MOLECULAR AND CELLULAR BIOLOGY, Feb. 1991, p. 864–871
0270-7306/91/020864-08$02.00/0

Vol. 11, No. 2

The Core Proteins A2 and B1 Exist as $(A2)_3B1$ Tetramers in 40S Nuclear Ribonucleoprotein Particles

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Received 7 June 1990/Accepted 31 October 1990

The six "core" proteins of HeLa cell 40S nuclear ribonucleoprotein particles (hnRNP particles) package 700-nucleotide lengths of pre-mRNA into a repeating array of regular particles. We have previously shown that the C proteins exist as anisotropic tetramers of $(C1)_3C2$ in 40S hnRNP particles and that each particle probably contains three such tetramers. We report here that proteins A2 and B1 also exist in monoparticles as $(A2)_3B1$ tetramers and that each monoparticle contains at least three such tetramers. Proteins A2 and B1 dissociate from isolated monoparticles as a stable tetramer upon nuclease digestion. In low-salt gradients, the tetramers sediment at 6.8S, which is consistent with a mass of 145 kDa. In 200 mM salt, the concentration which dissociates these proteins from RNA, only 4.2S dimers exist in solution. Tetramers of $(A2)_3B1$ possess the ability to package multiples of 700 nucleotides of RNA in vitro into an array of regular, 22.5-nm 43S particles. Unlike the in vitro assembly of intact 40S hnRNP, the $(A2)_3B1$ tetramers assemble by means of a highly cooperative process. These findings indicate that the $(A2)_3B1$ tetramers play a major role in hnRNP assembly and they further support the contention that 40S monoparticles are regular structures composed of three copies of three different tetramers, i.e., 3[$(A1)_3B2$, $(A2)_3B1$, $(C1)_3C2$].

The pre-mRNA transcripts present in isolated nuclei from actively growing HeLa cells are recovered from 0.1 M salt extracts or from nuclear sonicates as 40 to 250S ribonucleoprotein complexes (for reviews, see references 9, 12, 13, and 14). After mild RNase activity, the faster-sedimenting complexes are converted to monomer particles that can be recovered as a single peak centered at 40S in sucrose density gradients (4, 16, 23). When isolated by these procedures, 40S nuclear ribonucleoprotein particles (hnRNP) are primarily composed of multiple copies of six major nuclear proteins which are present in a fixed molar ratio and which migrate in sodium dodecyl sulfate (SDS)-containing gels as three groups of doublet bands (the A-, B-, and C-group proteins) with molecular weights between 30 and 40 kDa (1, 2, 4, 10, 15, 16, 22, 25, 26). These observations, together with electron micrographs of gently spread transcriptive units showing a contiguous array of 22-nm particles on nascent transcripts (18, 24), argue that pre-mRNA is packaged in vivo into a contiguous array of regular particles composed of repeating structural elements with a molar protein ratio of 3A1, 3A2, 1B1, 1B2, 3C1, and 1C2 (1, 10, 26). More recent evidence that nascent transcripts are packaged into a regular repeating structure has come from in vitro assembly studies. More specifically, the six major "core" particle proteins of HeLa 40S hnRNP self-assemble in vitro by means of a mechanism which is highly dependent on RNA length and protein/RNA ratio but not on nucleotide sequence or reactant concentration (10). Monoparticles reconstituted on 700 ± 20 nucleotides of RNA possess the same protein stoichiometry, sedimentation coefficient, ultrastructural morphology, UV light-induced protein-RNA cross-links, and salt-induced pattern of protein dissociation as native hnRNP particles. Dimers and oligomeric complexes form on integral multiples of this length, while RNA substrates of intermediate length support the assembly of nonstoichiometric complexes which sediment in a heterodisperse manner in sucrose density gradients. The particular character of this length dependence, whereby complete particles assemble on all available 700-nucleotide lengths of RNA, indicates an important role for RNA in the control of particle assembly and strongly suggests that RNP complexes form as regular repeating structures in vivo. Related work also supports this inference (22, 25).

To achieve a detailed view of pre-mRNA processing and to understand how the processing machinery gains access to packaged RNA, it will be necessary to understand the structural aspects of pre-mRNA packaging. A more-detailed view of packaged pre-mRNA has begun to emerge as the result of efforts to purify and characterize the structural components of hnRNP particles. We have recently purified the core proteins C1 and C2 and have shown that they exist as tetramers of $(C1)_3C2$ in native 40S hnRNP particles (1–3). It has also been shown that C1 and C2 are encoded by the same gene but that, through an alternate splicing event, C2 contains a 13-amino-acid insert sequence (7, 21).

In this report we describe the purification of the core proteins A2 and B1 and show that they also exist as tetramers in 40S hnRNP particles. The $(A2)_3B1$ tetramers sediment at 6.8S in low-ionic-strength sucrose density gradients with an apparent mass of 144 kDa. This compares favorably with a calculated mass of 145 kDa based on the amino acid sequence of proteins A2 and B1 (7). At 0.09 M NaCl, the tetramers exist in equilibrium with A2-A2 and A2-B1 dimers, while at 0.2 M NaCl, a salt concentration which completely dissociates these proteins from RNA (4), only the 4.2S dimers exist in solution. The $(A2)_3B1$ tetramer alone possesses the ability to package RNA into a repeating array of regular particles which, like native particles, contain 700 nucleotides of RNA. These particles, however, sediment slightly faster in sucrose density gradients (43S), they possess a higher protein/RNA ratio, and they appear larger in electron micrographs than do native or reconstituted 40S hnRNP particles. The 3:1 ratio of proteins A2 and B1 in

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native hnRNP is preserved in the 43S structure. A significant difference between the in vitro assembly of hnRNP and the 43S structure is that the latter assembles in a highly cooperative manner. For example, under conditions of RNA excess, complete 43S monoparticles assemble on a small percentage of the RNA molecules. The excess RNA, but no protein, remains at the top of gradients. Under conditions of excess protein, all RNA is packaged into 43S particles, leaving the unincorporated $(A2)_3B1$ tetramers near the top of the gradients. These findings suggest that RNA-activated protein-protein contacts between tetramers of $(A2)_3B1$ may play a major role in the assembly of hnRNP particles in vivo and they extend the evidence that 40S hnRNP particles are composed of three different tetramers, i.e., $n[(A1)_3B2, (A2)_3B1, (C1)_3C2]$, where n is probably 3.

