RNA–Protein Interactions in a Cell-Free System with Isolated Nuclei from GH_3 Cells†

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ABSTRACT: Previously it was shown that nuclei from GH_3 cells synthesized RNA in a cell-free system for an extended period of time (Biswas, D. K., et al. (1976) *Biochemistry 15*, 3270). Experiments reported here showed that cell-free synthesized RNA polymerase II products interacted with proteins contained in these nuclei resulting in the formation of RNA–protein complex (RNP). Cell-free RNA prepared from the nucleoplasm and the postnuclear supernatant was fractionated on oligo(dT)-cellulose as two distinct radioactive peaks. About 70–75% of the nucleoplasmic [^{3}H]RNA and 85–90% of the postnuclear supernatant [^{3}H]RNA did not bind to oligo(dT)-cellulose at 0.5 M KCl. The rest of the [^{3}H]RNA remained bound to the cellulose at 0.5, 0.25, 0.1, 0.0 M KCl and could only be eluted with buffer containing 50% formamide. A small fraction of ^{14}C-labeled protein derived from prelabeling intact cells with [^{14}C]leucine coeluted with 50% formamide elutable [^{3}H]RNA. Inhibition of cell-free RNA synthesis with 0.1 μg per mL of α-amanitin also inhibited the cell-free formation of RNP as evident by the absence of 50% formamide elutable ^{3}H and ^{14}C radioactivity. Inhibition of protein synthesis by cycloheximide pretreatment of cells also inhibited the cell-free appearance of RNP in the postnuclear supernatant. The differential kinetics of release of cell-free RNA products into the postnuclear supernatant indicated that the isolated nuclei performed a process resembling nuclear cytoplasmic RNA transport. Isopycnic banding of the cell-free RNP on CsCl density gradient revealed that particles having RNA to protein ratio similar to that of cellular RNP particles were formed in the cell-free system. It is concluded that cell-free RNA products of RNA polymerase II interact with protein contained in the nuclei and appear in the postnuclear supernatant as RNP particles, whereas the RNA polymerase III products were transported across the nuclear membrane as free RNA.

The primary transcripts of the RNA polymerase II, HnRNA,[1] are possible precursors of cytoplasmic mRNAs (Darnell et al., 1973). Both putative precursor and product were found to be present in the cell as RNP particles. The association of RNA with cellular proteins to form a RNA–protein complex (RNP particles) (Spirin & Nemer, 1965; Perry & Kelley, 1968; Henshaw, 1968; Darnell et al., 1973) is believed to be important for the subsequent transport of mRNA across the nuclear membrane and for its function and metabolism. Pederson (1974), Kumar & Pederson (1975), and Kish & Pederson (1975) made comparative studies of the protein associated with HeLa cell nuclear RNA, and with polysomal mRNA and observed qualitative differences in the proteins associated with HnRNP and polysomal RNP. In addition to the mRNP particles released from the polysomes there were also nonpolysomal, cytoplasmic RNP particles (Bag & Sarkar, 1975). The role of these particles and their relation to the other type of cytoplasmic RNP particles are not yet clear. Recently Barrieux et al. (1976) suggested that some of the proteins present in mRNP particles may act as translational factors. Several laboratories have studied the transport of RNA synthesized in the whole cells in isolated nuclei (Yannarell et al., 1976; Chatterjee & Weissbach, 1973; Stuart et al., 1977; Ishikawa et al., 1970). Schumm et al. (1973) have implicated a role for protein factors in transporting presynthesized RNA across the nuclear membrane.

We have reported earlier (Biswas et al., 1976) that isolated GH_3 nuclei synthesized RNA in a cell-free system for an extended period of time. Characterization of the cell-free RNA products revealed that all three RNA polymerases were active in this system and that posttranscriptional processing, such as poly(A) addition took place in this system. In the present investigation it was ascertained whether the cell-free synthesized RNA polymerase II products occurred as free RNA or RNP complex. The results presented here demonstrate that cell-free synthesized polymerase II RNA products interact with a small fraction of the cellular proteins contained in the isolated nuclei, leading to the formation of RNP particles. The results indicate that these were transported across the nuclear membrane into the postnuclear supernatant as RNP particles.

Experimental Procedure

Materials. Materials used in these experiments were obtained as follows: sucrose, grade I, cesium chloride, grade I, ATP, GTP, CTP, UTP, phosphocreatine, creatine phosphokinase, cycloheximide (Sigma Chemical Co.); oligo(dT)-cellulose, T_3 (Collaborative Research Inc.); formamide, reagent grade (Fisher Scientific Co.), before use formamide was treated with ion-exchange resin (AG 501-x8D), Bio-Rad Lab, and activated charcoal as described by Kumar & Pederson (1975), Nonidet P-40 (Shell Oil Co.), [U-^{14}C][leucine (296 mCi/mmol), [8-^{3}H]GTP (13.2 Ci/mmol) (New England Nuclear), α-amanitin (Calbiochem), glass fiber filters, GF/C (Whatman). Rat liver ribonuclease inhibitor was a previous stock of G. D. Searle & Co. GH_3 cells used in this investigation are hormone-producing rat pituitary tumor cells in culture (Martin & Tashjian, 1977). Culture media and sera were obtained from Grand Island Biological Co.

