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Purification and Characterization of Cytoplasmic Protamine Messenger Ribonucleoprotein Particles from Rainbow Trout Testis Cells†

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ABSTRACT: Poly(A)-containing protamine messenger ribonucleoprotein particles [poly(A+) pmRNP particles] have been isolated from the polysomal and free cytoplasmic subcellular fractions of trout testis cells by a two-step isolation procedure. Ethylenediaminetetraacetic acid (EDTA) treated particles from both cytoplasmic fractions were first fractionated by sucrose gradient centrifugation and the putative pmRNP particles localized by utilizing ^{3}H-labeled protamine complementary DNA (pcDNA) probes. In addition, particles present in these fractions were characterized by their translational activity in the heterologous, rabbit reticulocyte cell-free system and the protein components of crude mRNP complexes analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoesis. The final purification step involved affinity chromatography of pooled gradient fractions on oligo(dT)–cellulose from which intact pmRNP could be eluted with distilled water at 40 °C. Highly purified particles from both polysomal and free cytoplasmic fractions prepared by this procedure had buoyant densities of 1.35–1.37 g/cm^3 in CsCl or a protein content of approximately 82%. Particles isolated from EDTA-dissociated polysomes were actively translated in vitro, while their free cytoplasmic counterparts were not. High salt washed pmRNP particles or the RNA extracted from pmRNP preparations, however, directed the synthesis of trout protamines in this system. A model of the activation of stored pmRNP particles in vitro and in vivo is presented.

Spermatogenesis in rainbow trout (*Salmo gairdnerii*) testes is characterized by several well-defined events which occur during the terminal differentiation of germ cells to highly specialized spermatozoa. The most striking change in morphology and molecular composition of developing sperm cells, however, occurs during the final phase of sperm cell maturation (spermiogenesis) with the synthesis of a set of highly basic sperm-specific nuclear proteins: the protamines (Ling et al., 1971). Both the protamines and the messenger RNAs coding for these polypeptides have been well characterized biochemically (Gedamu & Dixon, 1976a,b), and a complementary cDNA [specific for protamine messenger RNA (pmRNA) sequences] has been synthesized (Iatrou & Dixon, 1977; Iatrou et al., 1978).

A major question which is central to an understanding of protamine gene expression is how the synthesis of protamine is triggered precisely at the spermatid stage of cell differentiation at a time when the transcriptional activity (Marushige & Dixon, 1969) and diversity of cytoplasmic mRNA sequences in the tissue were rapidly declining (Levy W. & Dixon, 1977). A ^{3}H-labeled protamine cDNA probe has been particularly useful in this regard for elucidating the distribution of such sequences in fractionated testis cells (Iatrou et al., 1978). These studies indicated that pmRNA synthesis began early in sperm cell development (about the primary spermatocyte stage) well before protamine synthesis in these cells. These data were consistent with, although they did not prove, a hypothesis in which newly transcribed pmRNA sequences were stored in the cytoplasm until the spermatid cell stage, at which time they moved to the polysomes and the synthesis of protamines on diribosomal complexes was initiated.

In many other systems, the processing, transport, and translation of mRNA in vivo are thought to involve the packaging of mRNA into specific ribonucleoprotein complexes (Neissing & Sekeris, 1971; Irwin et al., 1975; Beyer et al., 1977; Jenkins et al., 1978; Brunel & Lelay, 1979; Bag & Sells, 1979a,b; Rose et al., 1979; Liautard et al., 1976). A previous report from this laboratory (Gedamu et al., 1977) indicated the pmRNA might also be complexed with proteins in the cytoplasm of developing sperm cells; however, there was little information on the precise nature of these complexes or whether the translational control of protamine synthesis in vivo might be exerted through storage and subsequent activation of these cytoplasmic particles. In the present study, high specific activity [^{3}H]poly(U) and [^{3}H]pcDNA probes were employed to detect these complexes and thus aid in their isolation and analysis. Poly(A+) pmRNA containing RNP complexes from the steady-state free cytoplasmic and polysomal subcellular compartments of testes tissue have been characterized by this means. Data are presented indicating that the control of protamine synthesis involves the activation of stored protamine messenger ribonucleoprotein (pmRNP) particles by processes which can be mimicked in vitro by treatment of purified free cytoplasmic pmRNP with high ionic strengths.