MATERIALS AND METHODS

Cell culture and nuclear isolation. HeLa cells were grown in suspension culture in minimal essential medium containing penicillin G (25 U/ml) and streptomycin (25 μg/ml) supplemented with 5% calf serum. Cells in log-phase growth were harvested at densities of 6×10^5 cells per ml and stored at −70°C in 50% glycerol. For nuclear isolation, 1.5×10^9 to 2.0×10^9 thawed cells were washed twice in 360 ml of a solution containing 2.0 mM Tris hydrochloride (pH 7.5), 0.1 mM $MgCl_2$, and 0.002% Triton X-100. The cells were then suspended in 180 ml of the same buffer and lysed by 1 or 2 strokes of a loose-fitting hand-operated Dounce homogenizer. All solutions were at 0°C. The nuclear pellet was obtained by centrifugation in 50 ml of conical-bottom polycarbonate tubes using an IEC-PRJ refrigerated centrifuge at 1,800 rpm for 10 min. As judged by phase-contrast microscopy, this procedure yields nuclei essentially free of plasma membrane and cytoplasmic material.

hnRNP extraction and isolation. The nuclear pellet obtained as described above was washed once in 180 ml of a solution containing 90 mM NaCl, 1 mM $MgCl_2$, and 10 mM Tris hydrochloride, pH 7.2 (STM). The nuclear pellet clumped in this solution and was dispersed with a Dounce homogenizer. After centrifugation at 1,800 rpm for 8 min in an International PR-J centrifuge, the pellet was washed a second time in 90 ml of pH 7.2 STM. The nuclear pellet was then suspended in 2 to 4 volumes of pH 8.0 STM. This preparation was then disrupted with ultrasound by using a microtip transducer for three 10-s bursts at 60 to 80 W. The sample was held at 0°C during sonic disruption. The sonic extract was incubated at 37°C for 20 min to facilitate endogenous RNase activity and then centrifuged in a 15-ml Corex glass tube in the HB-4 rotor of the Sorvall RC2-B at 7,000 rpm for 10 min to remove chromatin and nuclear debris. The chromatin-clarified supernatant was then loaded on linear 15 to 30% sucrose density gradients (pH 8.0 STM) and centrifuged in a Beckman SW28.1 rotor at 25,000 rpm for 16 h. Typically, a preparation of 1.5×10^9 cells yields an optical density peak near 2 at 260 nm. The 40S hnRNP particles were positioned midway in the gradient following centrifugation.

Preparation of proteins A2 and B1. We have previously shown that if nuclear sonic extracts or gradient-purified 40S hnRNP are digested with RNase A under limited conditions, a 43S rearrangement product originally termed the nuclease-resistant structure forms spontaneously (16). With optimal digestion times, the 43S rearrangement structure contains essentially pure A2 and B1 in a 3:1 molar ratio and fragments of RNA about 200 nucleotides in length. Trace amounts of A1, B2, and the C-group proteins sediment with the 43S structure if nuclease digestion is overly brief. Aggressive digestion of all RNA completely prevents the formation of the 43S complex and will dissociate the particles once they are formed (16). If preparations of the 43S complex are warmed to room temperature, a second rearrangement occurs. This rearrangement is the formation of insoluble helical filaments with widths of 18 nm, pitches near 60 nm, and indeterminate lengths (16). This event occurs much more readily if the 43S complexes are previously digested with RNase A and dialyzed against a low-salt solution containing 5 mM Mg^+ (i.e., 0.1× pH 8.0 STM containing 5 mM Mg^+).

For the studies described here, 43S complexes were obtained by adding RNase A (5 μg/10^8 nuclei) to crude nuclear sonic extracts prepared as described above. The preparation was incubated at 37°C for 10 to 15 min, chilled to 0°C, centrifuged to remove chromatin and nuclear debris, and then centrifuged in 15 to 30% gradients. The 43S complex was obtained by pooling the gradient fractions containing the 43S peak. Soluble A2-B1 used in the sucrose gradient experiments was obtained following RNase A digestion (10 μg/ml, 0°C, 1 h). For 43S particle reconstitution on in vitro-synthesized RNAs, 43S particles were dialyzed overnight against pH 8.0 STM to remove sucrose and adjusted to an optical density at 260 nm of 0.3/ml with pH 8.0 STM containing 1 mM dithiothreitol and 1 mM $CaCl_2$. The complex was then dissociated by digesting the endogenous RNA with micrococcal nuclease (Boehringer Mannheim) at a concentration of 300 U/ml. The digestion reaction was performed for 1 h at 0°C. Digestion at 37°C results in the formation of a precipitate, as previously reported (16). Since we have obtained from various sources nuclease which is contaminated with protease, each supply of enzyme was tested for protease activity prior to use. After complete digestion of endogenous RNA, the enzyme was inactivated by adding EDTA to a final concentration of 10 mM.

