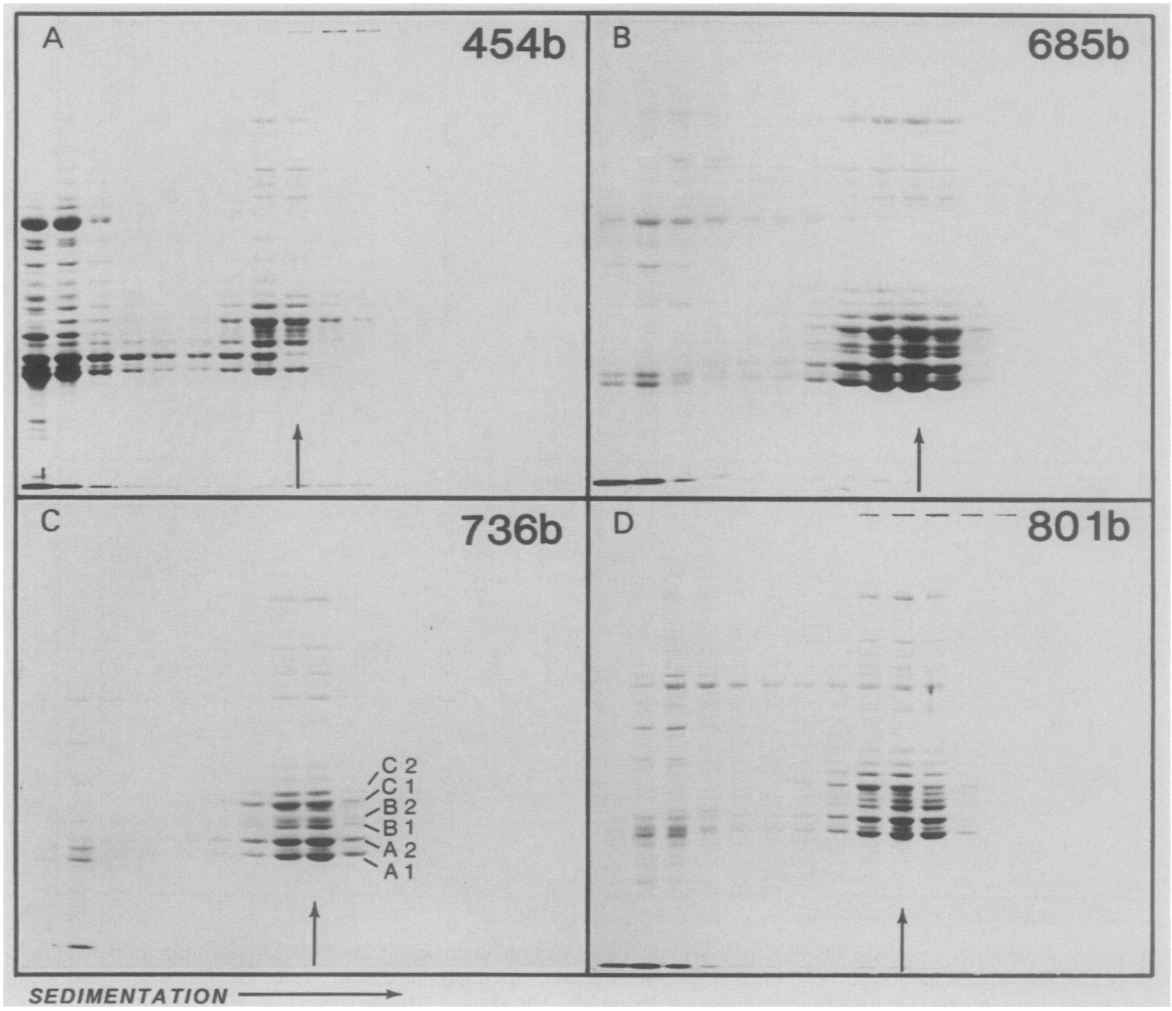

FIG. 4. hnRNP assembly on RNA substrates with lengths less than, nearly equal to, or slightly longer than monoparticle length. (A to D) Sedimentation properties and protein compositions of hnRNP complexes formed on RNA substrates of increasing length transcribed from the templates shown in Fig. 2. The gradients were 10 to 20% sucrose, and the top of each gradient is to the left. RNA lengths are shown in the upper right of each panel (454b, 454 nucleotides). The vertical arrows indicate the 40S position in each gradient. The six major proteins are labeled in panel C. All reconstitutions were performed at a protein/RNA ratio of 10:1.

reassemble under the conditions employed. In previous studies on the in vitro assembly of hnRNP particles, Wilk et al. (49) observed that most of the minor high-molecular-weight proteins fail to resediment with core particles after the suspension of pelleted material.

