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POLY(A)-PROTEIN INTERACTIONS AND TRANSPORT OF mRNA IN ISOLATED NUCLEI

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Summary. RNA-protein complexes (RNP), formed in a cell-free system using nuclei from GH cells, prelabelled, with [^{3}H] leucine,were isolated from nucleoplasm and from postnuclear supernatant (PNS). Radioactive proteins,associated with newly synthesized HnRNP and mRNP-like material in the PNS were examined.On SDS gels, the [^{3}H] protein pattern of poly(A)HnRNP was found to be more complex than that of PNS poly(A)-RNP.Only three radioactive proteins (78K,100K and 120K) were associated with the poly(A) segments of cell-free formed HnRNP and PNS poly(A)-RNP.Cordycepintriphosphate and α-amanitin reduced the transport of cell-free formed poly(A) RNP associated [^{3}H] proteins to the extent of 52% and 46% respectively. It may be concluded that the poly(A) segment of newly synthesized mRNA-like material is directly or indirectly (by providing binding sites for specific proteins) responsible for the transport of poly(A) containing mRNA.

Among the several steps involved in the process of eukaryotic mRNA biogenesis are the 3' end addition of poly(A) segments to the primary products of RNA-polymerase II and the interaction of proteins with such RNA molecules (1). Both heterogenous nuclear RNA(HnRNA) and cytoplasmic mRNA have been shown to exist as RNA-protein complexes (RNP). Although there is no definitive evidence for the assignment of specific roles to the proteins associated with either HnRNA or mRNA, several possible functions,including a role in the transport of mRNA,have been postulated (2-4). The 78K protein, which is found to be present in both HnRNP and mRNP (5),has been found to be associated with the poly(A) segment of mRNA (3,6-9).Schwartz and Darnell (3) have suggested that this protein is involved in the transport of mRNA. We have reported previously (10,11) that extended RNA synthesis,poly(A) synthesis and the interactions of newly synthesized RNA with nuclear proteins take place in the cell-free system with isolated nuclei from GH-cells. In this report we provide experimental evidence to suggest that interaction of specific proteins with the poly(A) segment of mRNA-like molecules is obligatory for the transport of newly synthesized, poly(A) containing RNA species across the nuclear membrane.

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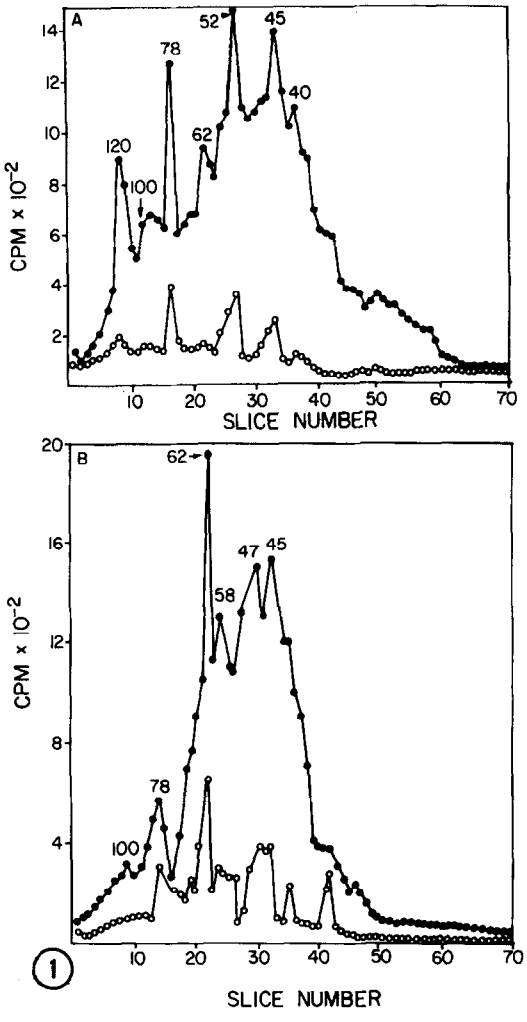
Methods: GH-cells used in this investigation are hormone producing pituitary tumor cells in culture (10). Procedures for growing, harvesting and washing of cells were described previously (10). Cellular proteins were prelabelled by growing cells in MEM minus leucine in the presence of 10 μCi/ml [^{3}H] leucine for 4 hr at 37°. Preparation of the nuclei, optimum conditions for RNA synthesis with isolated nuclei, cell-free RNP formation and fractionation by oligo(dT) cellulose chromatography has been described before (11). Postnuclear supernatant (PNS) was isolated after incubation by centrifugation of the cell-free reaction mixture at 1000 rpm for 10 min. in an IEC B-20 refrigerated centrifuge. Nucleoplasm was isolated from the pelleted nuclei after incubation, and cell-free formed poly(A) HnRNP were isolated from the nucleoplasm by oligo(dT) cellulose chromatography (5,6).Native HnRNP and native mRNP from salt washed polysomes were isolated according to the method of Pederson (5) and poly(A) HnRNP and poly(A) mRNP were isolated by subsequent oligo(dT) cellulose chromatography of these fractions. SDS polyacrylamide gel electrophoresis was carried out in preformed 10% polyacrylamide gels prepared according to the method of Laemmli (12). Samples were electrophoresed at 1mA/cm of gel and were sliced into pieces of 2mm in length. Radioactive proteins were extracted with 0.5ml of 50% Protosol in toluene at 37° for 16-18 hr and counted in 5ml of Omnifluor. Electrophoretically separated proteins were stained (as indicated in specific experiments) with coomassie blue and destained with gentle shaking in 10% isopropanol mixture.