Sucrose density gradient centrifugation. The conditions of centrifugation and determination of sedimentation coefficients have been described elsewhere (19). Sucrose gradients (5 to 20%) were prepared in 20 mM Tris hydrochloride (pH 8.0)–1 mM dithiothreitol. Other components (NaCl and $MgCl_2$) were varied as described below in Results. The 43S rearrangement products were dialyzed against the appropriate buffer and then digested with RNase A (10 μg/ml). The gradients were centrifuged at 188,753 × g for 35 h at 2°C. Samples (0.5 ml) were loaded onto 11.5-ml gradients. Bovine gamma-globin (7.0S), bovine serum albumin (4.3S), and RNase A (2.0S) were used as standards. Fractions were collected, trichloroacetic acid or ethanol precipitated, and subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

In vitro synthesis of RNA substrates. The 736-nucleotide monoparticle-length RNA substrate used for particle assembly was transcribed in vitro with T7 RNA polymerase and, as template, a construct of the mouse β^{maj}-globin gene (pMBG2020) inserted into the *Bam*HI site of the polylinker in the vector pGEM1. [^{3}H]uridine-labeled transcripts were synthesized and employed in the experiments described in Fig. 5. Details of gene construction and cloning are described elsewhere (10). The 736-nucleotide transcript containing exon 1, intervening sequence 1 (IVS 1), exon 2, and more than half of IVS 2 was obtained by linearizing the plasmid with *Hin*dIII. The 1,509-nucleotide dimer-length substrate was transcribed from the adenovirus pAD2MLT 1487-IVS construct, which contains a 491-nucleotide deletion of the intervening sequence between leaders 1 and 2 of

the adenovirus type 2 major late transcript region (10). The cloned adenovirus gene construct was cut with *Bam*HI to generate the 1,509 RNA. In vitro transcription and RNA purification were performed as described elsewhere (10). Prior to use for in vitro particle assembly, the RNA was assayed for correct length and absolute homogeneity by electrophoresis of 1 μg of RNA in 8 M urea sequencing gels followed by staining in ethidium bromide and visualization with UV light. The purified φX-174 single-stranded DNA (ssDNA) was a gift from Su Yun Chung of the Laboratory of Experimental Carcinogenesis, National Cancer Institute.

Reconstitution of the 43S RNP complex on RNA transcripts of defined length. In experiments to determine if proteins A2 and B1 alone possess the ability to package RNA in vitro in a length-dependent manner, transcripts of defined length were used as the assembly substrate. This RNA was mixed with A2 and B1 proteins in pH 8.0 STM at the desired protein/RNA ratio and reactant concentration, and the reconstitution was allowed to proceed for 1 h at 0°C. Following reconstitution, samples were analyzed by sedimentation in linear 15 to 30% sucrose density gradients.

Electron microscopy. A detailed description of sample preparation for electron microscopy is presented elsewhere (27). Briefly, native and reconstituted 43S complexes were fixed with 0.1% glutaraldehyde for 10 min. The samples were then absorbed to polylysine coated grids, stained with 5% uranyl acetate, and shadowed with platinum.

RESULTS

Proteins A2 and B1 exist as tetramers. We have recently determined that the core particle proteins C1 and C2 exist as a highly stable anisotropic $(C1)_3C2$ tetramer in solution and that the C-protein tetramer is a fundamental structural component of 40S hnRNP particles. We now demonstrate that the core particle proteins A2 and B1 also exist as tetramers. Evidence that protein A2 exists in native monoparticles in a homotrimeric association was previously obtained by using the homobifunctional protein cross-linking reagent DTBP (dimethyl 3,3′-dithiobispropionimidate) (16). It was also noticed that upon release from 40S monoparticles by RNase digestion, proteins A2 and B1 had sedimentation coefficients between 3S and 10S, as estimated from 30% sucrose gradients. These preliminary observations suggested that A2 and B1 existed as dimers or tetramers. This observation is confirmed and expanded upon here.

At 50 mM NaCl, the majority of proteins A2 and B1 sediment as a single peak with a sedimentation coefficient of 6.8 (Fig. 1). The dissociation of tetramers to stable dimers over the course of the 35-h sedimentation experiment may explain the peak broadening of the A2 and B1 proteins seen in Fig. 1. The calculated molecular weight of the complex, based on its sedimentation coefficient, is 144 kDa (19). This agrees well with a tetramer molecular weight of 145,436 based on the recent sequence-determined molecular weights of proteins A2 and B1 (36,002 and 37,430 respectively) (7). These molecular weight estimates could be improved by determining their Stokes radii. However, like the purified C-protein tetramer (1, 2), proteins A2 and B1 bind to dextran and agarose gel filtration matrixes at NaCl concentrations below 500 mM, but, unlike the C-protein tetramer, the proteins are not stable above 90 mM NaCl (data not shown). Because of uncertainties caused by not knowing the Stokes radii of these complexes, there is the possibility that the complexes are trimers and hexamers. On the basis of the sedimentation properties of the internal standards (RNase A [12.6 kDa], bovine serum albumin [BSA; 66 kDa], and immunoglobulin G [150 kDa]), a small amount of proteins A2 and B1 and also a low level of protein A1 (a contaminant present in the purified 43S complexes) can be seen to sediment slower than the BSA standard, as is expected for a free monomer. Any $(A2)_2$ or A2-B1 dimers present in these gradients should be at or near the position of BSA. At 90 mM NaCl, the $(A2)_3B1$ tetramers partially dissociate into dimers which move as a slower-sedimenting peak superimposed on the position of BSA (Fig. 2B). The slower-sedimenting peak has a sedimentation coefficient of 4.2, which corresponds to a molecular weight of 64 kDa. Judged by sequence analysis, the molecular weight of an $(A2)_2$ dimer is 72,004. The molecular weight of an A2-B1 dimer is 73,432. In the gradients shown in Fig. 2, a small amount of monomer can be seen as a slow-sedimenting shoulder (fractions 10 to 13) to the dimer peak (fractions 13 to 17). At 200 mM NaCl, the $(A2)_3B1$ tetramers are completely dissociated and sediment as dimers (Fig. 2A). An observation of some interest is the absence of a significant increase in the amount of monomer at the elevated salt concentrations which dissociate the tetramer into dimers. The absence of A2 and B1 monomers is presumably due to the high stability of the dimers under these conditions. The possibility that significant quantities of nucleic acid could account for the presence of dimers and tetramers was excluded by measuring the A_{260}/A_{280} ratio, which was below 1.8. There were also only minor amounts of nucleic acid detected following phenol extraction, labeling with [^{32}P]ATP and polynucleotide kinase, and denaturing gel electrophoresis.

