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Assembly of nuclear ribonucleoprotein particles during *in vitro* transcription*

(isolated nuclei/RNA polymerase II/heterogeneous nuclear RNA/RNA–protein crosslinking)

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ABSTRACT **The assembly of heterogeneous nuclear RNA (hnRNA) into ribonucleoprotein (RNP) particles has been investigated during *in vitro* transcription in isolated nuclei. Approximately 80% of the *in vitro* transcription observed in mouse Friend erythroleukemia cell nuclei is attributable to the activity of RNA polymerase II. *In vitro* hnRNA transcripts are assembled into particles having the same properties as the nuclear ribonucleoprotein (hnRNP) particles in which hnRNA is found *in vivo*. Direct contact of hnRNP proteins with newly transcribed hnRNA was demonstrated by nuclease protection experiments and by the covalent transfer of ^{32}P-labeled nucleotides from [α-^{32}P]UTP-labeled hnRNA transcripts to specific proteins by RNA–protein crosslinking followed by nuclease digestion and electrophoresis of the nucleotide-bearing proteins. The availability of an *in vitro* system for hnRNP assembly opens a new route for investigating the functional relationship between nuclear structure and mRNA processing.**

Isolated nuclei are capable of transcription when incubated under the proper conditions (1–4). For example, nuclei isolated from HeLa cells infected with adenovirus serotype 2 (Ad2) synthesize RNAs of the size expected for primary transcripts from the major late promoter at 16.5 map units on the Ad2 genome (5–7). Some of these *in vitro* Ad2 transcripts are correctly capped, polyadenylated, and spliced (6, 8, 9), indicating that the mRNA processing machinery is at least partially active in the isolated nucleus.

The RNA polymerase II transcripts of eukaryotic genes, known collectively as heterogeneous nuclear RNA (hnRNA), are complexed with a specific set of nuclear proteins in the cell (10–18, reviewed in ref. 19). The assembly of hnRNA transcripts into ribonucleoprotein (RNP) particles (hnRNP) appears to be a very early posttranscriptional event, as indicated by the RNP-like ultrastructure of nascent hnRNA (20) and by the presence of very briefly pulse-labeled hnRNA transcripts in RNP structures (17). This raises the question of whether hnRNA transcripts synthesized *in vitro* in isolated nuclei become assembled into hnRNP particles resembling those found *in vivo*.

To examine this issue, we have investigated the RNP status of hnRNA transcripts synthesized *in vitro* by endogenous RNA polymerase II in isolated nuclei, using the system of Manley *et al.* (8). Our results show that hnRNP assembly takes place *in vitro*, that the particle proteins make direct contact with the newly synthesized hnRNA, and that the *in vitro*-assembled particles are identical to native hnRNP by several criteria. This opens up an experimental system for exploring the relationship between nuclear RNP assembly and mRNA processing.

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METHODS

Cell Fractionation, *in Vitro* Transcription, and hnRNP Isolation. Stocks of mouse Friend erythroleukemia cells (clone 745) were maintained in monolayer cultures and grown in suspension culture for each experiment (13). Erythroid differentiation was induced for 84 hr with 2% dimethyl sulfoxide. In some cases, cells were pulse labeled for 15 min with [^{3}H]uridine in the presence of actinomycin at 0.08 μg/ml as described (13). For *in vitro* transcription, cells were first treated with actinomycin (0.08 μg/ml) and nuclei were then isolated by the method of Mory and Gefter (21) and incubated in the reaction mixture described by Manley *et al.* (8). Freshly isolated nuclei were used for all experiments. Reaction mixtures contained 1–5 $\times$ 10^8 nuclei per ml and either [^{3}H]UTP or [α-^{32}P]UTP. Transcription was allowed to proceed for 2 hr at 26°C unless otherwise noted.