The effect of reactant concentration was examined over a 1,000-fold range. Reconstituted monomers that sedimented at 40S and that possessed the same protein stoichiometry as isolated hnRNP were obtained whether the protein and RNA concentrations were 12 and 1 μM, respectively, or 12 and 1 nM, respectively. This insensitivity to reactant concentrations was observed when RNAs of various lengths were used. The studies described below were performed near a target of 1.2 μM protein and 100 nM RNA (80 to 200 μg of protein per ml and 8 to 20 μg of RNA per ml).

To determine the effect of the 5′-cap structure on the fidelity of hnRNP assembly, capped (^{7}mG and unmethylated) and uncapped substrates of 685, 962, and 1,509 nucleotides were compared. The presence of a cap had no detectable effect upon reconstitution for the substrates tested. All further studies were carried out with uncapped RNA.

Effect of RNA length on hnRNP assembly. The effect of RNA length on in vitro particle assembly was examined by using 15 RNAs transcribed in vitro from gene constructs cleaved with restriction enzymes (Fig. 2). Figures 4 and 5 show the sedimentation characteristics and the protein compositions of the complexes formed on 8 of the 15 transcripts tested. For the shorter RNA molecules (Fig. 4), 10 to 20% gradients were used, and all were subjected to the same spin conditions. For the dimer and oligomeric structures (Fig. 5), various gradients and spin conditions were used to obtain optimal separations (see legends to Fig. 4 and 5 and Materials and Methods). Arrows in these figures denote the 40S position as determined by the sedimentation of *E. coli* ribosomal subunits in parallel gradients.

Although all lengths of RNA supported the assembly of RNP complexes, only multiples of approximately 700 nucleotides supported the assembly of stoichiometric structures. For example, short RNA molecules of 36, 56, or 73 nucleotides transcribed from mouse β-globin or pGEM constructs supported the assembly of nonstoichiometric structures which sedimented in a heterodisperse fashion from the top of gradients to a 30S peak. Complexes which formed on these short RNA molecules contained mostly proteins A1, C1, and