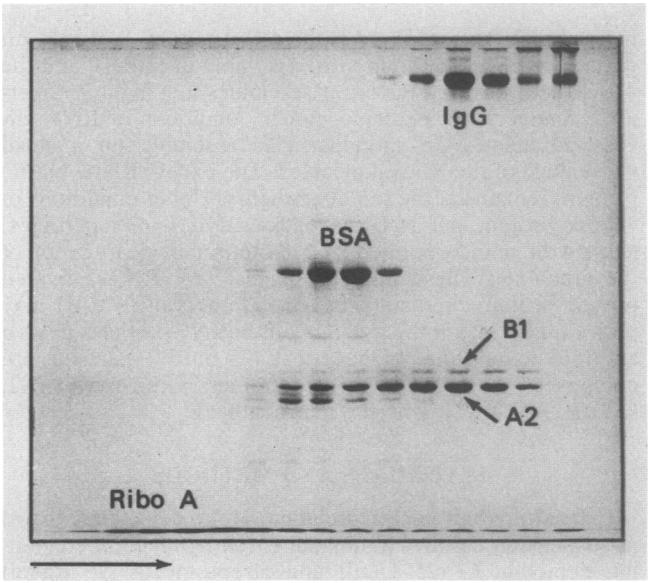

FIG. 1. Coomassie-stained 8.75% SDS-PAGE gel of the proteins in successive 1.0-ml fractions of a 5 to 20% sucrose density gradient (50 mM NaCl). Sedimentation of the proteins freed from purified 43S complexes by RNase A digestion is shown from left to right. The internal standards are immunoglobulin G (IgG) (molecular mass, 150 kDa, 7.0S), BSA (molecular mass, 66 kDa, 4.3S), and RNase A (Ribo A) (molecular mass, 12.6 kDa, 2.0S). The RNase standard is not resolved in these 8.75% gels but can be seen in the dye front in fractions 3 to 7. The $(A2)_3B1$ tetramer (6.8S) is labeled.

$(A2)_3B1$ tetramers package RNA and ssDNA in a length- and sequence-independent manner. It has previously been demonstrated that when RNA substrates of 736 and 1,509 nucleotides are added to the soluble proteins of 40S hnRNP

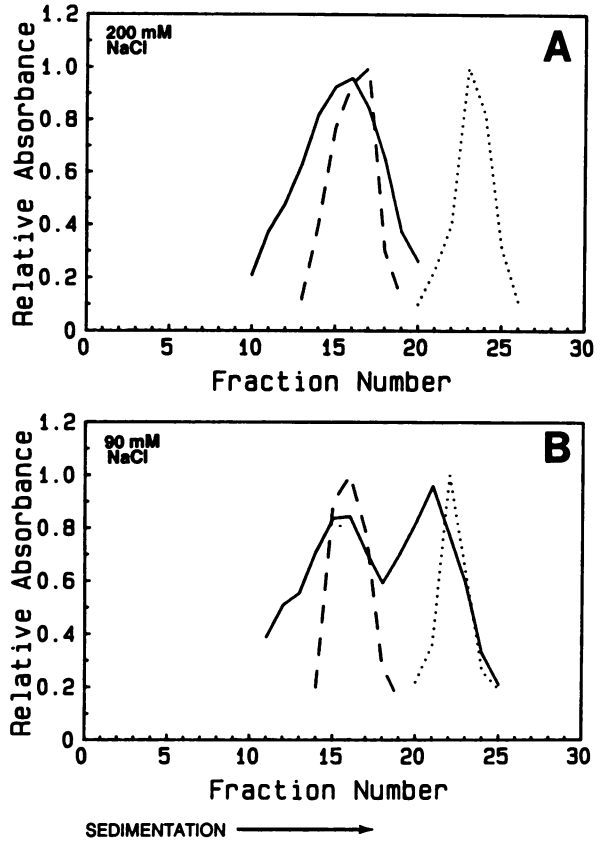

FIG. 2. Distribution of Coomassie-stained proteins in a 5 to 20% sucrose density gradient. As in Fig. 1, the 43S rearrangement products, composed almost entirely of proteins A2 and B1, were digested with RNase A to free the protein. Equal amounts of protein were added to gradients made to contain either 200 mM (A) or 90 mM (B) salt as described in Materials and Methods. Sedimentation was from left to right. The distributions of proteins A2 and B1 were determined by quantitating the amount of bound dye in each band from an SDS-PAGE gel for each gradient fraction. The band in each gel fraction with the largest amount of bound dye was set equal to 1.0. Because of this, the amounts of dimer and tetramer cannot be compared between the two panels. The relative A_{560} of the eluted dye is indicated on the y axis. The solid line shows the distribution of proteins A2 and B1, the dashed line shows the position of the BSA standard (4.3S), and the dotted line is the immunoglobulin G standard (7.0S).