Materials and Methods

Oligo(dT)–cellulose (T_3) was purchased from Collaborative Research Inc. RNase A was from Worthington Biochemical Corp., S1 nuclease was from Miles Laboratories, and AMV reverse transcriptase was a generous gift of Dr. J. Beard, Division of Cancer Cause and Prevention, National Cancer Institute. [^{3}H]dCTP of specific activity 23 Ci/mmol, [^{3}H]-

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arginine of specific activity 15 Ci/mmol, [^{3}H]lysine of specific activity 6 Ci/mmol, [^{3}H]poly(U) of specific activity 6 Ci/mmol of UMP, and Na^{125}I of specific activitiy 17 Ci/mg of NaI were all purchased from New England Nuclear. Rainbow trout testes were obtained from Brande, Denmark, in late August. Following their collection, they were quick frozen in dry ice and stored at −70 °C. Tissue was thawed in homogenization buffer [50 mM Tris-HCl, pH 7.5, 0.14 M KCl, 5 mM $MgCl_2$, 1 mM dithioerythritol (DTE), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 100 μg/mL heparin], scissor minced, and then homogenized at 4 °C with a Tekmar "Tissuemizer" equipped with a 5–500-mL probe at 20% power output.

Fractionation of Testes Cells. Trout testes homogenates were made 1% in Nonidet NP-40 (BRL) and fractionated according to the following scheme. Polysomes were pelleted from the postmitochondrial (30000*g*) supernatant by centrifugation at 225000*g* for 2.5 h in a 60 Ti rotor (Beckman) through a double layer of 1 M/2 M sucrose in homogenization buffer. The postribosomal RNP fraction was prepared from the supernatant above the 1 M sucrose pad by pelleting with a 24-h centrifugation at 200000*g* over a 10% sucrose pad.

Release of mRNP Particles. mRNP particles were released from polysomal and postribosomal supernatant pellets by gentle homogenization at 4 °C in homogenization buffer made 50 mM in ethylenediaminetetraacetic acid (EDTA). Routinely, 4 mL of buffer was used for each pellet derived from 25 g of tissue. After 30 min at 4 °C, the suspensions were clarified by centrifugation at 10000*g* for 10 min.

Fractionation of mRNP Particles by Sucrose Density Gradient Centrifugation. Aliquots of mRNP particles were fractionated on 15–35% sucrose gradients [made in 10 mM Tris-HCl (pH 7.5), 140 mM KCl, 1 mM EDTA, 1 mM DTE, and 0.5 mM PMSF] at 50 000 rpm in a Beckman VTi 50 vertical rotor for 4.75 h. Under these conditions, the EDTA-derived small ribosomal subunit sedimented near the tube bottom. Gradients containing particles were fractionated from the top on an ISCO Model 640 density gradient fractionator by puncturing the tube at the bottom and pushing sucrose up through the tube. The absorbance (A_{254}) of the tube contents was continuously monitored during the fractionation by an ISCO UA-5 monitor equipped with a Model 6 detection unit.

Poly(U) Hybridization Assay. Aliquots from fractionated gradients were assayed for their hybridization with [^{3}H]-poly(U) as described by Gedamu et al. (1977). After treatment with RNase to degrade any unhybridized [^{3}H]poly(U), samples were precipitated with 5% trichloroacetic acid (Cl_3-CCOOH) and filtered through glass fiber filters (GFA, Whatman). Filters were extensively washed with 2.5% Cl_3-CCOOH–0.5 M NaCl and then 95% ethanol and air-dried before counting in toluene-based scintillant (Gedamu & Dixon, 1976a).