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[1] Abbreviations used: RSB, reticulocyte standard buffer (Biswas & Tashjian, 1974); TBS buffer and TKM buffer (Biswas et al., 1976); RI, ribonuclear inhibitor; CK, creatine phosphokinase; HnRNA, heterogeneous nuclear RNA; BSA, bovine serum albumin; HnRNP, HnRNA-protein complex.

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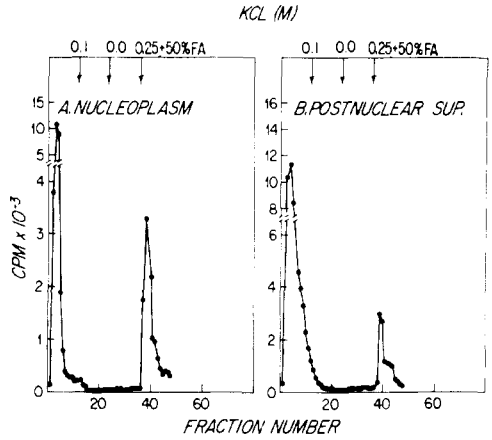

FIGURE 1: Oligo(dT)-cellulose chromatography of cell-free [^{3}H]RNA products. Nuclei were incubated in complete reaction mixture containing [^{3}H]GTP under conditions described previously (Biswas et al., 1976). Nucleoplasm and postnuclear supernatant fractions were isolated from the reaction mixture after the incubation period of 60 min at 29 °C. Details of the preparation of the samples and subsequent analysis by oligo(dT)-cellulose chromatography have been described in Experimental Procedure. These fractions were then applied to oligo(dT)-cellulose column equilibrated with 0.01 M sodium phosphate buffer containing 0.5 M KCl and eluted with the same buffer. At the arrow the elution buffer was changed to the one containing indicated concentrations of KCl. FA = formamide. (A) Fractionation profile of nucleoplasmic fractions; (B) fractionation profile of postnuclear supernatant fraction.

Preparation of Nuclei. GH_3 cells grown as suspension culture were harvested and washed with ice cold TBS buffer as previously described (Biswas & Tashjian, 1974). The washed cells were suspended in RSB at 10^7 cells/mL containing 2 units of RI and allowed to swell for 10 min at 0 °C. The cells were then homogenized in loose-fitting Dounce homogenizer and lysed with ten strokes. All the subsequent operations were carried out at 0–4 °C unless stated otherwise. The ruptured cell suspensions were sedimented at 500*g* for 10 min. The pelleted nuclei were washed three times with TKM buffer (Biswas et al., 1976) and finally resuspended in the same buffer at 5×10^6 to 1×10^7 nuclei/mL. These nuclei were found free of cytoplasm upon examination by phase contrast and electron microscopy (courtesy of Dr. George Szabo).

Formation of RNP in the Cell-Free System and Fractionation by Oligo(dT)-Cellulose Chromatography. RNA synthesis with isolated nuclei in the presence of [^{3}H]GTP was carried out in complete reaction mixture as previously described (Biswas et al., 1976). After the desired incubation time the reaction mixture was centrifuged at 500*g* for 10 min. Supernatant (referred to as postnuclear supernatant) was saved and the nuclear pellet was resuspended in TKM and centrifuged again; the second supernatant was added to the first. The KCl concentration of the pooled supernatant was adjusted to 0.5 M and applied to oligo(dT)-cellulose column preequilibrated with 0.01 M sodium phosphate buffer (pH 7) containing 0.5 M KCl. Preequilibration of the column and subsequent operation of fractionation of the cell-free RNA products were carried out at room temperature (Kumar & Pederson, 1975). All the eluting buffers contain 500 μg/mL of heparin. The sample (1–2 mL) was applied to the column and unbound radioactivity at 0.5 M KCl was removed from the cellulose with 10 mL of the same buffer. Subsequent elution of radioactivity from oligo(dT)-cellulose was carried out by applying 10 mL each of 0.01 M sodium phosphate buffer, pH 7, containing different amounts of KCl (as indicated in the figures). RNP was eluted from oligo(dT)-cellulose with buffer containing 0.25 M KCl and 50% formamide. Thirty-drop fractions were collected (approximately 12–14 fractions). Radioactivity in different fractions was measured by addition of 2 mL of 10% Cl_3CCOOH (cold) containing 50 mM sodium pyrophosphate in the presence of 0.1 mL of 1 mg/mL yeast RNA as carrier. Insoluble radioactivity was quantitated by filtration on GF/C filters which were counted at 30% efficiency in omnifluor. Recovery of ^{3}H and ^{14}C radioactivity from the oligo(dT)-cellulose column was in the order of 95–100%.