At the end of the incubation period, reactions were terminated by centrifuging the nuclei at 1000 $\times$ *g* for 5 min at 5°C and then suspending them in ice-cold reticulocyte standard buffer (RS buffer; 10 mM NaCl/1.5 mM $MgCl_2$/10 mM Tris·HCl, pH 7.2). The nuclei were then fractionated as detailed (13). The material banding at the 0:30% sucrose interface, containing the hnRNP particles and $\approx$12% of the chromatin (13), was used in some experiments while, in other cases, the hnRNP particles were further purified on sucrose gradients as described (13). Analysis of the *in vitro*-assembled particles by Cs_2SO_4 isopycnic banding and nuclease digestion followed described procedures (13, 15–17, 22). In some experiments, for purposes of comparison, hnRNP particles were isolated directly from *in vivo*-labeled cells as described (13).

Transfer of ^{32}P-Labeled Nucleotides from *in Vitro* Transcripts to hnRNP Particle Proteins. To identify proteins in direct contact with newly transcribed hnRNA, a photochemical RNA–protein crosslinking technique was used (15). Nuclei were incubated as usual with [α-^{32}P]UTP, centrifuged, and suspended in RS buffer. Half of the sample was then irradiated with 254-nm light at 3.6 $\times$ 10^5 ergs/mm^2 (1 erg = 0.1 μJ) as described (15). These and control (unirradiated) nuclei were then fractionated as usual and the hnRNP particles were purified in sucrose gradients. The hnRNP sedimenting faster than 20S was pooled and collected by ultracentrifugation. The pelleted particles were dissolved in RS buffer/1 mM $CaCl_2$ and digested at 37°C for 1 hr with pancreatic ribonuclease (25 μg/ml) and micrococcal nuclease (400 units/ml). The proteins were

Abbreviations: Ad2, adenovirus serotype 2; hn, heterogeneous nuclear; RNP, ribonucleoprotein.

* This is paper no. 18 in a series entitled "Ribonucleoprotein organization of eukaryotic RNA." Paper no. 17 is ref. 35.

precipitated by addition of 9 vol of acetone containing 50 mM HCl, dried, and dissolved in sample buffer for gel electrophoresis (10% glycerol/1% $NaDodSO_4$/2.5% 2-mercaptoethanol/62.5 mM Tris·HCl, pH 6.8/0.05% bromophenol blue). Electrophoresis was in 11% polyacrylamide/0.1% $NaDodSO_4$ gels (23).

RESULTS

hnRNA Transcription *in Vitro*. Nuclei were purified from Friend erythroleukemia cells and incubated in the reaction mixtures described by Weber *et al.* (5), Yang and Flint (7, 9), and Mory and Gefter (21) as modified by Manley *et al.* (8). The latter system displayed the highest rate of incorporation of [^{3}H]UTP into trichloroacetic acid-insoluble material and was therefore used throughout this investigation. The time course of UTP incorporation in the presence and absence of α-amanitin, a specific inhibitor of RNA polymerases II and III (24), is shown in Fig. 1. Incorporation is reduced to 20% of normal by a low concentration of inhibitor (0.5 μg/ml) and to 10% of normal by a high concentration (200 μg/ml). By this criterion, 80% of the incorporation reflects the activity of RNA polymerase II. hnRNA that had been pulse labeled *in vivo* with [^{3}H]uridine is completely stable, as trichloroacetic acid-precipitable radioactivity, during incubation of nuclei in the *in vitro* system for 2 hr, and both 15S and 9S β-globin RNA transcripts remain present as shown by gel blot hybridization with cloned β-globin DNA (data not shown).

The sizes of the hnRNA molecules into which UTP is incorporated in the *in vitro* transcription system were examined by centrifuging phenol-deproteinized RNA in sucrose gradients containing 99% dimethyl sulfoxide. Radioactivity was found in hnRNA molecules having sedimentation coefficients of 10–40S (data not shown), which is comparable with the previously reported size of mouse erythroblast hnRNA labeled *in vivo* (25). Hybridization of the *in vitro* transcripts to cloned mouse β-globin DNA showed that 0.04% of the labeled hnRNA was β-globin specific, as compared with a value of 0.01% for hnRNA labeled for 5 minutes *in vivo* (17).