Synthesis of Protamine cDNA. Protamine mRNA was isolated according to the procedure of Gedamu & Dixon (1976a). Aliquots were utilized for the synthesis of [^{3}H]-pcDNA following the method of Iatrou & Dixon (1977) and by utilizing [^{3}H]dCTP (23 Ci/mmol P, New England Nuclear) and 4 mM sodium pyrophosphate to assume a full-length cDNA copy (Myers et al., 1977). After the reaction was terminated and the RNA was hydrolyzed with 0.3 M KOH for 4 h, the whole reaction mixture was loaded onto and eluted from a Sephadex G-50 column packed in a glass wool plugged, sterile 2-mL glass pipet, with 50 mM ammonium acetate. cDNA in the column void volume was lyophilized and redissolved in double-distilled water. The specific activity of the [^{3}H]pcDNA prepared in this manner was approximately 6 × 10^6 cpm/μg.

cDNA:RNA Hybridization Assay. Aliquots of fractionated gradients were extracted with phenol–chloroform–isoamyl alcohol (50:49:1) as described by Gedamu & Dixon (1976b), and the RNA was precipitated overnight at −20 °C with 70% ethanol.

Ethanol-washed precipitates were dissolved in 50 μL of hybridization buffer [0.18 M NaCl, 1 mM EDTA, 0.1% sodium dodecyl sulfate ($NaDodSO_4$), and 10 mM Tris-HCl, pH 7.5] containing 3000 cpm of [^{3}H]pcDNA and hybridized for 3–5 h at 70 °C in 1.5-mL microfuge tubes under a thin layer of mineral oil. At the end of the hybridization, the reactions were diluted into 1 mL of S1 buffer [100 mM NaCl, 70 mM sodium acetate (pH 4.5), 2.5 mM $ZnSO_4$, 25 μg/mL shear and heat-denatured calf thymus DNA, and 5% glycerol] and divided in three equal aliquots. The S1 nuclease assay for quantitation of RNA–DNA hybrids was carried out essentially as described by Iatrou & Dixon (1977) in 300-μL reaction volumes. After 1 h of incubation at 45 °C, control and enzyme-treated samples were precipitated onto glass fiber filters and counted. Values were compared with those of the internal controls (no S1 treatment).

Oligo(dT)–Cellulose Chromatography. Gradient fractions containing presumptive poly(A+) pmRNP particles were bound to oligo(dT)–cellulose resin in homogenization buffer containing 50 mM EDTA at 4 °C for 30 min (binding buffer). Bound mRNP was separated from the unbound material by centrifugation at 2000*g* for 5 min. The resin was subsequently washed 4 times with binding buffer and then twice with 0.1 M ammonium acetate before the bound RNP was eluted with distilled water at 40 °C as described by Bag & Sells (1979a).

Labeling of RNP Complexes with ^{125}I. RNP preparations were labeled in vitro with Na^{125}I in the presence of Chloramine T (Barrieux et al., 1975). Reaction mixtures contained 1–2 μg of RNP, 30 μg of Chloramine T, 0.05 M phosphate buffer (pH 7.0), and 0.25 mCi of ^{125}I (17 Ci/mg NaI) in a total volume of 20 μL. After 5 min at 4 °C, 90 μg of sodium metabisulfite was added and the reaction continued at room temperature for 2 min. A 50-μL sample of a 1% KI–0.01% NaN_3 solution was added to stop the reaction. The whole reaction mix was loaded onto and eluted from a Sephadex G-75 column packed in a 1-mL pipet with 10 mM sodium phosphate buffer (pH 7.0). Four-drop fractions were collected, and the radioactivity in the column void volume was pooled for further analysis.

CsCl Density Gradient Analysis of RNP. Iodinated particles were fixed by dialysis against 10 mM sodium phosphate, 1 mM EDTA, and 4% formaldehyde (pH 7.0) for 4 h at 4 °C. Aliquots of fixed particles were layered onto 4.8 mL of 1.37 g/cm^3 CsCl in the same buffer containing 0.8% BRIJ-35 and spun to equilibrium in the Beckman SW 65L rotor at 40 000 rpm. Gradients were fractionated from the tube bottom into chilled microfuge tubes. Every other fraction was counted and its CsCl concentration determined by refractometry.