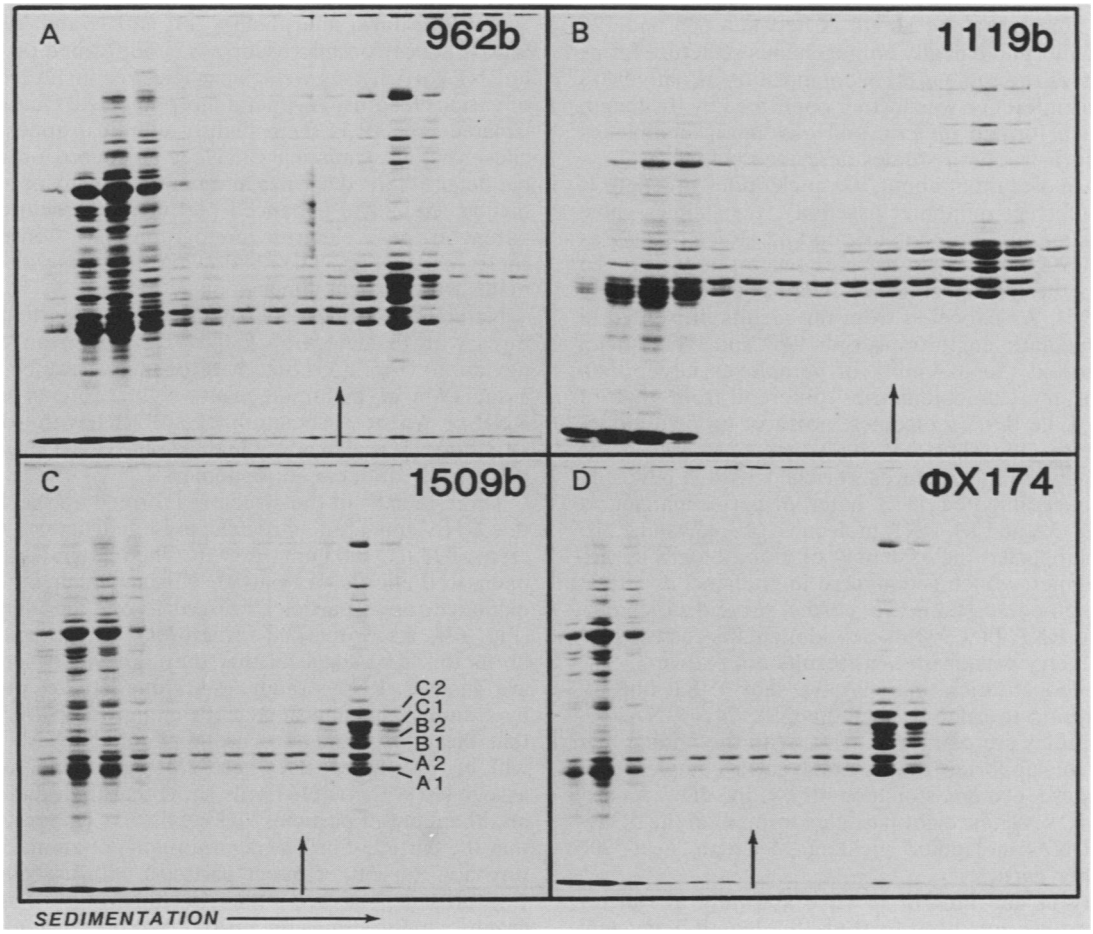

FIG. 5. hnRNP assembly on RNA substrates longer than the monomer. (A to D) Sedimentation properties and protein compositions of hnRNP complexes assembled on RNA substrates longer than those used for Fig. 4. Other details are as described in the legend to Fig. 4, except that for panels C and D the gradients were 15 to 30% and 15 to 40%, respectively.

C2 (data not shown). All of these short RNA molecules supported the assembly of complexes with similar sedimentation characteristics and protein compositions. In these experiments, greater than 90% of the added RNA sedimented with structures at 30S, a sedimentation coefficient which indicates that the complexes contain multiple RNA molecules. The formation of complexes with RNA fragments suggests that polymer continuity is not a requirement for particle assembly and stability. This observation is consistent with a previous report that particle-associated RNA can be cleaved to average lengths near 125 nucleotides before particle dissociation occurs (6).

With a 454-nucleotide RNA substrate, transcribed from the adenovirus major late region, two poorly resolved complexes were formed (Fig. 4A). One structure enriched in proteins A1, B1, and C sedimented at approximately 40S, while a complex enriched in A2 sedimented more slowly. In one fraction, the two protein stoichiometries overlapped, forming a ratio similar to that of native 40S hnRNP. However, as described below, this fraction possessed two morphologically distinct structures which may correlate with the two poorly resolved protein ratios. The results described here for the 454-nucleotide adenovirus transcript were also observed for a 512-nucleotide transcript of the mouse β-globin gene, except that the latter complexes sedimented slightly faster in gradients.

A 685-nucleotide transcript of gene 22/23 of T4 phage supported the quantitative assembly of complexes which sedimented at 40S in a peak as homodisperse as that of native 40S monoparticles. These particles contained the six core particle proteins in the same stoichiometry as that found in native hnRNP (protein A1/A2/B1/B2/C1/C2 ratio of 3:3:1:1:3:1; compare Fig. 4B with Fig. 1A and C). In contrast to the structures formed on shorter RNA molecules, the majority of the protein and RNA sedimented about a peak centered at 40S, and complexes above, within, and below the peak possessed the same protein composition. Nonstoichiometric structures enriched in proteins A1 and C or the A-group proteins did not form on this RNA length. The same results were obtained when a 726-nucleotide mouse β-globin transcript was used as the assembly substrate (Fig. 1C).

Slightly longer globin transcripts of 736 and 801 nucleotides supported the assembly of complexes which sedimented at 40S and which possessed nearly correct protein stoichiometry (Fig. 4C and D). However, on the heavier side of the 40S peak, structures enriched in proteins A1, A2, and B1 appeared. This effect was more pronounced with the 801-nucleotide RNA, and this finding suggests that the A-group proteins may be the first proteins to bind RNA during particle assembly.

The findings described above for the in vitro assembly of 40S hnRNP particles on RNA substrates of increasing length

demonstrate that when the length is between 685 and 726 nucleotides a morphologically homogeneous structure forms which possesses the same protein composition as native 40S particles. This inference was further confirmed by the length of RNA which formed dimeric and oligomeric complexes and by the ultrastructural studies described below.

If an RNA molecule of about 700 nucleotides in length is required for correct monomer assembly, then dimers possessing the same protein composition and stoichiometry as monomers should form on substrates twice this length. To test this hypothesis, longer RNAs were used in assembly studies (Fig. 5). As expected from the results presented in Fig. 4, intermediate-length transcripts (962 and 1,119 nucleotides) supported the assembly of complexes larger than 40S, with protein stoichiometries different from that of native RNP. Like RNA molecules too short to yield monomers, substrates too short to yield dimers supported the assembly of aberrant structures enriched with A1-A2 and A1-C, which sedimented in a heterodisperse manner in gradients (Fig. 5A and B). RNA molecules of 1,452 and 1,509 nucleotides supported the assembly of morphologically homogeneous dimers which sedimented in gradients as a tight band and which possessed nearly correct protein stoichiometry. With an RNA of 1,999-nucleotide length, correct protein stoichiometry was again lost (results not shown).

Previous reconstitution studies have shown that hnRNP proteins will bind to ssDNA, including φX174 ssDNA (39, 40). These studies are confirmed here, with the finding that φX174 ssDNA supported the assembly of a complex possessing standard protein stoichiometry (Fig. 5D). As described below, seven or eight particles formed on the 5,386-nucleotide DNA molecule, yielding a length near 700 nucleotides per particle.