particles dissociated by the removal of indogeneous RNA, monoparticles and dimers assemble, respectively (10). When the ϕX-174 genome, a ssDNA of 5,386 nucleotides, is used as the assembly substrate, seven or eight particles form. These and related results (22, 25) demonstrate that the six core particle proteins package 700 ± 20 nucleotide lengths of single-stranded nucleic acid into regular repeating structures. The electron micrographs in Fig. 3 show that similar results occur when these nucleic acid substrates are added to the nuclease-dissociated proteins of 43S complexes which consist almost exclusively of proteins A2 and B1 (see Fig. 4 through 8). The $(A2)_3B1$ tetramers package mouse globin pre-mRNA transcripts of 736 nucleotides into 43S, 22.5-nm monoparticles (Fig. 4A). Transcripts of 1,509 nucleotides are packaged into dimer particles (panel B), and the ssDNA genome of ϕX-174 is packaged into a polyparticle complex (panel C). The packaging of single-stranded nucleic acids of different sequences is the same according to electron microscopy, sedimentation, and protein composition. The protein composition and sedimentation properties of native 40S hnRNP particles and the 43S complex reconstituted on the 736-nucleotide transcript of the mouse β-globin gene are shown in Fig. 4.

$(A2)_3B1$ tetramers package RNA by means of a cooperative mechanism. Unlike the in vitro assembly of 40S hnRNP particles, the assembly of the 43S complex is insensitive to the ratio of protein and RNA in the reaction mix and is a highly cooperative process. As the protein/RNA ratio increases from 2.2 to 12, no difference is observed in the sedimentation rate of the resulting particles (Fig. 5). At the lower ratios (2.2 and 6.6), all of the protein assembles into 43S particles, leaving most of the excess RNA at the top of the gradients. The ratio of A2 to B1 in the particles formed within this range of protein/RNA ratios does not change (Fig. 6). At protein/RNA ratios above 12, the excess protein remains near the top of 15 to 30% sucrose density gradients (Fig. 7). The protein composition is the same for monoparticles formed on mouse globin transcripts of 736 nucleotides, for dimers assembled on the 1,509-nucleotide adenovirus RNA, and for the polyparticle complexes which assemble on the ϕX-174 ssDNA genome of 5,386 nucleotides (Fig. 8).

43S complex may contain 16 $(A2)_3B1$ tetramers. The amount of RNA added to 43S complex reconstitutions is equal to the amount of RNA in the 43S complex under conditions of protein excess. According to protein determinations (5, 17) and using an absorbance (optical density at 260 nm) of 1 for 40 μg of RNA, the ratio of protein to RNA in the reconstituted 43S complex is 9:1. This compares with 6:1 for reconstituted 40S hnRNP. On the basis of these ratios and the mass of a 736-nucleotide RNA, the respective masses of the 40S hnRNP particle and the 43S complex are calculated to be 1.67×10^6 and 2.38×10^6. By subtracting the mass of the 736-nucleotide RNA component (244 kDa) from the mass of the 43S complex (2.4×10^6) and dividing by the mass of the $(A2)_3B1$ tetramer (145.4 kDa), we have estimated that the 43S complex contains 14 to 16 $(A2)_3B1$ tetramers.

DISCUSSION

We have previously shown that a tetramer of the C1 and C2 proteins, $(C1)_3C2$, is a fundamental structural component of HeLa cell 40S hnRNP particles (1–3). Described above are the observations which argue that proteins A2 and B1 also exist as $(A2)_3B1$ tetramers and that they are fundamental structural components of hnRNP particles. This conclusion is based on four lines of evidence. (i) Homotypic trimers of protein A2 are among the major cross-linked products obtained when intact 40S hnRNP particles are exposed to the cross-linking reagent DTBP (16). (ii) Proteins A2 and B1 always copurify and are found in the ratio of 3 A2/1 B1. In this context, A2 and B1 have been studied in a purified form (this report), in native and reconstituted hnRNP particles, in the 43S rearrangement product, and in the highly insoluble fibers composed entirely of these proteins (16). (iii) Proteins A2 and B1 cosediment at 6.8S, which is consistent with a 145-kDa tetramer. A tetrameric form for A2 and B1 is also supported by the observation that 200 mM NaCl dissociates the complex into dimers (4.2S). (iv) The same salt concentration which dissociates proteins A2 and B1 from intact 40S particles also dissociates purified $(A2)_3B1$ tetramers into dimers. This fact argues that it is the $(A2)_3B1$ tetramer that is