Assembly of *in Vitro* hnRNA Transcripts into RNP Particles. Cells were pulse labeled *in vivo* with [^{3}H]uridine in the presence of actinomycin at 0.08 μg/ml to suppress ribosomal RNA synthesis (26). Nuclei were then purified and incubated in the *in vitro* transcription system with [α-^{32}P]UTP. The nuclei were fractionated as described (13), and the postnucleolar supernatant was centrifuged in a sucrose gradient to display the hnRNP particles. As shown in Fig. 2, the *in vitro* hnRNA transcripts (^{32}P) reside in structures whose sedimentation velocity is similar to that of hnRNP particles synthesized *in vivo* (^{3}H).

To explore further the RNP status of the *in vitro* hnRNA transcripts, postnucleolar fractions were analyzed by isopycnic banding in Cs_2SO_4 density gradients, in which the density of naked RNA is 1.65 g/cm^3 and that of hnRNP is 1.33–1.35 g/cm^3 (13, 27). Fig. 3 shows that most of the *in vitro* hnRNA transcripts band at 1.36 g/cm^3, as does the *in vivo*-labeled hnRNP. A small fraction of both the *in vivo* and the *in vitro* transcripts band as naked RNA (1.65 g/cm^3). The material banding at 1.36 g/cm^3 is estimated to be ≈80% protein and 20% RNA. These results, and those in Fig. 2, show that the majority of the hnRNA synthesized *in vitro* resides in material having the properties of hnRNP particles, including the diagnostic criterion of withstanding isopycnic banding in Cs_2SO_4 without prior aldehyde fixation (discussed in ref. 15). A previous report (28) indicates that hnRNA labeled in isolated nuclei becomes associated with proteins as defined by CsCl banding of formaldehyde-fixed material, which is a less diagnostic criterion for hnRNP then the results shown in Figure 3.

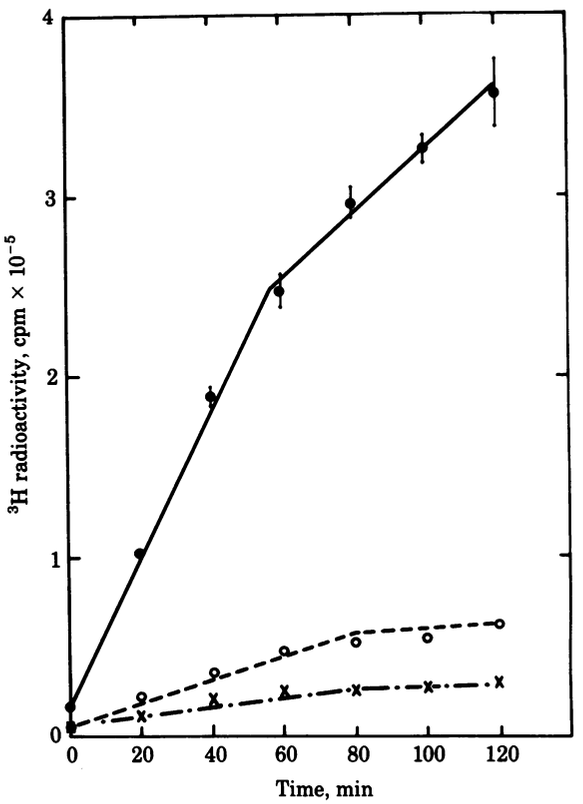

FIG. 1. *In vitro* transcription. Nuclei at 10^8/ml were incubated in the standard transcription system with [^{3}H]UTP at 140 μCi/ml (1 Ci = 3.7 × 10^{10} becquerels), and samples were removed as indicated (●); parallel reactions were run in the presence of α-amanitin at 0.5 μg/ml (○) or at 200 μg/ml (×). The amount of ^{3}H radioactivity incorporated into 10% trichloroacetic acid-precipitable material was measured. These data were obtained with nuclei isolated from cells that had been treated previously with actinomycin at 0.08 μg/ml *in vivo* to suppress ribosomal RNA synthesis (26). When *in vitro* transcription was done without prior actinomycin treatment *in vivo*, the level of incorporation was similar to that shown and the extent of inhibition by both low and high concentrations of amanitin were also the same, indicating that RNA polymerase I is not particularly active.