Insensitivity of 40S hnRNP in vitro assembly to splicing signals. The transcripts used to study the length-dependent patterns of RNP assembly (Fig. 2) also showed that RNA packaging in vitro occurred in a sequence-independent manner. If particle assembly depends on exon sequences, intron sequences such as the branch site or the polypyrimidine sequence, or exon-intron junctions, then transcripts with the same splice consensus sequences and exons should be assembled into identical RNP structures. The sets of RNAs transcribed from the adenovirus templates were identical with respect to these sequences, except for the presence or absence of a central region in the first intron, shown to be dispensable for splicing (15, 20). Despite identical consensus sequences and exons, each transcript yielded RNPs differing (as a function of length) in protein stoichiometry and sedimentation coefficient. This finding indicates that RNP does not assemble solely on exons or splicing signals. The possibility that nonconserved intron sequences direct RNP assembly was eliminated by reconstitutions on the 1,119- and 1,287-nucleotide globin transcripts. These RNAs were identical with respect to intron sequences but differed by the presence of an extra exon sequence in the 1,287-nucleotide RNA. Despite having identical introns, these RNAs yielded different hnRNPs. Further evidence for sequence-independent assembly are reconstitutions on 685- and 726-nucleotide transcripts of the gene 22/23 region of phage T4. These transcripts possessed no eucaryotic splicing signals, yet like the 736-nucleotide β-globin transcript, they supported efficient and stoichiometric RNP assembly (Fig. 1C and 4B). The observation that RNP assembly can occur on phage ssDNA also indicates that it is the single-stranded nature of nucleic acids which hnRNP recognizes and not the sequence or the presence of a 2′ hydroxyl group or a uridine moiety.

Ultrastructural morphology of native and reconstituted hnRNP. Electron microscopy was performed on native 40S hnRNP particles, as well as particles reconstituted on RNAs of various lengths. A general morphological characteristic of isolated hnRNP is their slightly elliptical appearance. Because of this, statistical analyses are based on an average diameter obtained by measurement of the major and perpendicular axes. The enhanced peripheral particle density apparent in these electron micrographs is a consequence of rotary shadowing. The shadowing technique also overestimates true particle dimensions.

Native 40S monoparticles appeared as particulate structures with diameters of 26.0 ± 2.1 nm (mean ± standard deviation) (Fig. 6A). Size heterogeneity may be due to the association of high-molecular-weight proteins with native RNP or to the association of snRNPs with some of the structures. It is also possible that some of the structures may be partially dissociated monomers.

Three-fourths of the structures formed on the 454-nucleotide RNA appeared as regular closed structures with diameters of 23.0 ± 0.95 nm (n = 80). The remainder appeared as open RNP fibrils, as particles from which an RNP fibril extended, or as particles bridged to one another by a fibril (Fig. 6B, arrow-marked structures). The diameter of the fibrils (6.0 nm) suggests that they are not naked RNA but are, instead, RNP strands. Additional studies on the fibrils by scanning transmission electron microscopy also indicates that these structures are protein-coated RNA. These data will be presented elsewhere. Measurements of particles associated with visible fibrils show that their diameter is not less than that of particles lacking fibrils. This result suggests that the particle-fibril configuration is not an unfolded structure but consists of intact particles with associated fibrils. The presence of free fibrils in numerous electron micrographs supports this conclusion. The presence of two structures in the peak fraction of particles assembled on the 454-nucleotide RNA may correlate with the appearance of the two complexes poorly resolved in sucrose gradients (described above). Fibrils were observed with the 454-nucleotide RNA but not with longer transcripts. This discrepancy perhaps suggests that there was insufficient length for subunit assembly into a closed structure.

Particles formed on the 736-nucleotide RNA were 24.0 ± 0.84 nm in diameter (n = 80). In this case, the small standard deviation is consistent with the structural homogeneity apparent from the monodisperse pattern of sedimentation in gradients. These structures possessed nearly perfect monoparticle protein stoichiometry (Fig. 3C and 4C). Structures reconstituted on the 962-nucleotide RNA were similar (24.0 ± 1.0 nm in diameter; n = 80) to those formed on the 736-RNA but with smaller particles abutting the monomer (Fig. 6D, arrows).

As RNA length increased, the smaller particle associated with monomers increased in size, approaching the dimensions of a second complete particle. On an RNA with length approximately twice that of the monomer, i.e., 1,509 nucleotides (Fig. 7A), we found dimers of the same size (24.0 ± 1.3 nm; n = 80) indistinguishable from monomeric complexes formed on an RNA half this length (compare with monomers in Fig. 6C). These dimers possessed a protein stoichiometry similar to that of monomers (Fig. 4C).