Preparation of Nucleoplasm. Nucleoplasm was prepared by rupturing the nuclei after removal of the postnuclear supernatant, by sonic oscillation in a Branson sonifier, Model W-350 at 20 W by 3 × 15 s pulses. Most of the nuclei were ruptured under these conditions as determined by phase contrast microscopy. The sonicate was then layered on 10 mL of 30% sucrose in TKM buffer and centrifuged at 5000 rpm in SW41 rotor in a L3-50 Beckman ultracentrifuge, for 15 min. The nucleoli sedimented and nucleoplasm was collected from the top and interphase of the sucrose solution. Salt concentration of the nucleoplasm was then adjusted to 0.5 M KCl and then loaded on to the oligo(dT)-cellulose column and fractionated as described above.

Centrifugation in CsCl Density Gradients. The fractions comprising the first peak of the oligo(dT)-cellulose column (flow-through material) and the 50% formamide elutable material were pooled separately and dialyzed against 0.01 M sodium phosphate buffer to remove KCl and formamide. The dialyzed samples were then concentrated by alcohol precipitation. The concentrated material containing the RNP particles was fixed by making the solution 6% with freshly neutralized formaldehyde. The formaldehyde treated samples were kept at 0 °C for 1 h for fixation. The fixed samples were then layered on a CsCl gradient preformed by mixing 2.4 mL of CsCl solution having a density of 1.6 g/mL with 2.6 mL of CsCl solution having a density of 1.35 g/mL. The tubes were then filled with light mineral oil layered over the samples and centrifuged at 41 000 rpm for 16 h in SW 41 rotor at 4 °C. After the centrifugation 8-drop fractions were collected by puncturing the tubes at the bottom. Refractive indexes of alternate fractions were measured in a Bausch and Lomb refractometer. Cl_3CCOOH-precipitable radioactivity was then determined in each sample. Marker samples such as 80S ribosome were run under the same conditions. Refractive index and optical density at 260 nM were determined in the fractionated samples.

Isolation of RNA. Reaction mixtures were chilled following incubation and separated into nuclear pellet and postnuclear supernatant fractions. Nucleoplasm was isolated from the nuclear pellet as described above. To both fractions was then added proteinase K (50 μg/mL) and after 15 min at room temperature the mixtures were made 1% with $NaDodSO_4$. RNA from these mixtures was then extracted by the phenol-pH 9 method of Lee et al. (1971). For the preparation of poly(A) RNA, the total RNA extracted either from nucleoplasm or from postnuclear supernatant was subjected to oligo(dT)-cellulose chromatography under the conditions described earlier (Biswas et al., 1976). The 0.0 M KCl-elutable radioactive material was concentrated by alcohol precipitation.

Results

Formation of RNP Particles in a Cell-Free System Containing Isolated Nuclei. Results presented in Figure 1 show the fractionation profile on oligo(dT)-cellulose of cell-free [^{3}H]RNA products in the nucleoplasm (panel A) and that in postnuclear supernatant (panel B). Both postnuclear supernatant and nucleoplasmic fraction separated on oligo(dT)-

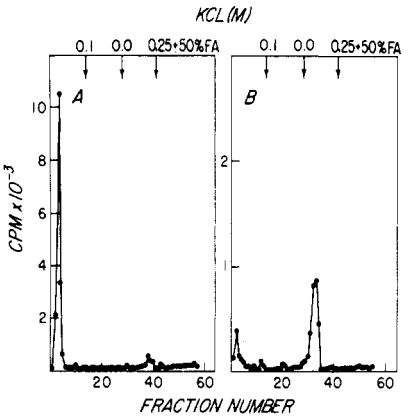

FIGURE 2: Oligo(dT)-cellulose chromatography of [^{3}H]RNA extracted from flow-through and 50% formamide elutable cell-free products of postnuclear supernatant. Incubations were carried out with isolated nuclei in the presence of [^{3}H]GTP under the conditions described in Experimental Procedure. Postnuclear supernatant was isolated from the reaction mixture after the incubation (60 min) and subjected to oligo(dT)-cellulose chromatography. The fractions comprising the flow-through material (0.5 M KCl wash) and those eluted with 50% formamide were pooled and RNA was extracted from each of these two radioactive fractions. The salt concentration in the [^{3}H]RNA solutions was then adjusted to 0.5 M KCl and the samples were applied to oligo(dT)-cellulose column and fractionated under the conditions described in Methods. Panel A shows the fractionation profile of [^{3}H]RNA in flow-through radioactive peak and panel B shows that of [^{3}H]RNA in the 50% formamide elutable radioactive cell-free products of postnuclear supernatant. FA = formamide.