Direct Contact of Proteins with *in Vitro* hnRNA Transcripts. The above results show that the *in vitro* hnRNA transcripts are incorporated into RNP structures, but they do not

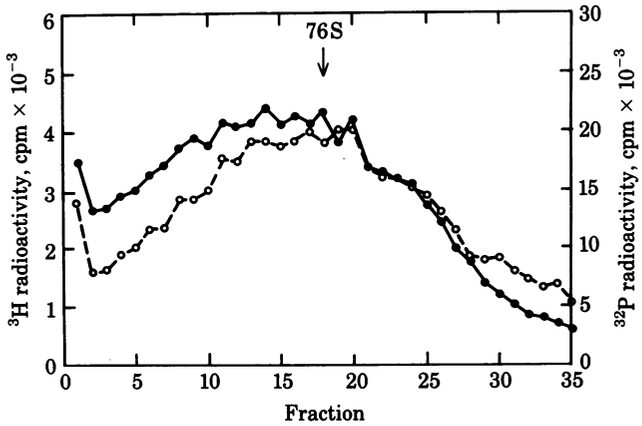

FIG. 2. Presence of *in vitro* hnRNA transcripts in RNP structures. Cells were labeled *in vivo* for 15 min. with [^{3}H]uridine, and then nuclei were isolated and allowed to transcribe *in vitro* for 2 hr in the presence of [α-^{32}P]UTP. The hnRNP fraction was isolated and sedimented in a 15–30% sucrose gradient (SW27 rotor, 15,000 rpm, 17 hr, 4°C), and trichloroacetic acid-precipitable ^{3}H [*in vivo* (●)] and ^{32}P [*in vitro* (○)] radioactivities were determined. Sedimentation is from right to left.