Analysis of complexes formed on φX174 ssDNA shows that this 5,386-nucleotide substrate is packaged in seven or eight particles (diameter, 23.0 ± 1.8 nm; n = 80). When eight particles were present, one was usually smaller than the others. The presence of seven to eight particles

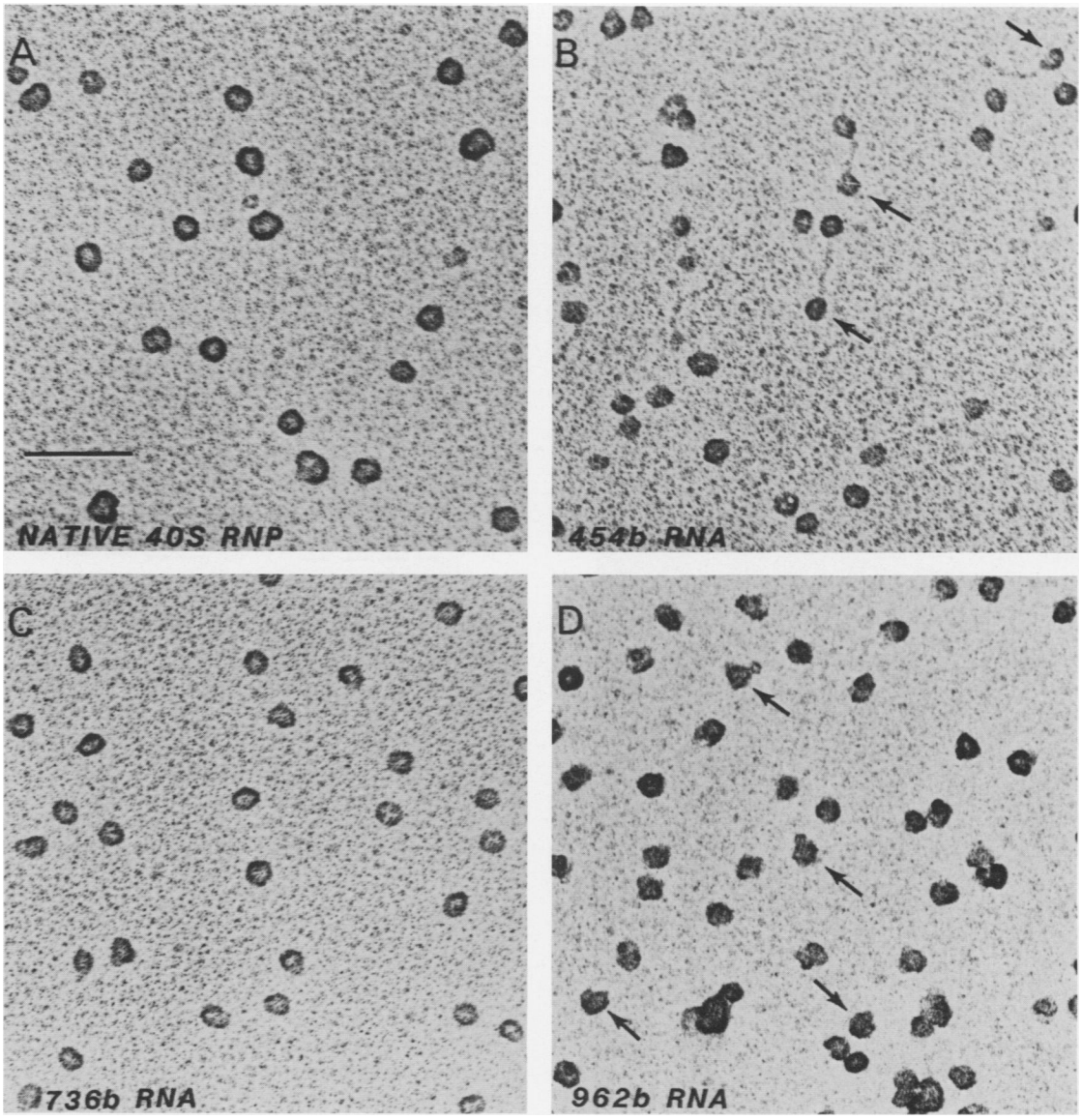

FIG. 6. Electron micrographs of native and reconstituted hnRNPs. Reconstitutions were performed as described in the legends to Fig. 4 and 5, and the products were analyzed by sucrose gradient centrifugation. The peak gradient fractions were pooled, and the samples were fixed with 0.1% glutaraldehyde. Samples were absorbed to polylysine-coated grids, stained with 5% uranyl acetate, and platinum shadowed. The RNA substrates used are indicated in the lower left corner of each panel. Native 40S hnRNP were prepared as described in the legend to Fig. 1A. The bar in panel A represents 100 nm. Particles associated with RNP fibrils are noted with arrows in panel B, and in panel D, arrows identify particles with smaller particles attached.

in this complex is consistent with there being approximately 700 nucleotides of RNA packaged in one 40S monomer.

