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Biochemistry

In vitro assembly of a pre-messenger ribonucleoprotein*

(adenovirus 2 major late promoter/transcription in HeLa cell extracts/RNA–protein crosslinking/mRNA processing)

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ABSTRACT **Transcription of the *Bal* I E restriction fragment of adenovirus DNA by RNA polymerase II in a HeLa cell extract produces a RNA transcript 1,712 nucleotides in length. This transcript contains the first two elements of the tripartite leader that, *in vivo*, is spliced onto the late mRNAs. We have found that this adenovirus 2 transcript forms a specific ribonucleoprotein complex (RNP) in this *in vitro* system. The RNP particle sediments in sucrose gradients as a monodisperse peak at 50 S and has a buoyant density of 1.34 g/cm^3 in Cs_2SO_4, indicating the same 4:1 protein/RNA composition as native nuclear RNPs that contain pre-mRNA sequences (hnRNP). Moreover, the *in vitro*-assembled RNP is resistant to concentrations of NaCl that are known to dissociate nonspecific RNA–protein complexes. The adenovirus 2 transcript is precipitated by a monoclonal antibody for hnRNP core proteins. In addition, RNA–protein crosslinking of [α-^{32}P]UTP-labeled transcript/RNP complexes reveals that the major proteins in contact with the RNA are the M_r 32,500–41,500 species known to be associated with hnRNA *in vivo*. These results demonstrate the *in vitro* assembly of a specific RNA polymerase II transcript into RNP. Moreover, because the 1,712-nucleotide adenovirus 2 transcript lacks poly(A) addition sites and because the leader sequences are not spliced appreciably in this *in vitro* system, it follows that RNP formation requires neither polyadenylylation nor splicing, nor is it sufficient to cause the latter.**

mRNA processing takes place in nuclear ribonucleoprotein particles (RNPs) known as heterogeneous nuclear RNP, or hnRNP (reviewed in ref. 1). These particles are variable in morphology (2, 3), RNA sequence (4, 5), and protein composition (6–10). For this reason, most previous studies of hnRNP organization have succeeded only insofar as revealing major structural features common to hnRNP particles as a whole.

To define hnRNA–protein interactions in greater detail, it would be useful to study a RNP particle containing a specific pre-mRNA transcript. One possibility is to isolate an especially prevalent pre-mRNA, as RNP, from the nuclei of cells producing abundant mRNAs. However, in most cases, the nuclear precursors of abundant cytoplasmic mRNAs turn out not to be particularly prevalent in the nuclear RNA (for β-globin mRNA, see ref. 11; for heat-shock mRNA transcripts in *Drosophila* nuclear RNA, see ref. 12). A second possibility is to generate a specific RNP by *in vitro* assembly from defined components, including a pure pre-mRNA transcript. In this paper, we describe the successful outcome of this second approach.

MATERIALS AND METHODS

Transcription. The *Bal* I E fragment of adenovirus 2 (Ad2) DNA cloned in pBR322 was kindly provided by P. Sharp (Massachusetts Institute of Technology), and the HeLa whole cell extract was prepared from uninfected cells as described (13). The standard transcription reaction volume was 25 μl and contained, in addition to 2.5 μl (1.25 μg) of *Bam*HI-restricted DNA, 15 μl of HeLa extract, 1.0 μl of [α-^{32}P]UTP (10 μCi; specific activity = 630 Ci/mmol; 1 Ci = 3.7×10^{10} Bq), 4 μl of water, and 2.5 μl of transcription buffer [containing 500 μM (each) ATP, GTP, and CTP, 50 μM UTP, 60 mM KCl, 0.2 mM EDTA, 1.3 mM dithiothreitol, 10% glycerol, and 12 mM Hepes (pH 7.9)]. The reaction mixture was incubated at 30°C for 90 min in all experiments.