$NaDodSO_4$ Gel Electrophoresis. Proteins were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis according to the method of Laemmli (1970) in a Bio-Rad slab gel apparatus. The molecular weight of the polypeptides was estimated by comparison with known standards run in adjacent wells of the slab gel.

In Vitro Translation Assay. Extracted RNA or purified mRNP samples were assayed for their biological activity in the messenger RNA dependent rabbit reticulocyte cell-free system as described by Pelham & Jackson (1976). Aliquots

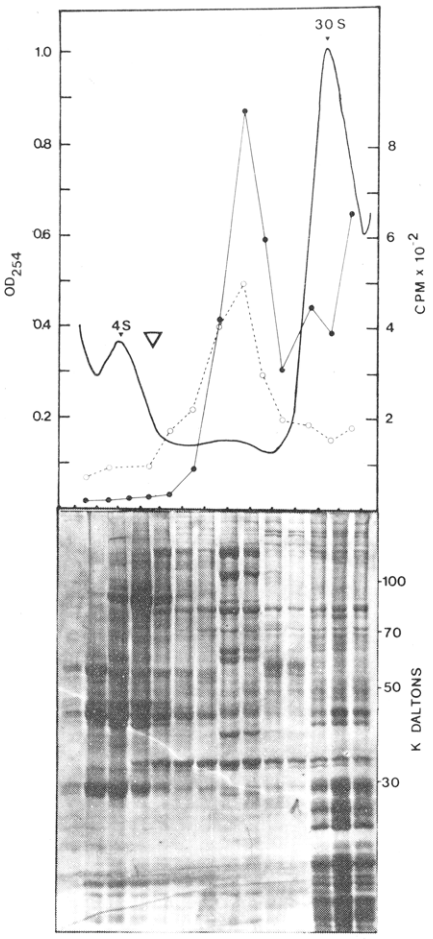

FIGURE 1: Gradient fractionation of EDTA-dissociated polysomes from trout testis cells. EDTA-dissociated polysomes were fractionated in 15–35% sucrose gradients in 10 mM Tris-HCl (pH 7.2), 0.14 M KCl, 1 mM EDTA, 0.5 mM PMSF, and 1 mM DTE, as outlined under Materials and Methods. Fractions were assayed for their absorbance at 254 nm (—), hybridization to [^{3}H]poly(U) (●), or hybridization to [^{3}H]pcDNA (○). Aliquots from every other fraction in the gradients were assayed directly for hybridization to [^{3}H]poly(U) under conditions described under Materials and Methods. Phenol-extracted RNA from aliquots of gradient fractions was used in RNA excess; [^{3}H]pcDNA hybridization reactions were essentially as described previously. The open triangle shows the relative sedimentation of protein-free pmRNA. Proteins present in gradient fractions were analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis as described previously. Molecular weights of the stained proteins were estimated by comparison with molecular weight polypeptide markers run on adjacent gel slots.