Results and Discussion: Analysis of proteins associated with cell-free formed poly(A)-HnRNP and PNS poly(A)-RNP. Results presented in Fig.1, panel A,show that radioactive proteins migrating with apparent molecular weights of 40K, 45K, 52K, 62K, 78K, 100K and 120K were present in the nucleoplasmic poly(A) HnRNP fraction. Similarly analyzed radioactive proteins, having electrophoretic mobilities corresponding to molecular weights 45K, 47K, 58K, 62K, 78K and 100K, could be detected in PNS poly(A)-RNP (Fig.1, panel B).When cell-free RNA synthesis was carried out in the presence of α-amanitin (0.5 μg/ml) there was a 70-80% reduction of radioactive proteins associated with poly(A) HnRNP and a 40-60% reduction of those associated with PNS poly(A)-RNP. These results demonstrate that the major fraction of the radioactive proteins associated with poly(A)-HnRNP and PNS poly(A)-RNP were formed under cell-free conditions by interaction of the newly synthesized RNA with [^{3}H] labelled nuclear proteins. The α-amanitin resistant fraction, 20-30% in the case of poly(A)HnRNP and 40-60% in the case of PNS-poly(A)-RNP, constitutes labelled proteins associated with RNP fractions formed in the intact cells (native RNP).The resemblance of poly(A) HnRNP and PNS poly(A) RNP,isolated from the nuclear system, to native HnRNP and native RNP can be seen in the comparison of HnRNP



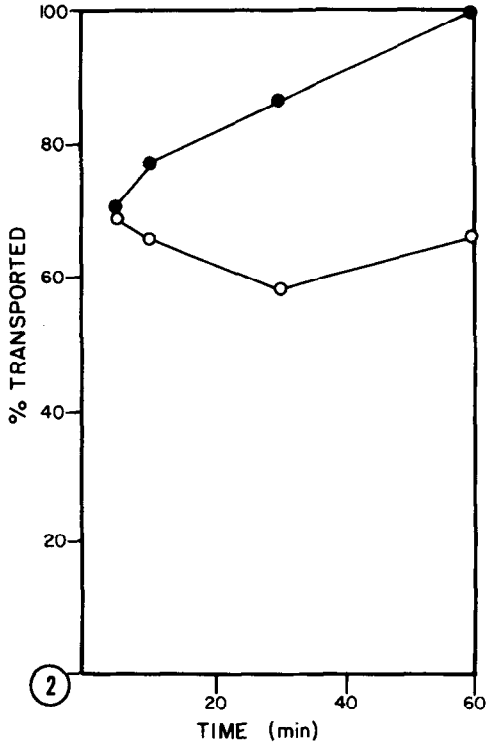


Fig. 1. SDS-polyacrylamide gel electrophoretic analysis of proteins assocated with cell-free formed RNP. Panel A shows the radioactive profile of proteins associated with nucleoplasmic poly(A)-HnRNP isolated after incubation of the nuclei (isolated from cells prelabelled with [^{3}H] leucine) in the complete reaction mixture in the absence (•-•) or in the presence (o-o) of α-amanitin (0.5 μg/ml). The numbers designate the approximate molecular weight x10^{-3} of the radioactive proteins as calculated from the mobility of several protein standards electrophoresed with each sample. Details of the gel electrophoresis procedure are described in "Materials and Methods". Panel B shows the radioactive profile of proteins associated with postnuclear supernatant poly(A)-RNP. PNS-poly(A)-RNP isolated from a reaction mixture incubated (60 min, 29°) in the absence (•-•) or in the presence (o-o) of α-amanitin (0.5 μg/ml).