RNA Analysis. The desired portion of the transcription reaction, usually 5–50 μl, was mixed with 2.4 ml of a solution containing 50 mM sodium acetate (pH 5.2), 1% $NaDodSO_4$, 10 mM EDTA, and *Escherichia coli* tRNA at 10 μg/ml; 2.4 g of CsCl was added and dissolved by heating to 60°C for 2 min. The solution was layered over 1.2 ml of 5.7 M CsCl/100 mM EDTA and centrifuged in a Beckman SW 50.1 rotor at 38,000 rpm for 24 hr at 20°C (14). The RNA pellet was dissolved in 0.3 M sodium acetate (pH 5.2) and precipitated with 2 vol of 95% ethanol at −20°C. The RNA was collected by centrifugation and dissolved in 3 μl of water, 4 μl of freshly deionized glyoxal, 8 μl of dimethyl sulfoxide, and 1 μl of 0.16 M sodium phosphate buffer (pH 7.0). The solution was heated at 50°C for 1 hr and loaded immediately on a 1.1% agarose vertical slab gel in 10 mM sodium phosphate buffer (pH 7.0). Electrophoresis was at 90 V for 2 hr (20–22°C). The gels were dried and exposed to x-ray film at −70°C by using Dupont Cronex Lightning Plus screens.

Analysis of RNP Assembly *in Vitro*. Several analytical approaches were used to investigate RNP formation *in vitro*, and the experimental details for nitrocellulose filter binding, sucrose gradient sedimentation, Cs_2SO_4 banding, reaction with monoclonal antibody, and RNA–protein crosslinking are all given in the respective figure legends.

RESULTS

Strategy. The experiments to be described were based on three considerations. (*i*) Specific transcription of cloned DNA sequences by RNA polymerase II can now be readily achieved in cell extracts (13, 15–17). (*ii*) In the procedure of Manley *et al.* (13), the transcription extract is prepared by exposing a whole cell homogenate to 0.6 M $(NH_4)_2SO_4$. We knew that this ionic strength (namely, 1.2 M NH_4^+ ion) is sufficient to solubilize many nuclear proteins, including those of hnRNP particles (6), and that after removal of the residual nuclei, the extract would

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Abbreviations: RNP, ribonucleoprotein; hnRNP, heterogeneous nuclear RNP particle(s); Ad2, adenovirus 2; Nt, nucleotide(s).

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contain soluble hnRNP proteins. Therefore, it seemed possible that such extracts might support hnRNP assembly when a specific RNA was transcribed. (*iii*) The level of mRNA splicing in such extracts is extremely low (18–20). It occurred to us that this situation, normally a disappointment, might actually favor one's ability to detect the formation of a stable RNP structure, to the extent that possible RNP dynamics related to splicing would not complicate the picture.

A 1,712-Nucleotide (Nt) RNA from the Ad2 Major Late Promoter Associates with Proteins in Transcription Extracts. Fig. 1*A* illustrates the adenovirus DNA *Bal* I E fragment (14.7–21.5 map units) used as a template in these experiments. It contains the major late promoter (16.45 map units) and the first two elements of the tripartite leader that is joined, by leader-to-leader splicing, to the 5′ termini of at least 13 different late mRNAs (21–26). Transcription of the *Bal* I E DNA fragment *in vitro* in the HeLa cell extract system yields a 1,712-Nt "run-off" transcript (ref. 13; Fig. 1*B*, lane 1). For the results on RNP formation to be described, it is important to keep in mind two features of this transcript in particular: (*i*) it contains neither mRNA-coding regions or polyadenylylation sites, and (*ii*) it does contain two splicing sites: the 5′ and 3′ sites between leaders 1 and 2 and the 5′ site for leader 2-to-3 splicing.