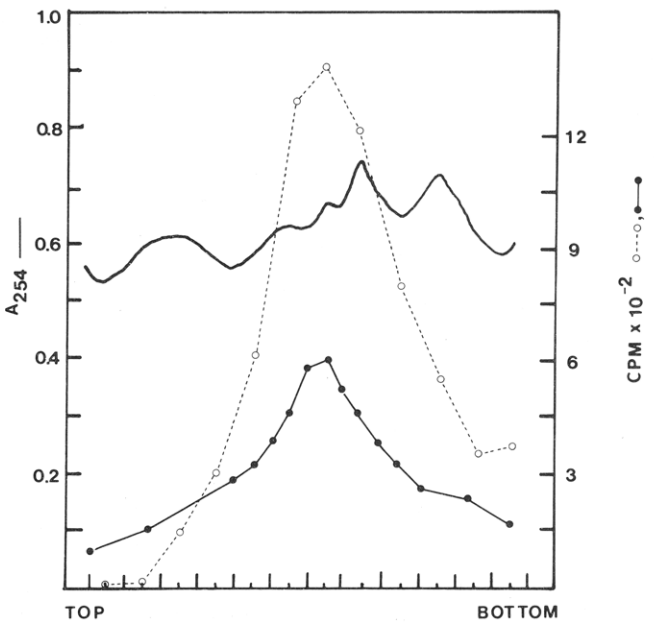

FIGURE 2: Detection of poly(A+) pmRNP particles in gradient-fractionated postribosomal pellets. Postribosomal pellets were suspended in 50 mM EDTA buffer and clarified by centrifugation, and the soluble fraction was loaded onto 15–35% sucrose gradients. After ultracentrifugation, gradients were fractionated and assayed for absorbance at 254 nm (—), hybridization to [^{3}H]pcDNA (●), or hybridization to [^{3}H]poly(U) (○). Direction of sedimentation is from left to right.

from the 20-μL reactions were either precipitated onto Whatman 3MM filters with acidified acetone and washed extensively with 5% Cl_3CCOOH–0.25% sodium tungstate (Gedamu & Dixon, 1976a) and counted or applied to polyacrylamide gels containing 6.25 M urea (Hurley, 1977). Alternately, products were analyzed by CM-52 column chromatography as described by Gedamu et al. (1979).

Fluorography of ^{3}H-Labeled Translates. Polyacrylamide gels were processed for fluorography following the procedure of Laskey & Mills (1975). Preflashed Kodak Royal X-OMat film was placed on the top of the dried gel and exposed for 2 weeks at −70 °C. Product identification was based on mobility of the radioactive bands in comparison with marker proteins run on adjacent gel slots.

Results

Detection of Gradient-Fractionated pmRNP Particles. EDTA-treated polysomal (Figure 1) and free cytoplasmic (Figure 2) mRNP preparations were fractionated on sucrose gradients, and the [^{3}H]poly(U) hybridization assay was used to detect poly(A)-containing particles in these gradients. Putative protamine mRNP particles were distinguished from other, faster sedimenting poly(A) complexes by utilizing a purified ^{3}H-labeled complementary DNA probe to pmRNA (Iatrou et al., 1978). In each case, the [^{3}H]pcDNA hybridization assay detected pmRNA sequences sedimenting at 14 S, coincident with a major peak of [^{3}H]poly(U) hybridization. No significant [^{3}H]poly(U) or [^{3}H]pcDNA hybridization was detected in the 6–7S region of these gradients where marker, protein-free pmRNA sedimented under identical conditions. In addition, [^{3}H]pcDNA did not hybridize to material sedimenting faster than 14 S when gradients were run for shorter periods of time (data not shown).

Polysomally derived pmRNP particles were localized to a region of the gradients corresponding to a broad peak of material absorbing at 260 nm (Figure 1). It has been previously demonstrated that up to 80% of this material represents cosedimenting aggregates of an 8S ribosomal RNP (5S rRNA + 34K protein) (Gedamu et al., 1977) which can be separated from the 14S pmRNP region by recentrifugation. Analysis of the protein components of putative pmRNP particles was carried out by $NaDodSO_4$–polyacrylamide slab gel electrophoresis (Laemmli, 1970). This technique allowed the analysis of proteins in fractions from across the entire gradient in one run and thus permitted the localization of those proteins specific to putative pmRNP complexes. Polysomal pmRNP complexes fractionated in buffers containing 140 mM KCl and 0.5 mM PMSF had a variety of polypeptide components with molecular weights ranging from 34 000 (34K) to 160 000 (160K) preferentially localized in those regions of the gradient containing [^{3}H]poly(U) and [^{3}H]pcDNA hybridizing sequences (Figure 1). Major polypeptide species with molecular weights of 150K, 120K, 90K, 85K, 73K, 60K, 50K, 43K, and 34K were repeatedly observed in these fractions. Upon recentrifugation of the pmRNP region, all but the 34K-dalton polypeptide remained associated with particles sedimenting