Fig.2. Kinetics of release of radioactive proteins from nuclei into PNS. Nuclei isolated from cells prelabelled with [^{3}H] leucine were incubated in the complete reaction mixture in the absence (•-•) or presence)o-o) or cordycepintriphosphate (5μg/ml). Aliquots of the reaction mixture were withdrawn at the indicated times and centrifuged to pellet the nuclei and to isolate the PNS. TCA insoluble radioactivity was then determined in the aliquot of PNS. The amount of radioactivity released from nuclei into PNS in the control reaction mixture incubated for 60 min. in the absence of the drug was considered as 100% release of radioactive proteins.

(Fig.1A) and PNS poly(A)-RNP (Fig.1B) isolated from the reaction in the absence (•–•) and in the presence (o–o) of α-amanitin.

Cell-free poly(A) Synthesis and Release of Radioactive Proteins and poly(A) RNP into the Post-nuclear Supernatant: We have previously demonstrated that poly(A) synthesis occurs in the cell-free system with isolated nuclei and that cordycepintriphosphate (5μg/ml ; Sigma Chemical Co.) inhibits poly(A) synthesis to the extent of 75-80% whereas only 5-10% of total RNA synthesis is inhibited (10). Results presented in Fig.2 demonstrate that roughly 65-70% of the total radioactivity of a 60 min control incubation was released into the post-nuclear supernatant within 5 min. After this initial burst,the release of radioactive proteins increased slowly with time and reached 100% after 60 min of incubation in the absence of the drug (Fig.2, •–•). However, in the presence of cordycepintriphosphate(Fig.2, o–o) only the initial burst of release of TCA insoluble radioactivity was observed. There was no further increase in postnuclear supernatant TCA insoluble radioactivity during the remainder of the incubation. These results show that the release of a major fraction of the [^{3}H] proteins from the nuclei is independent of poly(A) synthesis whereas there is a [^{3}H] protein fraction (30-35% of the total postnuclear supernatant radioactivity) whose release from the nuclei is dependent on poly(A) synthesis.

When either cell-free RNA synthesis or poly(A) synthesis was inhibited, there was a subsequent inhibition of the time dependent release of radioactive proteins from the nuclei into the postnuclear supernatant. Using α-amanitin (0.5 μg/ml), it was observed that the radioactive proteins whose release was inhibited up to 45%, were associated exclusively to the poly(A) RNP fraction (Table 1). In a parallel manner, the 52% inhibition of [^{3}H] protein release into PNS by cordycepintriphosphate, (Table 1), was also localized to PNS-poly(A)-RNP complexes. These results, coupled with the observation that the PNS does not contain either free poly(A) RNA or non-poly(A) containing products of RNA polymerase II, lead to the conclusion that the re-

Table I. Inhibition of poly(A)-RNP release into PNS by cordycepintriphosphate and α-amanitin

	[^{3}H] Protein in Nuclei	[^{3}H] Protein PNS-Poly(A)-RNP	o/o Inhibition
	CPM		
1. Control	9.9×10^5	3.5×10^4	0
2. + α-amanitin(0.5)	11.0×10^5	1.9×10^4	46
3. + cordycepin-triphosphate (5)	9.6×10^5	1.7×10^4	52

Prelabelling of the cells, isolation of nuclei, and incubation conditions for RNA synthesis and RNP formation are described in "Materials and Methods". Equal amounts of nuclear suspension were added to each reaction mixture. TCA insoluble radioactivity was determined in aliquots withdrawn from each reaction mixture at zero time of incubation. After incubation for 60 min at 28°, the reaction mixture was centrifuged, and poly(A)-RNP from PNS was isolated as described in "Materials and Methods". TCA insoluble radioactivity in the PNS-poly(A)-RNP fraction was determined. Numbers in parenthesis indicate the concentration (μg/ml) of the drugs in the reaction mixture.

lease of poly(A)-RNA from nuclei is dependent on prior interaction of nuclear proteins with poly(A).

Proteins Associated with the Poly(A) Segment of Cell-free Formed RNP Particles: To identify the proteins associated with the poly(A) segment of cell-free formed RNP, these fractions were treated with pancreatic RNase A and T_1. RNase resistant RNP were isolated by rechromatography on oligo(dT) cellulose. Results presented in Fig.3, panel A, show that, among the several proteins which were associated with poly(A)-HnRNP, only those in the molecular weight range of 78K, 100K and 120K were associated with the RNase resistant poly(A) segment of cell-free formed HnRNP particles. Several coomassie blue stainable proteins having approximate molecular weights of 44K, 52K, 62K, and 78K were identified in the RNase treated fraction of native HnRNP particles (Fig.3,panel B).