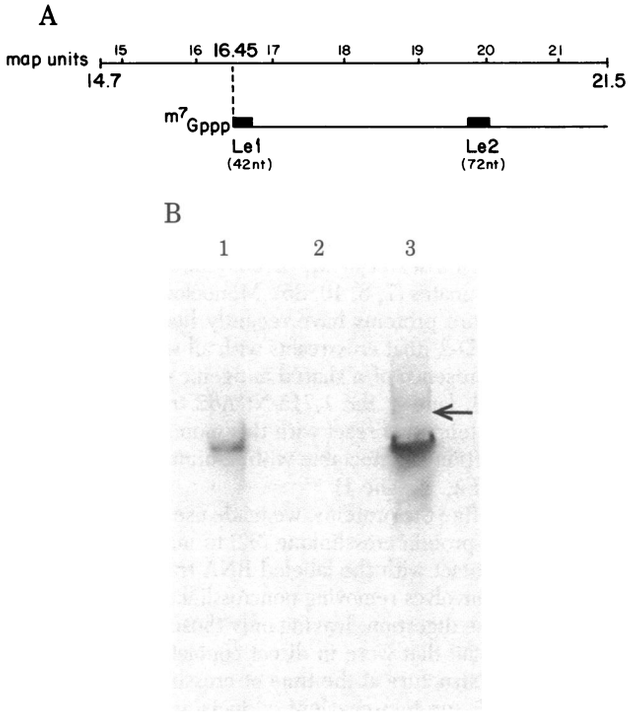

FIG. 1. (*A*) Organization of the Ad2 *Bal* I E DNA fragment and its transcript. (Le, leader.) (*B*) Analysis of RNP formation by nitrocellulose filter binding. Twenty-five-microliter reaction mixtures were diluted with binding buffer (20 mM KCl/1.5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.6) to a final volume of 1.5 ml. The solution was passed through a 0.45-μm pore diameter nitrocellulose filter disc (Millipore HAWP) at a slow flow rate, and the filter was washed with 30 ml of binding buffer. Controls established that under these conditions only 1–2% of a protein-free RNA sample was retained on the filter, whereas 80–100% of the *in vitro* transcription product was retained (based on trichloroacetic acid-insoluble radioactivity). Bound RNA was eluted by incubation of the filter with 1 ml of 0.1% $NaDodSO_4$ for 2 hr at 20°C (recovery ≈ 80%) and was further purified by incubation with proteinase K (37°C, 1 hr), followed by addition of sodium acetate (pH 5.2) to 0.3 M and ethanol to 67%. Lane 1, RNA transcribed *in vitro* on the *Bal* I E DNA fragment was deproteinized, glyoxalated, and displayed in a 1.1% agarose gel. Lane 2, the *Bal* I E DNA transcript was deproteinized and tested for nitrocellulose filter binding. Lane 3, an aliquot of the *Bal* I E DNA transcript was analyzed for filter binding activity, without prior deproteinization. The arrow indicates the position of HeLa cell 18S rRNA run in a separate lane.

The first clue that the 1,712-Nt *Bal* I E DNA *in vitro* transcript was associating with proteins came from nitrocellulose filter binding experiments, by using conditions in which protein-free RNA does not bind but RNA–protein complexes do (27, 28). Phenol-deproteinized transcript does not bind nitrocellulose filters in 10 mM KCl (Fig. 1*B*, lane 2), whereas the transcript does bind when tested directly from the transcription reaction without deproteinization (Fig. 1*B*, lane 3). Of course, these results do not establish that this RNA–protein interaction is specific.

The Transcript Forms a 50S RNP Complex. To begin examining the possibility that the transcript–protein interaction was specific, the complexes were sedimented on sucrose gradients and the amount of 1,712-Nt transcript in each region was determined. As shown in Fig. 2, the transcript sediments as a monodisperse peak at 50 S. The RNA itself would be expected to sediment at ≈18 S in these gradients. Compared to the very heterogeneous sedimentation (30–300 S) of total hnRNP (6, 29), the well-defined 50S peak of this specific Ad2 transcript is striking. In the currently accepted model of hnRNP organization (30) 30S heterotypic protein particles are thought to cover about 800 nucleotides of RNA. According to this model, the 1,712-Nt Ad2 transcript would be expected to contain two 30S