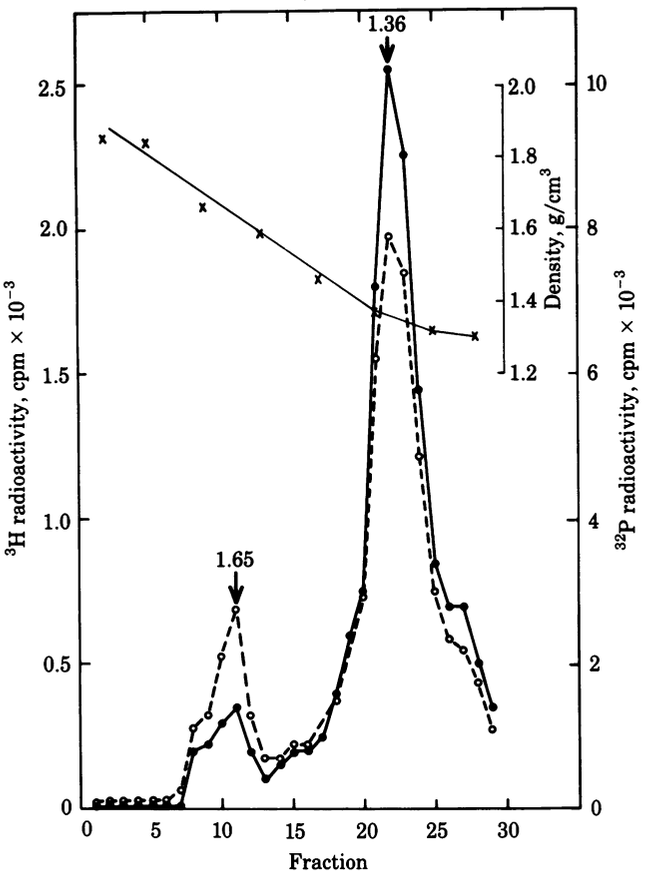

FIG. 3. RNP structure of *in vitro* hnRNA transcripts as shown by isopycnic banding in Cs_2SO_4. A portion of the hnRNP fraction from the experiment shown in Fig. 2 was layered on a preformed Cs_2SO_4 gradient (initial density, 1.25–1.75 g/cm^3) and centrifuged to equilibrium (SW50.1 rotor, 34,000 rpm, 64 hr, 20°C). Density and trichloroacetic acid-precipitable ^{3}H [*in vivo* (●)] and ^{32}P [*in vitro* (○)] radioactivities were measured as described (22, 27).

establish that the labeled hnRNA is itself directly complexed with protein. Two extreme possibilities are illustrated in Fig. 4: In one (*A*) the *in vitro* transcripts are added as naked RNA "tails" onto hnRNP particles previously assembled *in vivo*; in the other (*B*), the *in vitro* transcripts are similarly extended on preexisting hnRNP but are, in addition, assembled into RNP.

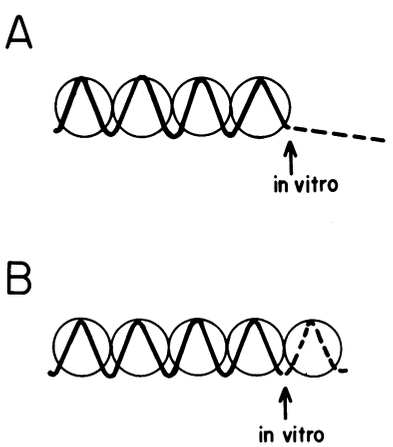

FIG. 4. (*A*) *In vitro* extension of naked hnRNA transcripts onto hnRNPs previously assembled *in vivo*. (*B*) *In vitro* assembly of hnRNP on newly transcribed hnRNA.

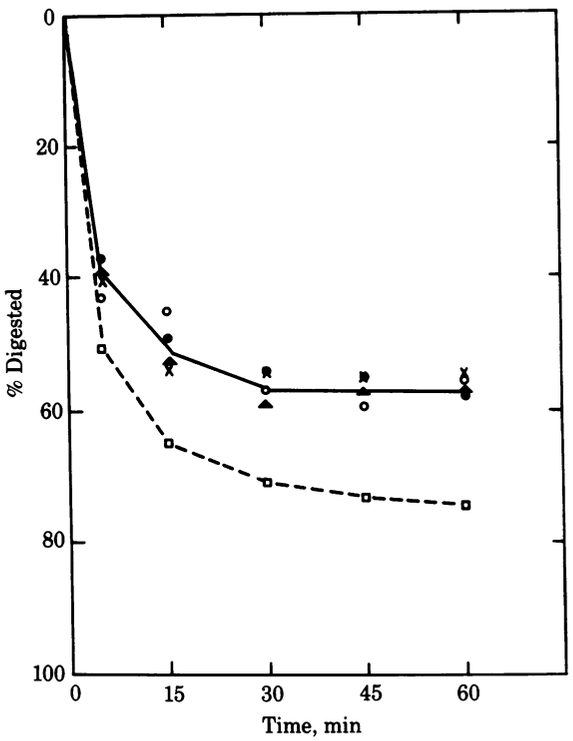

FIG. 5. *In vitro* hnRNA transcripts assemble into nuclease-protected structures. Transcription *in vitro* was allowed to proceed in the presence of [^{3}H]UTP, and nuclei were fractionated. hnRNP particles labeled *in vivo* with [^{3}H]uridine and deproteinized hnRNA were used for comparison. In all cases, the samples were adjusted to a final A_{260} value of 4.0 by adding unlabeled hnRNP (isolated directly from nuclei without incubation *in vitro*) to ensure that the nuclease/hnRNA ratio would be the same for all digestions. Pancreatic RNase was added to 0.05 μg/ml and the samples were incubated at 4°C. Aliquots were removed as indicated, and trichloroacetic acid-precipitable radioactivity was determined. x, hnRNP labeled *in vivo* for 20 min with [^{3}H]uridine; ▲, hnRNP labeled *in vitro* for 30 min with [^{3}H]UTP; ●, hnRNP labeled *in vitro* for 60 min; ○, hnRNP labeled *in vitro* for 120 min; □, deproteinized hnRNA.