Fidelity of in vitro hnRNP assembly. As shown above, when the RNA substrate was between 685 and 726 nucleotides in length and when the protein/RNA ratio in the reaction mixture was near the range from 10:1 to 20:1, monomers assembled in vitro which possessed the same sedimentation coefficient, protein stoichiometry, and ultrastructural morphology as those of native 40S hnRNP. Also like native particles, the particles which assembled in vitro dissociated upon RNA digestion and showed the same order of protein dissociation upon increasing ionic strength (3). As a further test for correct protein-nucleic acid interaction during in vitro assembly, we have examined the ability of the C proteins to preferentially cross-link to RNA upon moderate UV irradiation. It has previously been shown that the C proteins interact with RNA such that upon UV irradiation these proteins are preferentially cross-linked to RNA (6, 11, 33, 46). This situation is true for hnRNP cross-linked in vivo and also for isolated hnRNP.

Monomers were reconstituted on the 736-nucleotide mouse β-globin transcript, isolated by sucrose gradient sedimentation, and subjected to UV irradiation by the procedure of Choi and Dreyfuss (6). Following irradiation, the salt concentration was raised to 0.7 M to dissociate non-cross-linked protein (1). This material was analyzed on a 5 to 25% sucrose gradient containing 0.7 M NaCl (Fig. 8). A C protein-RNA complex was resistant to the conditions of high salt concentration and sedimented away from the non-cross-

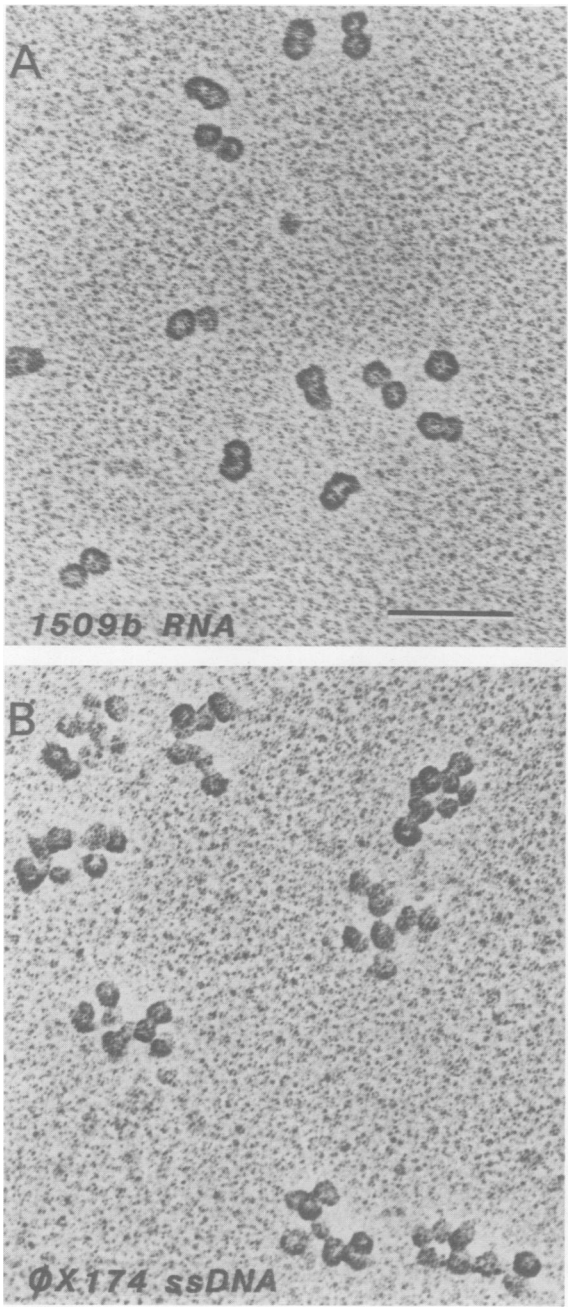

FIG. 7. Electron micrographs of dimers and oligomeric complexes. (A) Dimers which assemble on 1,509-nucleotide RNA substrate. (B) Oligomeric complexes which assemble on ϕX174 ssDNA. Details are as described in the legend to Fig. 6. The bar in panel A represents 100 nm.