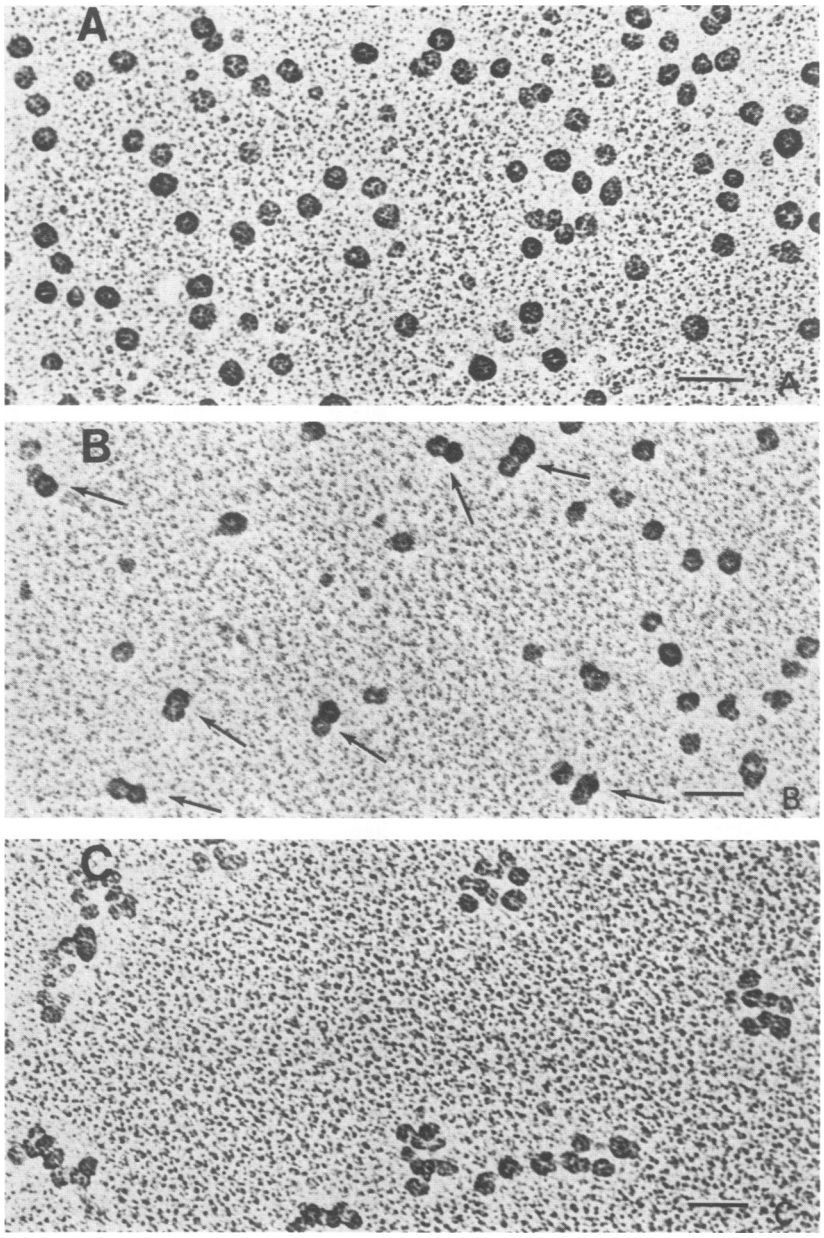

FIG. 3. (A) Electron micrographs of 43S monoparticles which assemble in vitro on 736-nucleotide transcripts of the mouse β-globin gene. (B) Dimers which assemble on transcripts of the same gene which are 1,509 nucleotides in length. (C) Polyparticle complexes which assemble on the φX-174 ssDNA genome of 5,386 deoxyribonucleotides.

the structurally significant component of 40S hnRNP particles.

A 43S RNP particle composed mostly of proteins A2 and B1 forms by dissociation and rearrangement of the major hnRNP when nuclear sonic extracts are digested with nuclease (16). The present results show that $(A2)_3B1$ tetramers assemble to form 43S complexes which, like intact 40S hnRNP, package multiples of 700-nucleotide lengths of RNA into monomer, dimer, and oligomeric complexes. The packaging of 700 ± 20 nucleotides of RNA into regular repeating structures either by all six of the 40S hnRNP core particle proteins (10) or by two of the proteins alone is unexpected but informative. The ability of multiple copies of just one type of tetramer [$(A2)_3B1$] to assemble into a stable particle which contains the same length of RNA as reconstituted hnRNP particles suggests that $(A2)_3B1$ tetramers may replace $(C1)_3C2$ and $(A1)_3B2$ tetramers in the particle structure. Because two different particles (43S rearrangement particle and the 40S hnRNP particle) do not form when all the proteins are present, the characteristic stoichiometry of native particles must be determined by regulatory interactions among the different tetramers. This possibility is supported by the finding that the interactions among the $(A2)_3B1$ tetramers show some distinct differences from the protein-protein interactions which occur when all the tetramers are present. For example, during hnRNP particle assembly in