One way of discriminating between the two possibilities is to probe the nuclease sensitivity of the *in vitro* hnRNA transcripts, using conditions in which hnRNP and naked hnRNA are digested at different rates (13, 16, 17). The results of such an analysis are shown in Fig. 5. *In vitro* hnRNA transcripts are more resistant to ribonuclease than naked (deproteinized) hnRNA and their ribonuclease sensitivity profile is identical to that of hnRNP particles labeled *in vivo*. This indicates that the *in vitro* transcripts are indeed covered by protein. The *in vitro* assembly of hnRNP continues for considerable periods of time; if nuclei are allowed to transcribe for 1 hr without labeled UTP and then for an additional 90 min with [^{3}H]UTP, the hnRNA transcripts labeled between 60 and 150 min *in vitro* are found to be nuclease protected in measurements of the kind shown in Fig. 5.

To examine the RNP status of the *in vitro* hnRNA transcripts at a finer level of resolution, we exploited the ability of 254-nm light to crosslink hnRNA–protein associations, as described in our studies of hnRNP structure *in vivo* (15, 18). If nuclei are allowed to transcribe *in vitro* in the presence of [α-^{32}P]UTP and the resulting hnRNP is crosslinked and then digested to completion with nuclease, any proteins that had been in direct contact with the *in vitro*-synthesized hnRNA at the time of irradiation will carry ^{32}P-labeled nucleotides due to the covalent linkage established by the photochemical crosslinking of RNA nucleotides in direct contact with protein. The ^{32}P atoms then

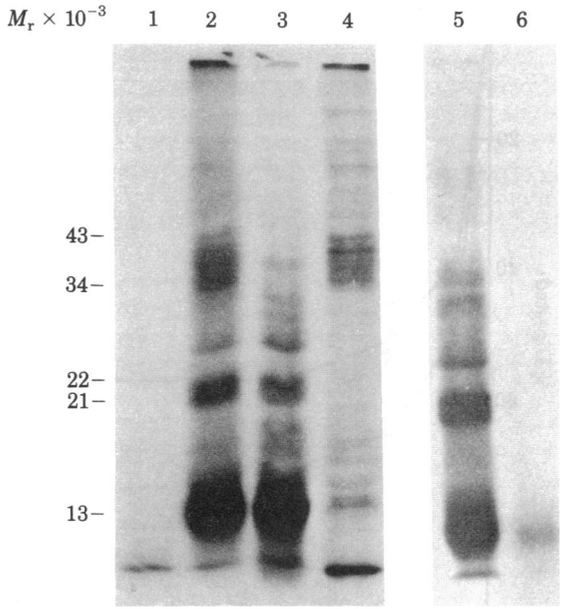

FIG. 6. Contacts between *in vitro* hnRNA transcripts and hnRNP proteins as shown by RNA–protein crosslinking. Lanes 1, proteins in gradient-purified hnRNP after *in vitro* transcription with [α-^{32}P]UTP but without RNA–protein crosslinking; 2, proteins after *in vitro* transcription with [α-^{32}P]UTP and crosslinking; 3, proteins crosslinked *in vivo* to [^{3}H]uridine pulse-labeled hnRNA in the postnucleolar supernatant fraction (hnRNP); 4, proteins crosslinked *in vivo* to [^{3}H]uridine pulse-labeled hnRNA in the chromatin/nucleolar fraction; 5, proteins in postnucleolar supernatant fraction (hnRNP) after *in vitro* transcription with [α-^{32}P]UTP and crosslinking; 6, same as lane 5 except that the sample was digested with proteinase K prior to electrophoresis. Numbers to the left of lane 1 apply to bands in lanes 2–4; lanes 5 and 6 are from a gel run on a separate occasion.

serve to mark the RNA-contacting proteins when the latter are displayed in polyacrylamide gels (29).