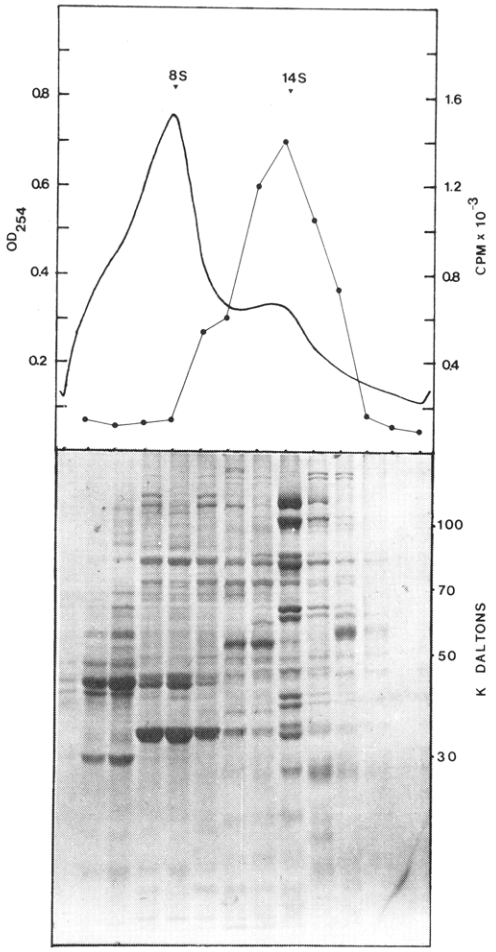

FIGURE 3: Resedimentation analysis of pooled fractions from gradients containing polysomal-derived protamine mRNP complexes. Gradient regions containing both [^{3}H]poly(U) and [^{3}H]pcDNA hybridization were resedimented through identical 15–25% sucrose gradients and the fractions assayed for absorbance at 254 nm (—), hybridization to [^{3}H]poly(U) (●), and protein composition by $NaDodSO_4$–polyacrylamide gel electrophoresis as described previously. The sedimentation values were estimated from 4S and 30S markers run on parallel gradients.

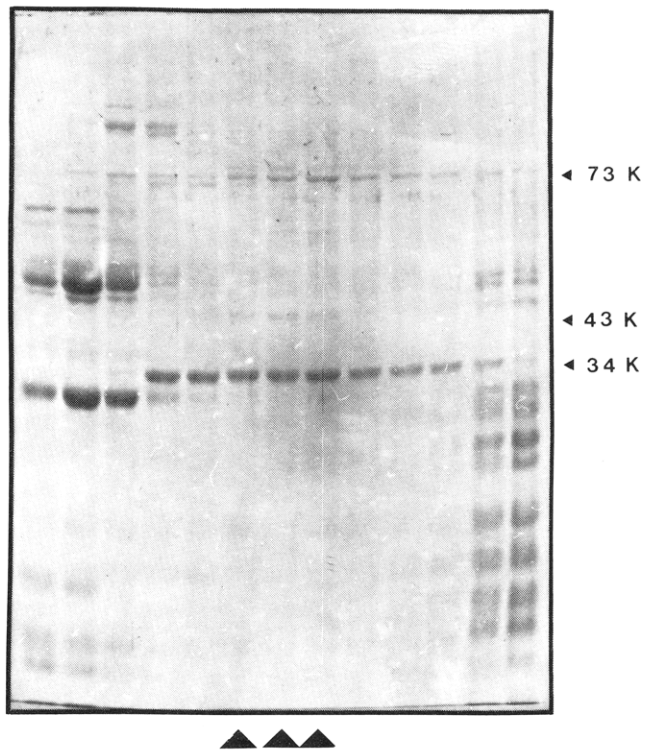

FIGURE 4: $NaDodSO_4$–polyacrylamide gel electrophoresis of the proteins of gradient-fractionated, EDTA-dissociated, and 0.3 M KCl washed testis polysomes. EDTA-released pmRNP's from 0.3 M KCl washed polysomes were fractionated by sucrose gradient centrifugation and aliquots from the fractions subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis. Proteins were visualized by staining with Coomassie Blue R-250. Arrows indicate the peak hybridization to [^{3}H]poly(U).

at 14 S (Figure 3). The 34K-dalton polypeptide is associated with 5S RNA and is not an integral component of the pmRNP particles (Gedamu et al., 1977). Prior treatment of polysomes with 0.3 M KCl before EDTA dissociation greatly reduced the number and quantity of proteins associated with the pmRNP regions of the gradients (Figure 4). Only the 73K-, 43K-, and 34K-dalton polypeptides were found as major components in fractions of the gradients where pmRNA sequences were detected.