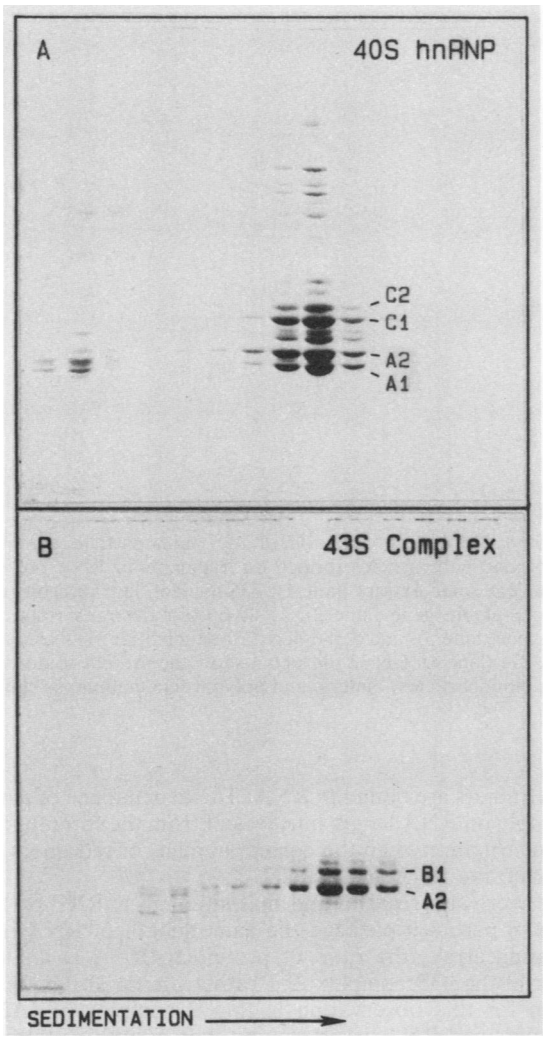

FIG. 4. Coomassie-stained 8.75% polyacrylamide slab gels of the proteins in successive 1.0 ml fractions of 15 to 30% sucrose density gradients. This figure shows the sedimentation (from left to right) and protein composition of 40S hnRNP particles assembled in vitro on phage T4 transcripts of 726 nucleotides (A) and 43S complexes assembled in vitro on 736-nucleotide transcripts of the mouse β-globin gene (B). In panel A, proteins B1 and B2 are not labeled but are the doublet between A2 and C1. The protein/RNA ratios were 15:1 (A) and 12:1 (B).

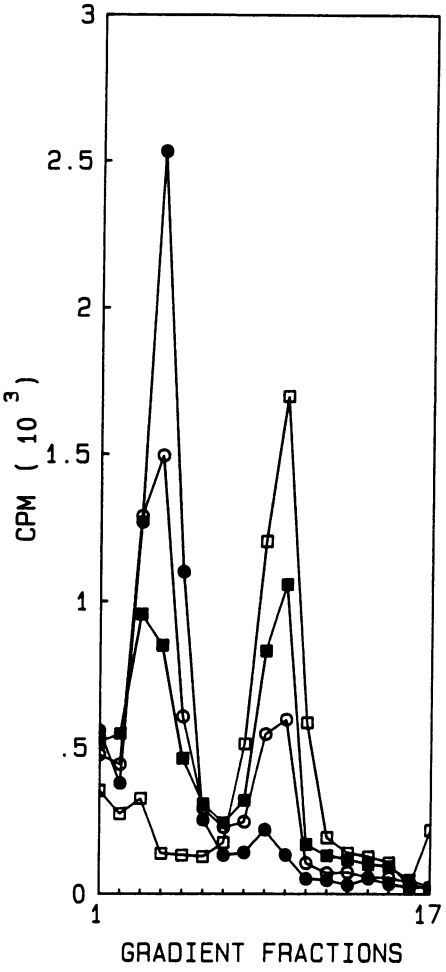

FIG. 5. Effect of the protein/RNA ratio on assembly and sedimentation of 43S complexes. The assembly and sedimentation of reconstituted 43S particles were monitored as a function of [^{3}H] uridine distribution in 15 to 30% sucrose density gradients. Closed circles show the sedimentation of particles formed at a protein/RNA ratio of 2:1. Note that at this ratio, most of the RNA remains at the top of the gradient, unbound by protein. Open circles represent a ratio of 6.6:1, and closed squares represent a ratio of 8:1. Note that at a ratio of 12:1 (open squares), almost all of the RNA sediments at 43S. Protein compositions are shown in Fig. 4.

vitro, correct particle assembly is dependent on appropriate protein/RNA ratios in the reaction mix. By contrast, assembly of the 43S particle occurs in a cooperative, all-or-none manner regardless of protein/RNA ratio.

Two different models for cooperative and nonspecific binding of proteins to single-stranded nucleic acids have been described. The unlimited-cooperativity model of McGhee and von Hippel (20) describes a situation in which ligand-ligand interactions are between nearest neighbors and the lattice is completely saturated at the stoichiometric point. GP32 exhibits this type of binding (11). The other model, developed by Bujalowski and Lohman to describe *Escherichia coli* single-stranded binding-protein binding, is limited cooperativity (6). In this model only two ligands (single-stranded binding-protein tetramers) interact, resulting in the presence of gaps along the lattice and a beads-on-a-string appearance (8). The cooperative and nonspecific binding of the $(A2)_3B1$ tetramers has been studied by electron microscopy of the complexes formed on different lengths of RNA (Fig. 3) and by sucrose gradient sedimentation of complexes formed under different ratios of protein to RNA (Fig. 5). The $(A2)_3B1$ tetramers completely coat the RNA in an all-or-none manner indicative of the unlimited-cooperativity model. If the binding was noncooperative or of limited cooperativity, all the RNA would be bound by some protein, resulting in a broad distribution of the RNA in the gradients. However, the cooperative binding of $(A2)_3B1$ tetramers to RNA may be complicated by additional tetramer-tetramer interactions which enable tetramers to form large 43S complexes on 700-nucleotide lengths of RNA.

Like native hnRNP particles, 43S particles completely dissociate following extensive treatment with nuclease.

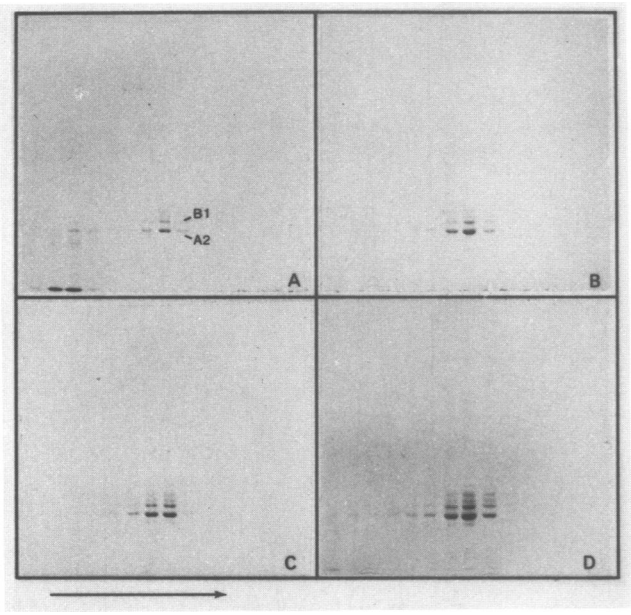

FIG. 6. Effect of the protein/RNA ratio on protein composition and sedimentation of 43S complexes. Protein/RNA ratios in the reaction mix were 2.2:1 (A), 6.6:1 (B), 8:1 (C), and 12:1 (D). Note that protein composition and sedimentation are the same regardless of protein/RNA ratio in the reaction mix. See Fig. 5 for the distribution of RNA in these gradients.