The results of such crosslinking experiments are shown in Fig. 6. Nuclei were allowed to transcribe *in vitro* as usual. Then, portions of the nuclei were irradiated at 254 nm as described (15), hnRNP particles were isolated on sucrose gradients (13) and digested with nuclease, and the proteins were visualized by gel electrophoresis and autoradiography. When the crosslinking step was omitted, the hnRNP proteins contained no ^{32}P derived from [α-^{32}P]UTP (Fig. 6, lane 1). In contrast, after crosslinking, several protein bands carried covalently attached ^{32}P (lane 2). These proteins have molecular weights of 13,000–43,000. The labeling of these proteins is dependent on concurrent transcription, because inclusion of a high concentration of actinomycin in the *in vitro* transcription system almost completely abolished the ^{32}P radioactivity associated with the gel bands (data not shown). This rules out the trivial possibility that the crosslinking technique is capturing nonpolymerized ribonucleotides bound to nuclear proteins in the *in vitro* system. One group of these proteins is a set of five bands having estimated molecular weights of 34,000, 35,000, 37,500, 39,000, and 43,000, which are similar to those reported for the group A, B, and C "core" polypeptides of mammalian hnRNP (30, 31). These are also among the proteins that we have shown to be in direct contact with hnRNA *in vivo* by RNA–protein crosslinking (18). Moreover, the facts that the labeled proteins (Fig. 6, lane 2) are recovered from purified hnRNP particles (13) and that the majority of RNA labeled *in vitro* is transcribed by RNA polymerase II (Fig. 1) lead to the conclusion that these proteins are in contact specifically with hnRNA. This is confirmed by the experiment shown in Fig. 6, lanes 3 and 4, in which cells were labeled with [^{3}H]uridine *in vivo*, crosslinked, and fractionated. In this case, the ability of a low concentration of actinomycin (0.08 μg/ml) to suppress ribosomal RNA synthesis *in vivo* (26) is exploited by treating the cells with the inhibitor prior to [^{3}H]uridine labeling. As shown in lane 3, the postnucleolar supernatant fraction, containing the hnRNP, contains a set of proteins that have covalently attached [^{3}H]uridine and these *in vivo*-crosslinked proteins correspond qualitatively to many of the bands observed in hnRNP assembled *in vitro* (lane 2). The proteins crosslinked *in vivo* to hnRNA in the subnuclear fraction that contains nucleoli and most of the chromatin (13) are shown in lane 4. This fraction, which is expected to contain nascent hnRNP, is specifically enriched in the M_r 34,000–43,000 proteins. As shown in lanes 5 and 6, protease digestion of *in vitro*-transcribed hnRNP prior to electrophoresis abolished the ^{32}P radioactivity.

DISCUSSION

We have demonstrated that hnRNA transcripts synthesized *in vitro* in isolated nuclei become incorporated into RNP structures that are similar to the nuclear RNP particles in which hnRNA is found *in vivo* (13). There is apparently sufficient RNP protein in the isolated nuclei to accommodate assembly of the hnRNA transcripts into particles. Previous reconstruction experiments in which labeled hnRNA was added to HeLa or Friend cell nuclei during hnRNP isolation have not shown the existence of a large available pool of soluble hnRNP proteins in the nucleus (12, 13). However, in the present experiments, the nuclei were isolated under isotonic conditions (21) and this may allow retention of more hnRNP protein than in the earlier reconstruction experiments (12, 13), in which the nuclei were isolated in hypotonic buffers. Moreover, the amount of hnRNA transcribed *in vitro* is small. For example, in a typical reaction with 10^8 Friend cell nuclei, we estimate that $\approx$6 fg of hnRNA is synthesized per nucleus. It is possible that the amount of hnRNP protein required to accommodate the assembly of such small amounts of transcript into hnRNP particles would have escaped detection in the previous reconstruction experiments. It is also possible that the physico-chemical conditions in the *in vitro* transcription system are more favorable for hnRNA–protein interactions than in the hypotonic buffers used in the previous experiments.