Postpolysomal supernatants were centrifuged until all particles sedimenting faster than 5 S were pelleted from solution, leaving behind the bulk of free protein in the supernatant. Despite removal of most of the contaminating protein, free cytoplasmic pmRNP particles were localized in a region of the gradients which did not consistently correspond to the major UV-absorbing peaks in those gradients (Figure 2), suggesting that the profiles of the gradients represented material other than specific pmRNP complexes. The protein composition of gradient-fractionated, postpolysomal supernatant preparations also confirmed the substantial contamination of pmRNP particles with other, non-pmRNA ribonucleoprotein complexes and/or aggregates. No specific proteins could be reliably assigned to pmRNP-containing regions of the gradients by utilizing solely this purification procedure. Similar results were obtained when parallel experiments were carried out with putative globin mRNP particles from postpolysomal supernatant fractions of rabbit reticulocytes (data not shown). Furthermore, treatment of supernatant particles with 0.3 M KCl had little effect on the distribution of major polypeptides in fractions of these gradients.

Translational Activity of Gradient-Fractionated pmRNP. (*a*) *Deproteinized mRNA Fractions.* The biological activity of gradient fractions containing crude pmRNP particles was assayed in the mRNA-dependent rabbit reticulocyte lysate as described by Pelham & Jackson (1976) to characterize further the distribution of translatable mRNA sequences across these gradients (Figure 5). RNA extracted from gradient fractions was assayed for the incorporation of [^{3}H]arginine (in the case of postpolysomal RNP gradients) or [^{3}H]arginine and [^{3}H]-lysine (in the case of polysomal RNP gradients) into hot Cl_3CCOOH–sodium tungstate precipitable material. Trout protamines are arginine rich but contain no lysine residues. Thus, while arginine incorporation is a good indication of protamine synthesis, lysine incorporation indicates the presence of nonprotamine mRNA sequences in these gradients. The major peak of [^{3}H]arginine incorporation in both these gradients corresponded to the regions of pmRNA sequences detected by [^{3}H]pcDNA hybridization. The [^{3}H]lysine incorporation, however, corresponded to a region in the EDTA-released and gradient-fractionated RNP which sedimented faster than putative pmRNP complexes.

(*b*) *Intact mRNP Particles.* When fractionated pmRNP particles were used to direct the synthesis of proteins in translation assays instead of the RNA extracted from these particles, quite different results were obtained (Table I). Polysomal pmRNP complexes directed the synthesis of