cellulose column as two distinct Cl_3CCOOH-precipitable radioactive peaks. About 70–75% of the nucleoplasmic [^{3}H]RNA product and about 85–90% of that present in the postnuclear supernatant did not bind to oligo(dT)-cellulose equilibrated with buffer containing 0.5 M KCl. The [^{3}H]RNA products which remained bound to oligo(dT)-cellulose at 0.5, 0.25, 0.1, and at 0.0 M KCl could be eluted by 0.01 M phosphate buffer containing 50% formamide. The second peak (referred to as formamide elutable fraction) constituted about 25–30% of the nucleoplasmic [^{3}H]RNA products and about 10–15% of the total postnuclear supernatant [^{3}H]RNA products. Very little radioactivity (1–2%) could be eluted from cellulose with 0.01 M phosphate buffer alone indicating that both nucleoplasmic and postnuclear supernatant poly(A)-containing RNA products were mostly present as RNA–protein complex.

To investigate the specificity of interactions of cell-free synthesized [^{3}H]RNA with proteins contained in the isolated nuclei, the following experiments were performed. Removal of externally added proteins such as BSA, RI, or CK from the reaction mixture one at a time did not effect the formation of RNP in the cell-free system. While removal of RI effected the net synthesis of RNA, it did not effect RNP formation. Incubation of [^{3}H]poly(A) RNA, extracted either from postnuclear supernatant or nucleoplasm with BSA, or RI or CK under the optimal conditions for RNA synthesis and RNP formation, in the absence of nuclei did not form RNP as judged by the absence of 50% formamide elutable radioactivity (data not shown). Synthesis of RNA and formation of RNP were also carried out by using nuclei prepared in three different ways: (1) by Dounce homogenization; (2) by mild detergent treatment (0.2% NP-40): and (3) by Dounce homogenization of NP-40 treated cells. Nuclei prepared by methods 1 and 3 were found to be free of cytoplasmic contamination upon examination by phase contrast and electron microscopy, whereas those from method 2 contained a rim of cytoplasm around the nuclei. Irrespective of which types of nuclei were used in the cell-free system, the formation of RNP was always evident. In subsequent experiments nuclei prepared by Dounce homogenization which were free of cytoplasm were used.

RNA Present in Formamide Elutable and Flow-Through Fractions of Postnuclear Supernatant. Rechromatography of the cell-free RNA products present in the 0.5-M KCl wash and that present in the formamide elutable fraction of postnuclear supernatant was carried out on oligo(dT)-cellulose. Most of the radioactivity in the flow-through material did not bind to oligo(dT) at 0.5 M KCl upon reapplication of the concentrated material to the column. Most of the radioactivity in the formamide elutable [^{3}H]RNA products, when reapplied to the oligo(dT)-cellulose column after extensive dialysis in 0.01 M sodium phosphate buffer and subsequent concentration by alcohol precipitation, was retained by the cellulose at 0.5 M KCl. This could be eluted with 50% formamide containing buffer (data not shown).

[^{3}H]RNA was extracted from the 0.5 M KCl wash and from the formamide elutable fraction and applied to oligo(dT)-cellulose column (Biswas et al., 1976). Essentially all the [^{3}H]RNA extracted from flow-through material did not bind to the column at 0.5 M KCl (Figure 2, panel A). However, the [^{3}H]RNA extracted from 50% formamide elutable cell-free products remained bound to the cellulose and could be eluted with buffer containing no salt (0.0 M KCl) (Figure 2, panel B). This result indicated that the cell-free synthesized RNA which interacted with the nuclear protein to form the RNP complex was a poly(A)-containing RNA.[2]

Buoyant Density Gradient Analysis of Cell-Free Formed RNP Complex. The presence of RNP particles in the cell-free products was further demonstrated by CsCl buoyant density gradient centrifugation. The 50% formamide elutable postnuclear supernatant and that present in nucleoplasmic material banded in CsCl gradient with a peak density of 1.39 g/mL (Figure 3, panel A) and 1.40 g/mL (Figure 3, panel B), respectively . The flow-through material in the postnuclear supernatant did not form a distinct radioactive peak in CsCl and most of the radioactivity was at the bottom of the tube, suggesting that this RNA contains little or no protein. The flow-through radioactive material in the nucleoplasmic fraction, however, banded at a density of 1.42 g/mL (Figure 3, panel C). These results suggest that both poly(A)-containing RNA and nonpoly(A) RNA in the nucleoplasm were present as RNP particles. The 80S ribosome banded at a density of 1.57 g/mL, when analyzed in CsCl gradient under identical conditions.