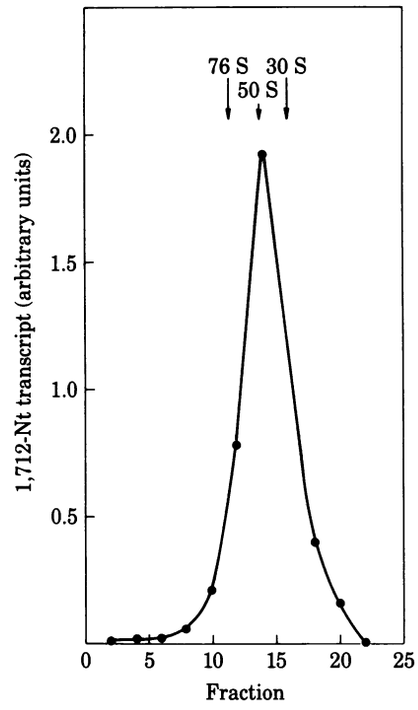

FIG. 2. The Ad2 transcript forms a 50S RNP *in vitro*. A 125-μl transcription reaction was diluted 1:5 with RSB (0.01 M NaCl/1.5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.0) and centrifuged in a 15–30% linear sucrose gradient in RSB at 14,500 rpm for 17 hr in a Beckman SW 41 rotor (4°C, without brake). Each two consecutive gradient fractions were pooled (volume ≈1 ml), diluted with 12.5 ml of RSB, and then centrifuged in a Beckman 50 Ti rotor at 50,000 rpm for 3 hr and 40 min at 4°C (*k* factor = 18 S). (Prior experiments had revealed that the majority of transcript was in structures sedimenting at 20 S or more.) The pelleted RNP was dissolved in 2.4 ml of a solution containing 50 mM sodium acetate (pH 5.2), 1% $NaDodSO_4$, and 10 mM EDTA. The RNA was purified by CsCl centrifugation, glyoxalated, and displayed in a 1.1% agarose gel. Autoradiograms obtained in the linear range of the film's dpm–grain exposure response were scanned to determine the relative amounts of 1,712-Nt transcript throughout the gradient.

particles, which is entirely compatible with the observed sedimentation coefficient of 50 S.

The *in Vitro* RNP Resembles Native hnRNP. Native hnRNP particles have a protein/RNA mass ratio of ≈4:1, as manifest by a buoyant density of 1.34 g/cm^3 in Cs_2SO_4 (29, 31–33). In addition to providing an analytical method for determining RNP composition, isopycnic banding in Cs_2SO_4 also constitutes a diagnostic criterion for the authenticity of hnRNP particles because, in contrast to ribosomes or artificial RNA–protein complexes, hnRNP is stable to Cs_2SO_4 banding in the absence of prior fixation (29, 31, 34). In Fig. 3*A* it can be seen that this is also the case for ≈70% of the *in vitro*-assembled Ad2 RNP. In addition to possessing this diagnostic resistance to Cs_2SO_4 banding, the *in vitro*-assembled RNP has a density of 1.34 g/cm^3, which is precisely the same as native hnRNP.