Moreover, when 43S particles are reconstituted in the presence of limiting RNA, excess protein remains near the top of gradients in a low-molecular-weight form. Both findings indicate that tetramer-tetramer interactions occur only when the tetramers are bound to RNA. The dependence of particle assembly on RNA length must result from the formation of a stable structure when the correct number of tetramers have bound to the RNA substrate.

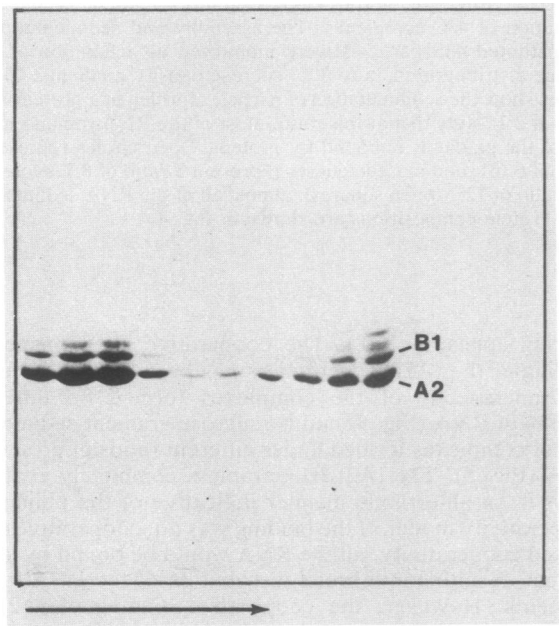

FIG. 7. Assembly of the 43S complex under conditions of limited RNA. This Coomassie-stained 8.75% polyacrylamide slab gel shows the proteins present in successive 1.0-ml fractions of a 15 to 30% sucrose gradient. The protein/RNA ratio was 24:1. Note that the excess protein remains near the top of the gradient (left).

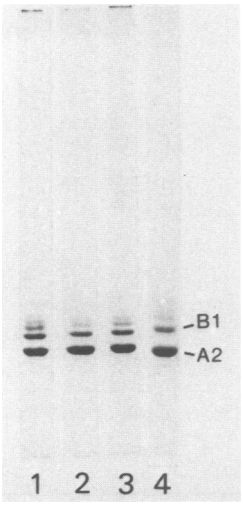

FIG. 8. Protein composition of 43S monoparticle complexes, dimers, and polyparticles formed on fragments of RNA present in the nuclear sonic extract (lane 1), 736-nucleotide transcripts of the mouse β-globin gene (lane 2), 1,509-nucleotide transcripts of the same gene (lane 3), and 5,386-deoxyribonucleotide ssDNA genome of ϕX-174 (lane 4). Gels 2 through 4 show the protein compositions of the monoparticles, dimers, and polyparticle complexes shown in Fig. 3.

We were surprised to find that although hnRNP particles and 43S particles package the same length of RNA into a repeating array, the ratio of protein to RNA is distinctly larger in the 43S complex (9:1 rather than 6:1). One explanation for this observation is that a complex of $(A2)_3B1$ tetramers and RNA assemble and then additional tetramers which are not bound to RNA add to the complex. Another possibility is that the average site size of the six core particle proteins is larger than the $(A2)_3B1$ tetramer site size. In this case, more $(A2)_3B1$ tetramers could bind per unit length of RNA.

In addition to the interactions which occur during intact 40S particle assembly, the $(A2)_3B1$ tetramers can interact in two additional ways. In the absence of the other core particle proteins, they package RNA in a cooperative, length-dependent fashion to form the 43S rearrangement product. In the absence of RNA and at temperatures above 10°C, the tetramers assemble to form highly insoluble fibers of indeterminate length (16). Previous studies have shown that proteins A2 and B1 are protected from protease when assembled in 40S hnRNP particles and that the other core particle proteins (C1, C2, A1, and B2) are preferentially released from intact particles upon nuclease treatment (16). When these findings are considered together with the various tetramer-tetramer and tetramer-RNA interactions described here, it seems plausible to suggest that $(A2)_3B1$ tetramer interactions play a major role in particle stability and in driving the closed structure of the 40S hnRNP particle.

The observation that proteins A2 and B1 can exist in five different forms (40 to 300S polyparticles, 40S monoparticles, 43S rearrangement complexes, insoluble helical filaments, and free tetramers), depending on the specific techniques used, illustrates a problem inherent in the study of large,

multicomponent complexes. The problem is the uncertainty of which complexes observed in vitro have relevance to actual biological processes. In all of the complexes so far observed, proteins A2 and B1 exist in a stable 3:1 ratio. It is our view that only detailed biochemical analysis of purified complexes and their components will eventually allow us to make these determinations.

ACKNOWLEDGMENTS

This work was supported by NSF grant DMB 8819051 to W.W.L.

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