Interaction of Cell-Free Synthesized [^{3}H]RNA with [^{14}C]Leucine Labeled Proteins. Intact cells were exposed to [^{14}C]leucine and subsequently RNA synthesis was conducted with isolated nuclei in a reaction mixture containing [^{3}H]GTP. The cell-free formation of a [^{3}H]RNA-^{14}C-labeled protein complex and its transport across the nuclear membrane was then studied by following both ^{3}H and ^{14}C radioactivity (in the postnuclear supernatant and in the nucleoplasmic fraction) by fractionation on oligo(dT)-cellulose. The [^{3}H]RNA products in the flow-through material and in the 50% formamide elutable fraction were associated with superimposable ^{14}C radioactive peaks (Figure 4A and 4C). About 75–85% of

[2] The terms poly(A) RNA and nonpoly(A) RNA were used in this report on an operational basis. The RNA products which remained bound to oligo(dT)-cellulose at high salt (0.5 M KCl) were designated as poly(A) containing and the RNA products which did not bind under the same conditions were referred to as nonpoly(A) RNA. Poly(A) RNA could be eluted from oligo(dT)-cellulose with buffer containing no salt.

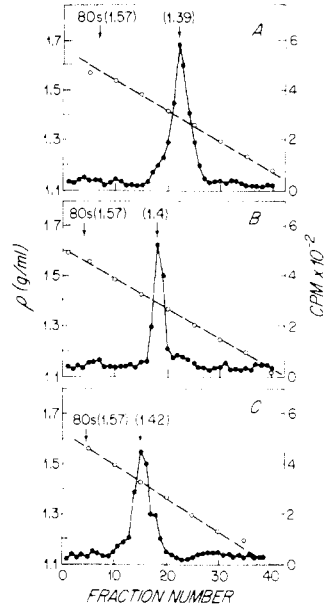

FIGURE 3: Isopycnic banding of cell-free products on CsCl density gradient. Nuclei were incubated in complete reaction mixture containing [^{3}H]GTP for 60 min at 29 °C. Nucleoplasm and postnuclear supernatant were isolated from the reaction mixture after the incubation and subjected to oligo(dT)-cellulose chromatography. The oligo(dT)-cellulose fractionated cell-free [^{3}H]RNA products in the flow-through material and 50% formamide elutable fractions were then concentrated by alcohol precipitation, dialyzed, fixed in formaldehyde, and applied to CsCl gradients as described in Experimental Procedure. (A) Postnuclear supernatant–50% formamide elutable [^{3}H]RNA products; (B) nucleoplasmic formamide elutable material; and (C) flow-through (0.5 M KCl wash) [^{3}H]RNA products in the nucleoplasmic fraction. The numbers in parentheses represent density (ρ = g/mL) in the peak tube. (O—O) Density; (●—●) ^{3}H.