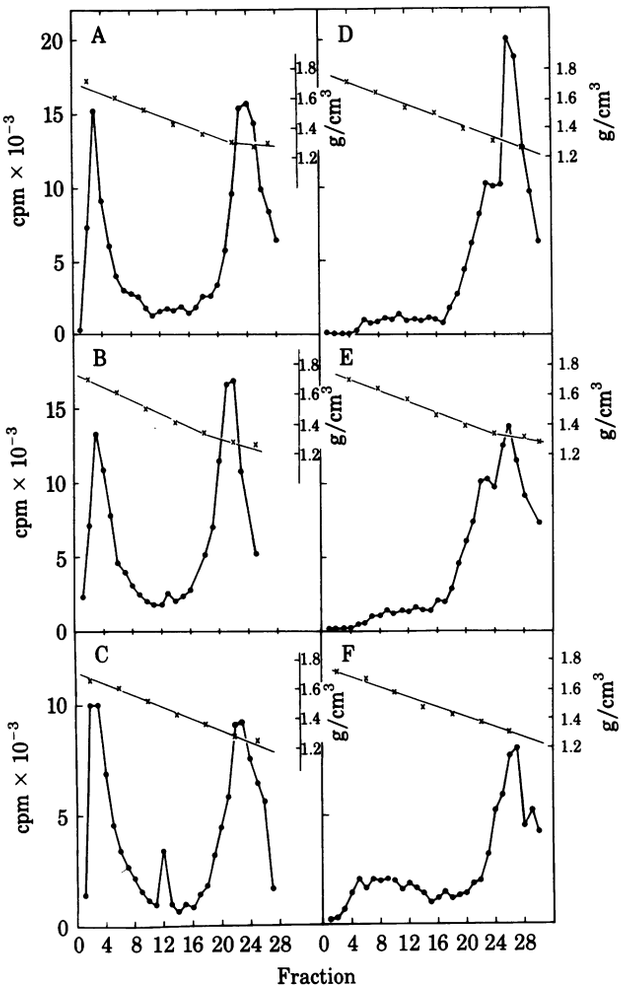

FIG. 3. Cs_2SO_4 banding of *in vitro*-assembled RNP. Twenty-five microliter transcription reactions were diluted to 400 μl with RSB and layered on a 4.8-ml preformed Cs_2SO_4 gradient (1.12–1.75 g/cm^3). In some experiments, the diluted transcription reaction was made 0.1 M or 0.5 M in NaCl prior to Cs_2SO_4 centrifugation. In other experiments, samples of transcription reactions in RSB, 0.1 M NaCl, or 0.5 M NaCl were subjected to RNA–protein crosslinking. In these cases, 400-μl aliquots were placed in 2.0-cm^2 wells of a Falcon 3047 tissue culture plate and irradiated for 15 min at 4°C with 3.6×10^5 ergs/mm^2 (1 erg = 10^{-7} J) of 254-nm light, by using the irradiation source described previously (32). The Cs_2SO_4 gradients were centrifuged in a Beckman SW 50.1 rotor at 34,000 rpm for 65–70 hr at 20°C. The profiles shown are 10% trichloroacetic acid-insoluble radioactivity. (*A*) 0.01 M NaCl; (*B*) 0.10 M NaCl; and (*C*) 0.50 M NaCl. (*D–F*) Same as *A–C* except that after addition of NaCl buffers, samples were irradiated before Cs_2SO_4 banding. In *C* and *F*, the transcription reaction aliquots were two-thirds the amount used in the other four gradients.

Approximately 35% of the transcripts band as naked RNA at 1.66 g/cm^3 (Fig. 3*A*). This must represent either transcripts that are not assembled into RNP or metastable complexes that dissociate in the Cs_2SO_4 gradient. To distinguish between these two possibilities, the *in vitro* transcripts were subjected to photochemical RNA–protein crosslinking with 254-nm light to stabilize RNP structure (32). As can be seen in Fig. 3*D*, Cs_2SO_4 banding now reveals no peak of naked RNA, and there is a corresponding increase in the amount of transcript banding at the density characteristic of native hnRNP (1.34 g/cm^3). Analysis of deproteinized RNA from the 1.34 g/cm^3 peak in Fig. 3*D*, and from both the 1.34 and 1.66 g/cm^3 peaks in Fig. 3*A*, revealed intact 1,712-Nt Ad2 transcripts (data not shown). Therefore, we conclude that the Ad2 transcripts assemble *in vitro* into RNP structures having the 4:1 protein/RNA composition characteristic of hnRNP (Fig. 3*D*) and that, furthermore, ≈70% of these particles possess a RNP structure that withstands Cs_2SO_4 banding without prior RNA–protein crosslinking (Fig. 3*A*).