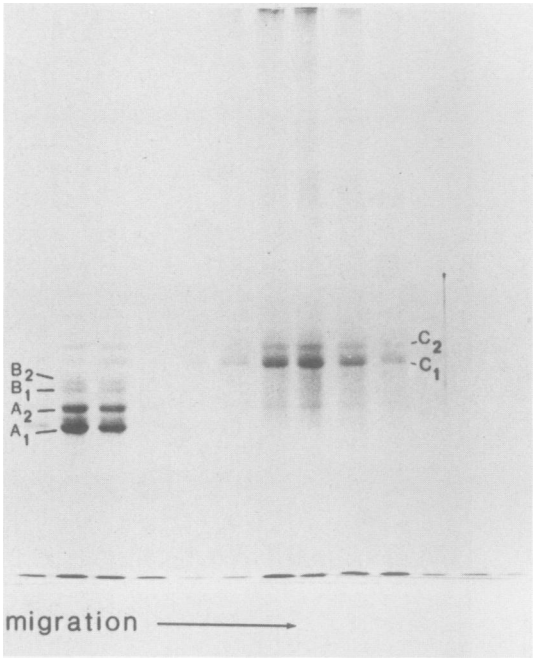

FIG. 8. Cross-linking of C proteins induced by UV irradiation. 40S particles were assembled on the 736-nucleotide transcript of mouse β-globin, collected from 15 to 30% sucrose density gradients, and irradiated as described in Materials and Methods. The salt concentration was increased to 0.7 M, and the irradiated particles were sedimented through a 10 to 20% gradient containing 0.7 M NaCl. Non-cross-linked protein is seen at the top of the gradient (left) in a dissociated state, and the C protein-RNA cross-linked complex is seen to sediment away from the dissociated protein.

linked A- and B-group proteins which remained at the top of the gradient. Identical results were seen with native 40S hnRNP particles.

DISCUSSION

The observations described above demonstrate that the six major core proteins of HeLa 40S hnRNP particles function to package approximately 700 nucleotides of RNA into a fundamental monomeric unit. If nascent transcripts are packaged in vivo by hnRNP to form a fundamental repeating structure, then homogeneous dimers should form in vitro on RNA with length twice that of the monomer, and on long substrates, the length should be divisible by the number of particles formed to yield a value near 700 nucleotides; the particles formed should also be morphologically identical to monoparticles and should look like those observed in gently spread transcriptive units. These predictions are confirmed in this by electron microscopy and by the observation that mono-, di-, and polyparticle protein compositions are the same. The finding that intermediate-length RNA supported the assembly of nonstoichiometric complexes which sedimented in gradients as structurally heterogeneous complexes further supports the conclusion that RNA functions as a vernier for the stoichiometric self-assembly of protein into a regular stable structure. Evidence which confirms this conclusion is the ability of the proteins to form 40S particles possessing the same stoichiometry (protein A1/A2/B1/B2/C1/C2 ratio of 3:3:1:1:3:1) as intact monomers even though the molar ratio of protein in the reaction mixture was intentionally skewed (i.e., when the protein mixture was deficient in A1 and the C-group proteins). This ability was observed whenever the RNA substrate was limiting and had a length that was an integral multiple of approximately 700 nucleotides.

These observations indicate that the primary structure of pre-mRNA is determined by hnRNP and that nascent transcripts exist as a contiguous array of repeating structural units which may self-associate to form larger aggregate structures, perhaps synonymous with very large RNP structures termed perichromatin granules. This contention is

supported by the high intranuclear concentration of the core hnRNP proteins, by electron micrographs of gently spread transcriptive units (36) showing a contiguous array of 22-nm particles, by electron micrographs of native hnRNP released from nuclei showing complexes (45) which look like the oligomeric complexes observed in this study, by the generation of 40S particles from very large hnRNP complexes, and by studies indicating that perichromatin granules probably contain hnRNP particles (9, 13, 14).

The findings reported here confirm the extend those of two previous studies on the in vitro assembly of hnRNP complexes. As in the present study, Wilk et al. (49) dissociated isolated monoparticles with micrococcal nuclease but used various ribopolymers and MS2 phage RNA as the assembly substrate. These investigators concluded that monoparticles package 900 to 1,200 nucleotides of RNA in a sequence-independent manner. In the studies of Pullman and Martin (39), the ionic detergent sodium deoxycholate was used to dissociate hnRNP complexes and the endogenous RNA was removed with micrococcal nuclease. After the addition of tobacco mosaic virus RNA, globin mRNA, ϕX174 ssDNA, or homoribopolymers, the detergent was sequestered with Triton X-100 to achieve particle assembly. As in the study of Wilk et al. (49), the length of nucleic acid in monoparticles was estimated from electron micrographs of oligomeric complexes to be near 1,000 nucleotides. In the study of Pullman and Martin (39), particle assembly was not reported to be dependent on the protein/RNA ratio. The length of RNA per monoparticle reported here (700 ± 20 nucleotides [mean ± standard deviation] was determined by identifying the length required for the assembly of monoparticles and dimers possessing correct protein stoichiometry and confirmed by counting the number of particles on long nucleic acid substrates.