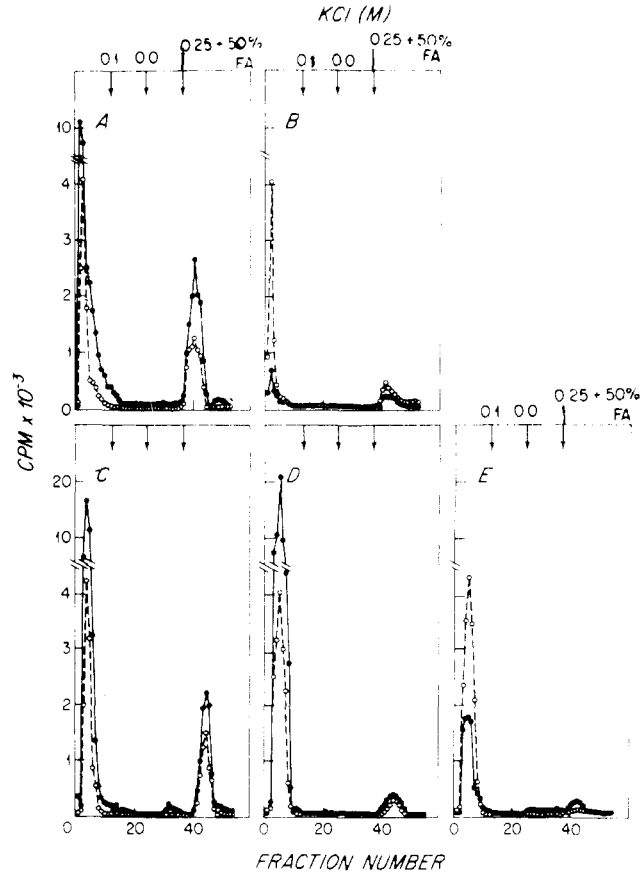

FIGURE 4: Oligo(dT)-cellulose chromatography of cell-free [^{3}H]RNA products synthesized by nuclei isolated from [^{14}C]leucine-labeled cells. Cells grown in suspension culture were harvested and resuspended in preequilibrated leucine-free Eagle's minimal medium containing 10% dialyzed fetal calf serum at a density of 2×10^7 cells/mL. Following incubation for 30 min at 37 °C in 5% CO_2 and 95% air, the cells were exposed to [^{14}C]leucine (1 μCi/mL) and incubated for 4 h more at 37 °C. The cells were then harvested, washed three times with TBS, and resuspended in RSB to make nuclei by Dounce homogenization as described in Experimental Procedure. Isolated nuclei were then incubated in complete reaction mixture containing [^{3}H]GTP for 60 min at 29 °C. Nucleoplasm and postnuclear supernatant fractions were isolated from the reaction mixture after the incubation and fractionated on oligo(dT)-cellulose. The ^{3}H and ^{14}C radioactivity in each fraction was measured by making proper window adjustments in the liquid scintillation counter. (A) Nucleoplasmic cell-free RNA products in the control; (B) nucleoplasmic fraction isolated from reaction mixture incubated in the presence of 0.1 μg/mL α-amanitin; (C) postnuclear supernatant cell-free products isolated from control incubation; (D) postnuclear cell-free RNA products isolated from reaction mixture containing 0.1 μg/mL α-amanitin; and (E) postnuclear supernatant cell-free RNA products from the reaction mixture incubated in the presence of 100 μg/mL α-amanitin. (●—●) = ^{3}H; (O—O) = ^{14}C. FA = formamide.

the postnuclear supernatant and nucleoplasmic ^{14}C radioactivity was present in the 0.5 M KCl flow-through peak and about 15–25% was present in the 50% formamide elutable radioactive peak. Addition of low concentrations of α-amanitin (0.1 μg/mL) to the cell-free RNA-synthesizing system reduced the 50% formamide elutable ^{3}H and ^{14}C radioactivity in the postnuclear supernatant (Figure 4, panel D), whereas ^{3}H and ^{14}C radioactivity in the flow-through cell-free fraction was unaffected. Low concentrations of α-amanitin inhibited the synthesis of both poly(A)-containing and nonpoly(A)-containing [^{3}H]RNA product in the nucleoplasmic fraction, indicating that both of these RNAs in the nucleoplasm were the products of polymerase II (Figure 4, panel B). α-Amanitin at low concentrations inhibited completely the appearance of ^{14}C radioactivity eluted with [^{3}H]RNA by 50% formamide in the nucleoplasmic fraction. These results suggest that ^{14}C-labeled proteins formed RNA–protein complex with poly(A)-containing RNAs. Addition of α-amanitin at high concentrations (100 μg/mL) (Biswas et al., 1976) inhibited the synthesis of flow-through [^{3}H]RNA of the postnuclear supernatant, suggesting that this class of RNA was a product of RNA polymerase III (Figure 4E). However, ^{14}C radioactivity in this fraction was not affected by the drug, indicating that the polymerase III products were not associated with ^{14}C-labeled proteins.

Kinetics of Release of Cell-Free Synthesized RNA Products from the Nuclei. Results presented in Figure 5 show the kinetics of appearance of flow-through and 50% formamide elutable [^{3}H]RNA products in the postnuclear supernatant. The flow-through material was released from the nucleus immediately (zero time) after synthesis, whereas no significant amount of 50% formamide elutable cell-free RNA products could be detected in the postnuclear supernatant within 15 min of incubation. A significant amount of flow-through [^{3}H]RNA products could be detected in the postnuclear supernatant as early as 5 min of incubation and this class of cell-free [^{3}H]-RNA product continued to be released into the postnuclear supernatant and reached a plateau after 30 min of incubation (Figure 5). (A small fraction of radioactivity representing less than 1% of the total postnuclear supernatant radioactivity applied to the column was always present in the 50% formamide elutable fraction. This may represent the extent of nonspecific RNA–protein interactions in this system.) The 50% formamide elutable [^{3}H]RNA products in the postnuclear supernatant could be detected at about 15 min of incubation. This material continued to be released from the nuclei after

TABLE I: Effect of Cycloheximide Pretreatment of Cells on the Cell-Free Formation of Nucleoplasmic and Postnuclear Supernatant RNA Products.[a]

Treatment	Nucleoplasmic fractions (%)			Postnuclear supernatant fractions (%)		
	0.5 M KCl wash	0.0 M KCl eluate	50% formamide eluate	0.5 M KCl wash	0.0 M KCl eluate	50% formamide eluate
None	69.9	1.8	29.3	85	2	13
Cycloheximide (12)	75.1	2.9	22.0	89	2.5	8.5
Cycloheximide (20)	82.4	5.6	12.0	96	1.5	2.2