Further evidence for the specificity of the Ad2 RNP particles assembled *in vitro* comes from experiments in which the complexes were exposed to either 0.1 M or 0.5 M NaCl prior to Cs_2SO_4 banding. These salt concentrations are known to disrupt nonspecific RNA–protein complexes (27). It can be seen in Fig. 3 *B* and *C* that these salt treatments had no effect on the Cs_2SO_4 profiles. In parallel experiments, the RNP complexes were crosslinked after exposure to 0.1 M or 0.5 M NaCl and then banded. Again, the exposure to NaCl had little effect on the integrity of the particles (Fig. 3 *E* and *F*).

The *in Vitro*-Assembled RNP Particle Contains *Bona Fide* hnRNP Proteins. *In vivo*, hnRNA is complexed with several proteins of which a sextet of M_r 32,000–42,000 major "core" proteins predominates (7, 8, 10, 35). Monoclonal antibodies to these hnRNP core proteins have recently been isolated (36), including one, iD-2, that crossreacts with all six core proteins, indicating the presence of a shared antigenic determinant. As shown in Fig. 4, lane 2, the 1,712-Nt Ad2 transcript is complexed with proteins that react with this monoclonal antibody, whereas no reaction is detectable with nonimmune mouse immunoglobulin (Fig. 4, lane 1).

To further define the proteins, we made use of 254-nm light-catalyzed RNA–protein crosslinking (32) to mark proteins that are in direct contact with the labeled RNA transcript (10, 37). This technique involves removing noncrosslinked regions of the RNA by nuclease digestion, leaving only those few nucleotides (<1% of the total) that were in direct contact with protein in the initial RNP structure at the time of crosslinking. These labeled nucleotide–protein covalent adducts are then detected by displaying the products on a standard NaDodSO$_4$/polyacrylamide gel, followed by autoradiography (10, 37).

Lane 1 of Fig. 5 shows the typical results of this experiment. It can be seen that a group of proteins having molecular weights between M_r 32,000 and 42,000 are ^{32}P-labeled, indicating that they were in contact with the Ad2 transcript in the RNP particle. For comparison, lane 2 illustrates the proteins that can be crosslinked to [^{3}H]uridine nucleotides in pulse-labeled hnRNA when intact mammalian cells are irradiated with 254-nm light *in vivo*. Here the six distinct hnRNP core proteins can be discerned as individual bands in the autoradiogram, owing to the superior resolution of ^{3}H over ^{32}P. In both the *in vitro* RNP (Fig. 5, lane 1) and the native material crosslinked *in vivo* (Fig. 5, lane 2), a group of low molecular weight bands also contains crosslinked nucleotides; these may be generated by photo-damage during the irradiation or by proteolysis during the nuclease digestion. These low molecular weight bands notwithstanding, the concordance of a simple set of M_r 32,000–42,000 proteins in both native hnRNP (Fig. 5, lane 2) and in the *in vitro*-assembled RNP (Fig. 5, lane 1) argues strongly for the specificity