As shown here, the correct in vitro assembly of hnRNP depends on the protein/RNA ratio and transcript length but not on reactant concentration, nucleotide sequence, or the presence or absence of exons, introns, a 5′-cap structure, a 3′-poly(A) moiety, or ATP. Scanning transmission electron micrographs of fixed but unstained 40S hnRNP reveal a particle diameter of 18 nm (51). This finding, together with the length of RNA in monoparticles, yields an RNA packaging ratio near 12:1 if one assumes that the spacings of nucleotides in RNA and in DNA are similar. It is interesting to note that the packaging ratio of DNA in nucleosomes is also 12:1. As reported previously (39, 49) and again in this paper, the core proteins also do not discriminate between ssDNA and RNA. The finding that proteolytic fragments of proteins A2 and A1 possess DNA-unwinding activities seems consistent with this lack of discrimination (21, 40). It has also been demonstrated that HD-40, an A-group-like RNP from *Artemia* species melts RNA secondary structure at appropriate protein/RNA ratios (44).

Several observations point to important protein-nucleic acid and protein-protein interactions during particle assembly. That RNA activates protein-protein binding domains is seen in the complete particle dissociation which occurred upon RNA removal with nuclease. The same result is achieved by placing isolated or reconstituted particles in 0.8 M salt (23, 25). Evidence of regular cooperative protein interactions is apparent in the existence of a minimum RNA length for stoichiometric subunit assembly and aggregation. Examples of unique protein interactions are also evident when in vitro assembly was attempted under conditions of excess RNA. In this case, two complexes were formed: a slower sedimenting structure composed of proteins A1, C1, and C2 and a faster sedimenting structure enriched in the A- and B-group polypeptides. This finding is consistent with a degree of limited cooperativity during protein assembly. Under conditions of protein excess (above 20:1), a faster sedimenting complex (60 to 80S) formed in which the A- and C-group proteins were partially displaced by the B-group polypeptides. This result indicates that the individual proteins possessed different affinities for RNA.

The nonstoichiometric complexes which formed on RNA with lengths other than multiples of approximately 700 nucleotides also reveal information which may relate to protein interactions during particle formation. When RNAs with lengths considerably different from monomer length were used as the assembly substrate (i.e., 454, 512, 962, 1,119 nucleotides), a common feature was the appearance of structures rich in the A-group proteins which sedimented throughout upper gradient fractions. This was especially true for RNAs with lengths below 454 nucleotides. This finding suggests that the A-group proteins may be the first to associate with RNA. An observation supporting this possibility is the enrichment of these proteins in a subset of particles which formed on RNA with length slightly longer than the monoparticle length. For example, the complexes formed on the 736- and 801-nucleotide RNAs showed A-group protein enrichment on the heavy shoulder of the 40S peak. This result suggests that the small blebs attached to the larger particles formed on the 962-nucleotide substrates may be enriched in the A-group proteins. However, the heavy shoulder in gradients of these structures was enriched in A1 and the C-group proteins. This observation, namely, the appearance of A1-, C1-, and C2-rich complexes which often smeared to the bottom of gradients, was actually a second feature common to complexes which formed on RNAs of incorrect length. These events suggest that two binding interactions may be involved in particle formation: an interaction involving the A-group proteins and one involving A1, C1, and C2. As pointed out above, the A1-A2 and A1-C1-C2 binding groups seem to bind separately under conditions of excess substrate and form separate complexes which overlap in gradients. Complexes enriched in A2 and the C-proteins were never observed.

Several questions regarding RNA metabolism arise if hnRNP packages RNAs of 700-nucleotide length in a sequence-independent manner. For transcripts with short introns and exons, both may be packaged in one 40S particle. If intron excision requires the association of snRNP with consensus sequences and branch sites (see references 30 and 42 for reviews), it is not likely that the regular cooperative forces involved in hnRNP assembly can accommodate these structures. Rather, hnRNP may be excluded from reactive sites by snRNP or from other phasing elements such that intact monomers may exist only on long runs of coding and noncoding RNAs. 40S monomers are composed of repeating structural units each containing three A1s, three A2s, one B1, one B2, three C1s, and one C2 (24, 25); thus if individual proteins were to bind RNA in a sequence-specific manner, the sequence must be repeated several times for each unit of 700 nucleotides. The absence of this phenomenon is consistent with the absence of a sequence requirement for hnRNP assembly.

Previous ultrastructural findings (1, 2, 4, 27, 28) and several observations reported here suggest that ribonucleoproteins are likely to be present on short RNAs separating phasing elements and that this RNA may exist as an open fibrillar structure or as an aggregated complex. For example, the 454-nucleotide adenovirus transcript which is spliced in

HeLa nuclear extracts (15, 16) supported the assembly of both open fibrils and aggregated particulate structures. Ribonucleoproteins (especially the A-group proteins) also associated with RNAs with lengths at least as short as 36 nucleotides in vitro and apparently with the excess short-length RNA present in transcripts with lengths intermediate between monomer and dimer lengths. The existence of a minimum intron length (48) may correlate with a structural unit of 40S hnRNP. In this context, intact 40S hnRNP complexes appear not to exist as a prerequisite for splicing since the 60S spliceosome assembled on the 454-nucleotide adenovirus transcript (16) probably contains only a partial hnRNP structure.

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