The present results have a bearing on the requirements for *in vitro* mRNA processing in isolated nuclei. Although we have not examined the polyadenylation or splicing of specific mRNA precursors in these experiments, it is known that Ad2 transcripts made in the *in vitro* system we have used can be polyadenylated at correct sites and accurately spliced (8). To the extent that these processing steps were measured specifically on the Ad2 hnRNA that is labeled *in vitro* (ref. 8, see also ref. 9), our demonstration that the *in vitro* hnRNA transcripts are assembled into hnRNP particles suggests that the mRNA processing events observed in this isolated nucleus system occur on RNP templates.

Among the evidence for *in vitro* hnRNP assembly is the photochemical transfer of ^{32}P-labeled nucleotides in the labeled hnRNA to contacting proteins by RNA-protein crosslinking followed by nuclease digestion (Fig. 6). This yields reproducible patterns that include proteins having the same molecular weights as the major A, B and C core polypeptides of hnRNP but, in other respects, the patterns are different from the protein profiles reported for mammalian hnRNP (see, for example, refs. 13, 18, 30, 31). Coomassie blue-stained or [^{35}S]methionine-labeled hnRNP proteins show few components of molecular weight lower than the A core proteins (32,000 and 34,000), whereas the ^{3}H- or ^{32}P-nucleotide-bearing hnRNP proteins

crosslinked either *in vivo* or *in vitro* include proteins in the M_r 13,000–26,000 range (Fig. 6). However, it is not surprising that the amount of ^3H or ^{32}P present per polypeptide chain in these RNA-contact experiments deviates from the hnRNP protein mass-distribution profile seen by staining or [^{35}S]methionine labeling, because the nucleotide labeling of a given protein via crosslinking is determined by the length of RNA with which it is in contact. Moreover, because different amino acids and nucleotides have different crosslinking efficiencies (32), the amount of nucleotide transferred also depends on protein and RNA sequence. The possibility that the M_r <32,000 proteins labeled with ^{32}P are proteolytic products of hnRNP proteins, produced during *in vitro* transcription, is ruled out by their appearance in lane 3 of Fig. 6, where the crosslinking was carried out *in vivo*. It is also worth noting that nuclear proteins in contact with DNA should not contribute to the *in vitro* analysis shown in lane 2 of Fig. 6 because the precursor is a ribonucleotide and because the rate of DNA synthesis is extremely low at the stage of erythroid induction (3.5 days) at which the Friend cells were harvested for these experiments. We have examined the possibility that the M_r <32,000 proteins labeled in the crosslinking experiments are components of RNP particles containing small nuclear RNAs (33, 34), which are known to be associated with hnRNP (35). However, these proteins are not recognized by antibodies specific for RNPs containing small nuclear RNAs (unpublished results). Moreover, the rate of labeling of small nuclear RNAs in the *in vitro* system is far too low to account for the amount of [^{32}P]UTP label observed in the M_r 22,000, 21,000, and 13,000 proteins in lane 2 of Fig. 6 (data not shown).

The demonstration that hnRNA transcripts assemble into specific hnRNP particles in an *in vitro* system opens the door to several lines of investigation. For example, it may be possible to determine the immediacy with which various hnRNP proteins are deposited on the nascent transcript by combining RNA-protein crosslinking and nuclear fractionation into chromatin-associated (nascent) hnRNP and completed nucleoplasmic hnRNP. Moreover, the details of hnRNP assembly for the transcript of a defined gene and the functional involvement of hnRNP structure in mRNA processing may now be more accessible to analysis.

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