Results presented in Fig.3, Panel C show the protein pattern of the PNS-poly(A)-RNP fraction isolated after pancreatic RNase A and T_1 treatment and rechromatography on oligo(dT) cellulose. Three radioactive proteins, presumably associated with poly(A) segments of PNS poly(A) RNP with apparent molecular weights of 78K, 100K and 120K,were detected in the RNase treated PNS-poly(A)-

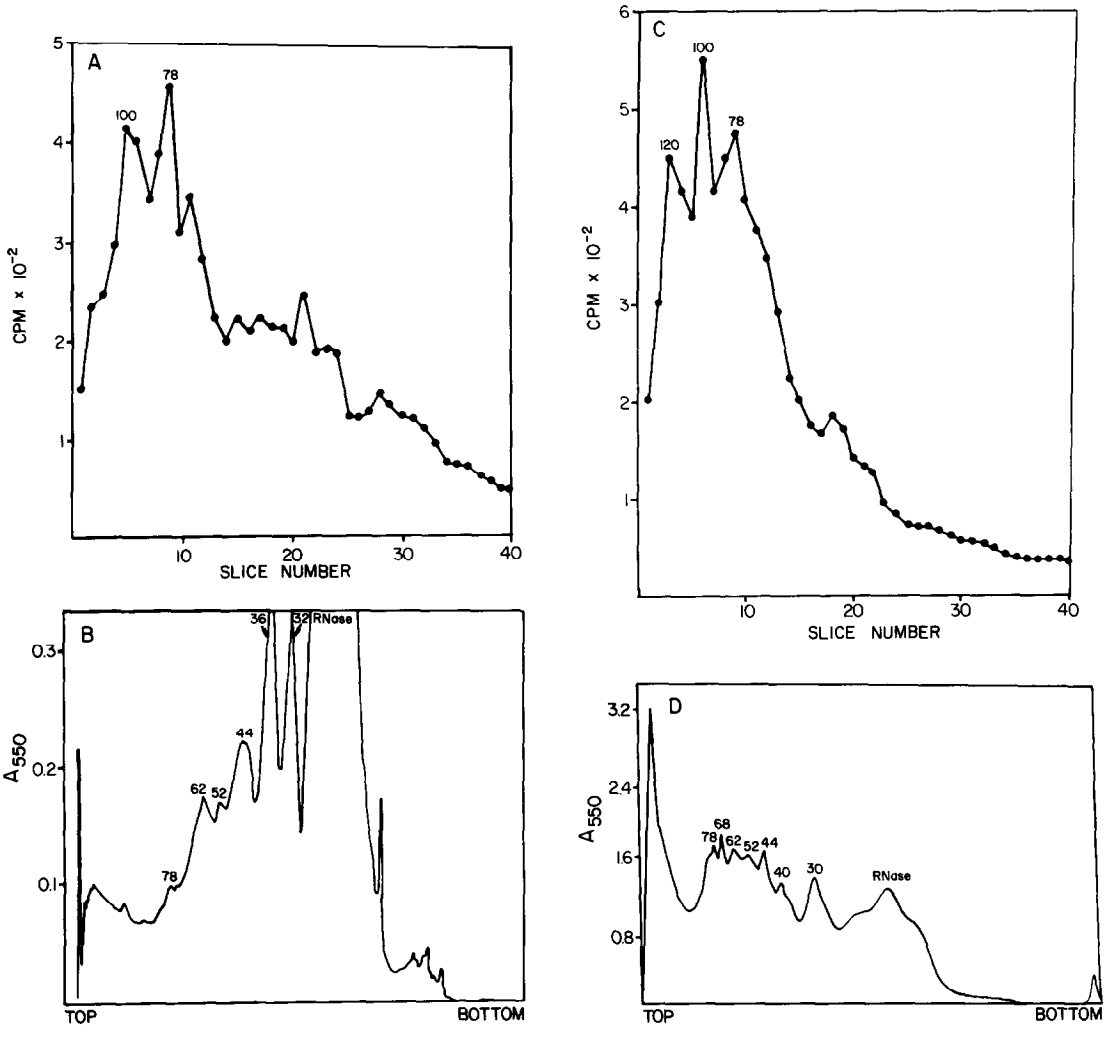


Fig. 3. SDS-polyacrylamide gel electrophoretic analysis of poly(A)-associated proteins of cell-free formed HnRNP, native HnRNP,PNS-poly(A)-RNP and native mRNP. Panel A shows the radioactive profile of proteins associated with the poly(A) segment of cell-free formed HnRNP. Poly(A) HnRNP was isolated from the nucleoplasmic fraction after incubation of nuclei under standard RNA synthesis and RNP formation conditions as described previously (10,11). The poly(A)-HnRNP was treated with pancreatic RNase A and T_1. RNase digestion with pancreatic RNase A (heat treated, 10 µg/ml) and T_1 (10 units/ml) was conducted in high salt buffer as described previously. The RNase resistant poly(A)-protein complex was isolated from the reaction mixture by oligo (dT) cellulose chromatography. The RNase resistant poly(A) segment was then dissolved in dissolving buffer and electrophoresed on 10% polyacrylamide gels under the conditions described in "Materials and Methods". Panel B shows the densitometric scan (at 550 nm) of poly(A) associated proteins isolated from native HnRNP particles after SDS-polyacrylamide gel electrophoresis and subsequent staining with coomassie blue. Panel C shows the radioactive profile of proteins associated with the poly(A) segment of cell-free formed and transported PNS-poly(A) RNP. Panel D shows the densitometric scan of poly(A) associated proteins of native mRNP. Isolation of PNS-poly(A)-RNP and mRNP is described in "Materials and Methods". Procedures for RNase treatment and for the isolation of [^{3}H] proteins associated with the poly(A) segment of cell-free

RNP fraction. This was in contrast with the coomassie blue stainable proteins associated with similarly isolated and RNase treated native mRNP particles (isolated from polysomes); Fig.3, panel D) which contained several coomassie blue stainable proteins with apparent molecular weights of 78K, 68K, 62K, 52K, 44K and 40K. Common to both native and cell-free formed RNP was a 78K protein which was detected as a radioactive protein peak in the poly(A) segment of cell-free formed PNS-poly(A)-RNP, and as a coomassie blue stainable protein band in the RNase resistant poly(A) segment of the native mRNP. The former contained two more radioactive protein bands (100K and 120K), which were not detected in the poly(A) segment of native mRNP. The poly(A) segment of the native mRNP particle contained several other lower molecular weight coomassie blue stainable protein bands which were not detected in the poly(A) segment of the cell-free formed PNS-poly(A)-RNP.