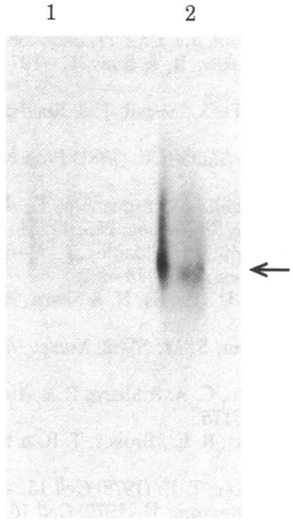

FIG. 4. The 1,712-Nt Ad2 *in vitro* transcript reacts with a hnRNP protein antibody. Two 125-μl transcription reactions were mixed with 875 μl of 0.1 M NaCl/1 mM $MgCl_2$/10 mM Tris·HCl, pH 8.5, and either 50 μl of monoclonal antibody iD-2 (36) or 50 μl of mouse IgG, both in phosphate-buffered saline. After 30 min at 4°C, 200 μl of protein A-Sepharose (Pharmacia) was added and the incubation was continued for another 30 min. One-half milliliter of blank Sepharose was then added, and the mixture was loaded into a disposable polypropylene column; 1.5 ml of 0.1 M NaCl/1 mM $MgCl_2$/10 mM Tris·HCl, pH 8.5, was added and the first 1.5 ml of eluate was collected. The column was then washed with 30 ml of 0.15 M NaCl/5 mM EDTA/0.5% Nonidet P-40 (Particle Data, Elmhurst, IL)/50 mM Tris·HCl, pH 7.5. Antibody and antibody-bound material were then eluted with 1.5 ml of 0.1 M glycine·HCl buffer (pH 3.0). RNA was recovered from eluates by adding $NaDodSO_4$ to 0.2% and Tris·HCl to 20 mM (final pH = 7.5), followed by phenol/chloroform extraction and ethanol precipitation. The RNA was glyoxalated and electrophoresed in a 1.1% agarose gel. Lane 1, mouse IgG, and lane 2, hnRNP protein monoclonal antibody.

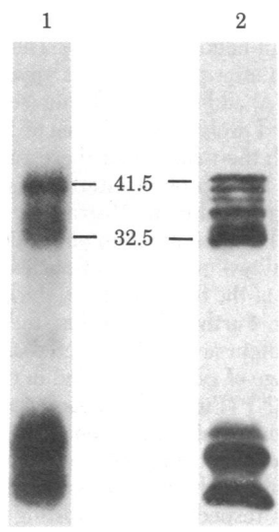

FIG. 5. Proteins associated with the Ad2 transcript *in vitro*. Twenty-five-microliter transcription reactions, run with [α-^{32}P]UTP, were diluted to 14 ml with RSB and centrifuged in a Beckman 50 Ti rotor at 50,000 rpm for 2 hr and 12 min (4°C) to pellet material larger than 30 S. The pellet was resuspended in RSB, NaCl was added to 0.5 M, and the sample was irradiated for 15 min at 4°C with 254-nm light (3.6×10^5 ergs/mm^2). Pancreatic ribonuclease was then added to 25 μg/ml, micrococcal nuclease to 400 units/ml, and $CaCl_2$ to 1 mM and the sample was incubated at 37°C for 1 hr. Protein was recovered by precipitation with 90% acetone containing 50 mM HCl, lyophilized, and dissolved in $NaDodSO_4$ gel sample buffer. Electrophoresis was in 11% polyacrylamide gels containing 0.1% $NaDodSO_4$. Lane 1, proteins in contact with the Ad2 transcript *in vitro*, and lane 2, proteins crosslinked to [^{3}H]uridine nucleotides in hnRNA from cells irradiated *in vivo* (taken from ref. 10). Molecular weights were determined from the migration of ^{14}C-labeled protein standards in parallel and are shown as $M_r \times 10^{-3}$.

of the latter, especially taken together with the antibody results (Fig. 4).

DISCUSSION

The experiments described here demonstrate that a defined RNA polymerase II transcript, produced from the adenovirus major late promoter, assembles into a specific RNP particle *in vitro*. This particle appears to be similar to native hnRNP by several criteria, including its protein/RNA ratio, stability in Cs_2SO_4 isopycnic banding, and the presence of *bona fide* hnRNP proteins. Of course, we cannot be certain at present that the *in vitro*-assembled particle has the same structure as native hnRNP in every respect.

These results establish several points concerning hnRNP assembly. Although these particles are normally formed in the nucleus, it is clear from the present results that nuclear structure is not essential for their assembly. This does not necessarily mean that hnRNP particles are not associated with other nuclear elements *in vivo*, but it does indicate that the first level of hnRNP structure can assemble in a soluble *in vitro* system. This conclusion is compatible with the previous demonstration that hnRNP, dissociated into RNA and protein by 2 M NaCl, can reassociate after dialysis to low ionic strength (38–40). Reconstitution of hnRNP-like structures from naked RNA has also been reported with an *Artemia* embryo protein, HD-40, that shares several properties with vertebrate hnRNP core proteins (41, 42). The present results extend these previous reconstitution experiments by demonstrating spontaneous *in vitro* RNP assembly from a specific gene transcript.