[a] Cells were grown in suspension in the presence of 1 μg/mL cycloheximide for periods indicated in parentheses (h). Cells were then harvested and washed, and nuclei were isolated and RNA was synthesized in complete system containing [^{3}H]GTP in a 60-min incubation at 29 °C. Nucleoplasm and postnuclear supernatant were isolated from the reaction mixture after the incubation period and cell-free RNA products in these fractions were analyzed on oligo(dT)-cellulose column as described in Experimental Procedure. Cl_3CCOOH-precipitable radioactivity in 0.5 M KCl wash, in 0.0 M KCl elute, and in 50% formamide elutable fractions obtained after oligo(dT)-cellulose chromatography was determined. Numbers indicate percent of total nucleoplasmic and postnuclear supernatant radioactivity.

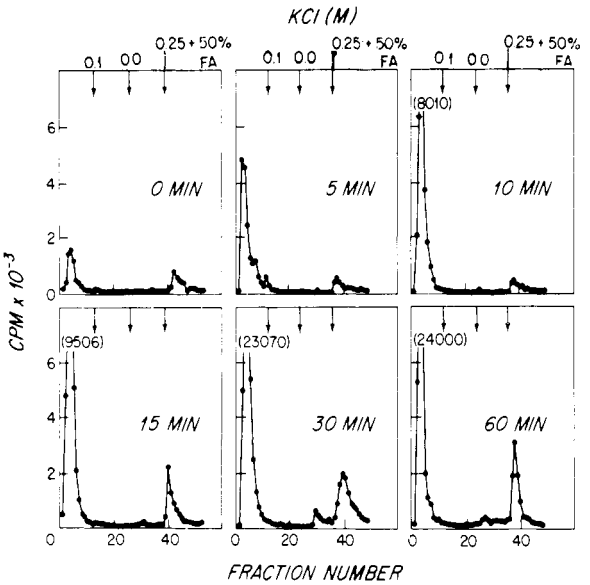

FIGURE 5: Kinetics of release of cell-free [^{3}H]RNA products into the postnuclear supernatant. Nuclei isolated from cells grown in suspension culture were incubated in complete reaction mixture containing [^{3}H]GTP at 29 °C. Aliquots of the reaction mixture were withdrawn at specified times and postnuclear supernatant was isolated and fractionated on oligo(dT)-cellulose, under the conditions described in Experimental Procedure, and Cl_3CCOOH-precipitable radioactivity was measured. Numbers in parentheses represent cpm in the peak fraction of the flow-through material; FA = formamide.

the initial 15-min lag period, as reflected by the increased amount of radioactivity in the 50% formamide elutable fraction with time. These results indicate that one class of cell-free RNA was released from the nucleus immediately after synthesis, whereas the other class was retained by the nuclei for 15 min and subsequently released into the postnuclear supernatant as an RNA–protein complex.

Effect of Cycloheximide on the Release of Cell-Free Synthesized RNA–Protein Complex. Cycloheximide at a concentration of 1 μg/mL inhibited protein synthesis in GH_3 cells (as judged by [^{3}H]leucine incorporation into hot Cl_3CCOOH-precipitable radioactivity) completely within 10 min (T. F. J. Martin, personal communication). Examination of cycloheximide-treated cells by phase contrast microscopy did not show significant morphological difference in comparison with control cells. Though growth of the cells was inhibited upon addition of cycloheximide (1 μg/mL) to the culture medium, the cells resumed growth after removal of the drug from the medium. When nuclei from control and cycloheximide-treated cells were incubated for 60 min, the distribution of [^{3}H]GTP incorporation in nucleoplasm, nucleolar pellet and postnuclear supernatant was 25, 35, and 40%, respectively. Cell-free [^{3}H]GTP incorporation into RNA was inhibited to the extent of 4, 28, and 86% upon pretreatment of the cells with cycloheximide for 12, 20, and 48 h respectively. The fraction of α-amanitin-resistant (polymerase I), 0.1 μg/mL α-amanitin-inhibited (polymerase II), and 300 μg/mL α-amanitin-inhibited RNA synthesis was the same in nuclei from control and cycloheximide-treated cells (data not shown). The effect of inhibition of cellular protein synthesis by cycloheximide on the cell-free formation of RNP was studied by analyzing the cell-free RNA products from the nucleoplasm and postnuclear supernatant after incubating nuclei in a reaction mixture containing [^{3}H]GTP. Cell-free RNP formation in nuclei isolated from cycloheximide-treated cells was inhibited. Poly(A)-RNP (50% formamide elutable radioactivity) represented 12% and 2.2% of nucleoplasmic and postnuclear supernatant [^{3}H]RNA fractions respectively in nuclei isolated from cycloheximide-treated cells as compared with 29% and 13% respectively in control nuclei (Table I). Protein-free poly(A)-containing RNA, which represented a small fraction (1–2%) both in nucleoplasm and postnuclear supernatant, increased considerably (from 1.8% to 5.6%) in the former, but not in the postnuclear supernatant, following cycloheximide treatment.