Since inhibition of poly(A) synthesis also inhibits release of cell-free formed mRNA-like material into the PNS, it may be concluded that the poly(A) region of mRNA is involved directly or indirectly in the transport of this class of RNA. If only the poly(A) segment is necessary for the transport of mRNA, without obligatory prior interaction with a specific class of proteins, free poly(A)-RNA would appear both in the PNS (in the case of the cell-free system) and in the cytoplasm (intact cell). However, experimental evidence from several laboratories suggests that mRNA in the cytoplasm or mRNA-like material in the cell-free system exists predominantly as RNP particles. Kinetic studies of the release of mRNA-like material into the PNS did not demonstrate the presence of free mRNA-like material in the PNS (at anytime) whereas mRNP-like material was detected between 10-15 min of incubation. Furthermore, it was also observed that when intact cells were treated with cyclohexamide, poly(A) RNA accumulated inside the nucleus without any trace

formed PNS-poly(A)-RNP and coomassie blue stainable proteins associated with native mRNP are identical to those described in the legends to Fig. 3. Numbers designate the molecular weight x 10^{-3} of protein bands as calculated from the mobility of protein standards coelectrophoresed with the samples.

of free poly(A) RNA in the PNS (11). It therefore appears that the poly(A) segment provides a site for the binding of specific proteins, leading to the formation of RNP complexes and that mRNA is transported across the nuclear membrane in this form. Of course this mechanism of transport of mRNA molecules in eukaryotic cells is not universal since eukaryotic cells contain some mRNA's which do not have poly(A) at the 3'-end.

In agreement with the observed involvement of the 78K protein in the process of transport of mRNA in intact cells (3), this study in the cell-free system with isolated nuclei provides further evidence to suggest that the 78K protein (if not alone) along with two other proteins interacts with the poly(A) segment of the cell-free synthesized mRNA-like material and is subsequently involved in the process of transporting this class of RNA across the nuclear membrane. Results presented in this investigation not only demonstrate that a process similar to nuclear cytoplasmic transport occurs in the cell-free system with isolated nuclei as faithfully as that which occurs in intact cells, but also provides evidence for the role of the poly(A) segment and its interaction with specific proteins in the eukaryotic mRNA transport process.

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