The experiments reported here do not establish that the hnRNP proteins bind only hnRNA and not RNA in general. Proteins that recognize specific DNA or RNA sequences will, under appropriate solvent conditions, also display a general affinity for nucleic acid (e.g., ref. 43 for the case of the cro repressor). This point has been addressed in detail by von Hippel and colleagues (44–46). In this respect it is of interest that hnRNP-like proteins have been shown to bind synthetic polyribonucleotides (41). According to the precedents mentioned above, this does not eliminate the possibility that hnRNP proteins bind specific hnRNA sequences *in vivo*, such as intron–exon borders (*vide infra*).

We do not know if the Ad2 *in vitro* transcript forms RNP while still a nascent RNA chain or does so only after the polymerase has moved off the DNA template. However, we found (to our complete surprise) that addition of deproteinized Ad2 RNA to the transcription extract did not lead to as much RNP formation as when the transcript was produced in the first place (unpublished data), suggesting that the polymerase–template–transcript complex may be a favored local environment for the deposition of hnRNP proteins on the growing RNA chain. In this regard, the recent finding that the *Bal* I E DNA template forms nucleosomes in this *in vitro* transcription extract may be pertinent (47).

Because the Ad2 transcript we have examined does not contain sites for polyadenylylation, these results demonstrate that RNP formation does not depend on poly(A) addition. Similarly, because splicing does not occur appreciably in this *in vitro* system (18–20), it is clear that hnRNP assembly neither depends on prior splicing nor is sufficient to cause it. These considerations are all compatible with the view that hnRNP assembly takes place on nascent transcripts, which was originally suggested by ultrastructural analysis of spread chromatin (48–50),

and is further supported by recent RNA–protein crosslinking studies of chromatin-bound hnRNA (10). Therefore, the normal schedule of post-transcriptional events appears to be hnRNP formation → poly(A) addition → splicing (see also ref. 51).

A major unsolved problem in the area of hnRNP function is the extent to which the structure of these particles is based on sequence-specific RNA–protein interactions. Recently, a detailed study has appeared of RNP structure on an adenovirus E2 transcript, the DNA-binding protein mRNA precursor (52). This study revealed two major sites of nuclease protection: one at the 5′ splice site of the first leader and the other in the center of the large intron. Further data in this study implicated a M_r 38,000 hnRNP protein at these protected sites, which is also within the size range of proteins we find in the *in vitro*-assembled Ad2 *Bal* I E RNP (Fig. 5). Moreover, the Ad2 hnRNP particle whose *in vitro* assembly has been described here displays a sedimentation coefficient compatible with the presence of two hnRNP subunits (although we have not directly demonstrated this point). In this respect, it is interesting that, like the adenovirus E2 RNP structure studied by Ohlsson *et al.* (52), the *Bal* I E transcript also contains two splice sites (between leaders 1 and 2 and the 5′ site for leader 2-to-3 splicing). We have recently proposed, on the basis of very different experiments, that hnRNP proteins may be located only on transcripts of intron-containing genes (33). This again would imply that hnRNP assembly is based, at least in part, on intervening sequences or splice junctions, or both.

The topography of hnRNP proteins on specific pre-mRNA sequences can be defined by use of RNP particles containing a single well-defined transcript, such as we have reported here. In addition, the *in vitro* assembly approach based on transcription of cloned DNA lends itself attractively to analysis of RNP formation on transcripts of intron-containing versus intron-lacking genes, as well as on transcripts of genes manipulated with respect to promoter and termination sites, intron location and polarity, and single nucleotide modifications, through the general vehicle of *in vitro* mutagenesis.

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