Discussion

Results presented in this report demonstrate that cell-free synthesized RNA interacts with a small fraction of protein contained in isolated nuclei, resulting in the formation of RNP particles. This was studied: (a) by fractionation of nucleoplasmic and postnuclear supernatant cell-free RNA products by oligo(dT)-cellulose chromatography; (2) by following the association of cell-free synthesized [^{3}H]RNA with ^{14}C-labeled protein (pre-labeled by incubation of intact cells with [^{14}C]-leucine); and (3) by isopycnic banding on CsCl density gradients. Poly(A)-containing RNA polymerase II products of the postnuclear supernatant and poly(A) and nonpoly(A) RNA products of the nucleoplasm formed RNP particles. Kumar & Pederson (1975) reported that both oligo(dT)-nonbound and -bound fractions of HeLa cell HnRNA were found as an RNA protein complex. The low α-amanitin-resistant, residual ^{14}C-radioactivity in the 50% formamide elutable fraction (Figure 4, panel B) may represent RNP formed in the intact cells during the incubation of the cells with [^{14}C]leucine.

Banding of the cell-free formed RNP particles on CsCl density gradients suggested that RNA to protein ratio of these particles was different from that of 80S ribosomes and comparable to RNP particles isolated from eukaryotic cells (Irwin et al., 1975). The bulk of the postnuclear supernatant RNA (polymerase III products) did not interact with nuclear proteins to form RNP and occurred in the cell-free system as free RNA. Only RNA products of RNA polymerase II and not of RNA polymerase III interacted with nuclear proteins to form RNP particles in the cell-free system.

[^{3}H]Poly(A) RNA isolated either from postnuclear supernatant or from nucleoplasm did not form RNP when incubated with proteins like BSA, CK, and RI. Inhibition of RNA polymerase II directed RNA synthesis completely abolished both nucleoplasmic and postnuclear supernatant RNP formation, suggesting that cell-free formation of RNP was a result of interactions between a specific class of newly synthesized [^{3}H]RNA and a small fraction of ^{14}C-labeled nuclear proteins (Figure 4).

Results presented in Figure 5 confirm our previous observation that there is a differential rate of release of cell-free synthesized RNA products into the postnuclear supernatant. RNA polymerase III products emerged from the nuclei immediately after synthesis, a finding that resembles the immediate appearance of pre-4S and 5S RNA into the cytoplasm of intact cells (Weinberg, 1973). In contrast, RNA polymerase II products synthesized in the cell-free system emerged into the postnuclear supernatant after a lag period as RNP particles. These results suggest that RNA polymerase II products underwent nuclear processing, including interaction with proteins before transport across the nuclear membrane. Thus these nuclei retained their capacity to perform a process resembling nuclear cytoplasmic transport of RNA.

When protein synthesis was inhibited by incubation of the cells with cycloheximide prior to isolation of the nuclei, formation of RNP in the cell-free system was inhibited suggesting that prior synthesis and presence of some of the nuclear proteins were essential for the cell-free formation of RNP particles. Pretreatment of cells with cycloheximide reduced the fractional amount of poly(A) RNP both in nucleoplasm and in postnuclear supernatant (Table I). Although there was a significant increase in poly(A) RNA in nucleoplasm it did not totally account for simultaneous reduction in poly(A) RNP fraction. There was no detectable increase in the poly(A) RNA in the postnuclear supernatant, suggesting that poly(A) RNA was transported from the nuclei mostly as RNP particle and not as free RNA. These results do not completely rule out the possibility of rapid degradation of released poly(A) RNA in the postnuclear supernatant. However, in control experiments 80–90% of cell-free synthesized [^{3}H]poly(A) RNA could be recovered following incubation in complete reaction mixture without the nuclei, suggesting that there was not much degradation of [^{3}H]poly(A) RNA taking place in the postnuclear supernatant.

It is concluded from the results presented in this report that cell-free poly(A)-containing polymerase II products interacted in the nuclei with a small fraction of nuclear proteins to form RNP particles. The RNA protein ratio of these particles resembles those isolated from different eukaryotic cells. It is suggested that this class of RNA emerges from the nuclei into postnuclear supernatant as RNA–protein complex. The nature of the proteins associated with these cell-free synthesized RNP particles is not known yet. Their characterization and the study of the interaction of these proteins with RNA should clarify the involvement of these proteins in mRNA stability, transport, function, and metabolism. The possible involvement of these proteins in the expression of a gene via modulation of any one or more of the processes of mRNA biogenesis and mRNA metabolism may be elucidated.

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