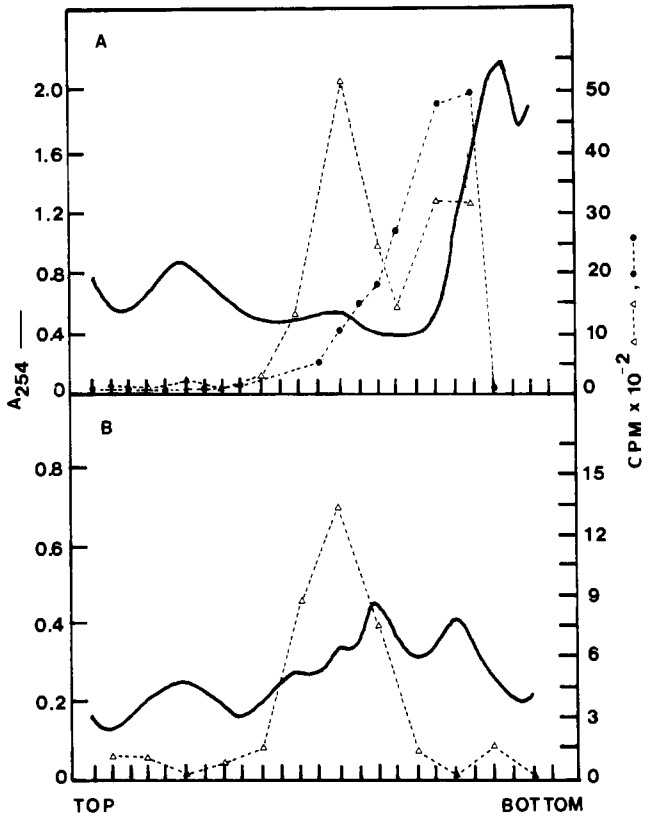

FIGURE 5: Translational activity of RNA from gradient-fractionated, EDTA-dissociated polysomes and postribosomal supernatant of trout testis cells. Phenol–chloroform-extracted RNA from gradient fractions was assayed for translational activity in the mRNA-dependent rabbit reticulocyte lysate by the incorporation of [^{3}H]arginine (△) or [^{3}H]lysine (●) into Cl_3CCOOH–tungstate-precipitable material. Reactions were carried out in sealed microfuge tubes in 20-μL volumes. After incubation at 30 °C for 1 h, 5-μL aliquots were removed and precipitated onto 3MM filters as described under Materials and Methods. (A) Polysomal-derived mRNP; (B) postribosomal supernatant mRNP; absorbance at 254 nm (—).

Table I: Translational Activity of Gradient-Fractionated pmRNP

source of RNP	rel amount of RNP	Cl_3CCOOH–tungstate [^{3}H]arginine precipitable (cpm incorporated)	minus control (cpm)
polysomal pmRNP	5	3000	1000
	10	6800	4800
	20	12000	10000
supernatant pmRNP	2	3000	1000
	5	900	
	10	840	
	20	600	
control (no RNP)		2000	

Cl_3CCOOH–tungstate-precipitable proteins in a manner which showed linear incorporation with increasing amounts of RNP added. Free cytoplasmic pmRNP particles, however, failed to direct the synthesis of such products in a linearly increasing fashion and even appeared, at higher concentrations, to inhibit the endogenous levels of activity.

(*c*) *Protamine mRNP's Purified by Binding to Oligo(dT)–Cellulose.* In order to achieve further purification of poly(A)-containing pmRNP particles from trout testis cytoplasm, regions of the gradient containing putative pmRNP complexes were fractionated by affinity chromatography on an oligo(dT)–cellulose matrix. It has been previously demonstrated that oligo(dT)–cellulose columns can be used to

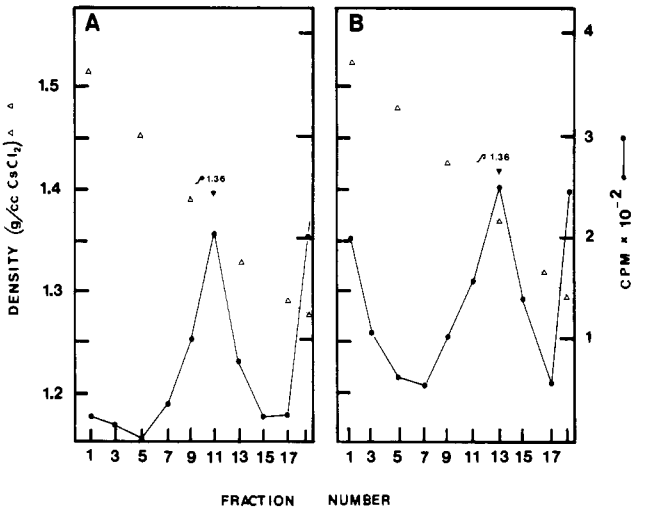

FIGURE 6: Buoyant density of oligo(dT)–cellulose-bound, ^{125}I-labeled pmRNP complexes in CsCl. ^{125}I-Labeled pmRNP purified from postpolysomal (A) or polysomal (B) cell fractions by oligo(dT)–cellulose was fixed with formaldehyde and centrifuged to equilibrium in CsCl as described previously. Fractions were assayed for radioactivity by liquid scintillation counting (●) and [CsCl] by refractometry (△).

isolate mRNP complexes containing poly(A) tracts by selective elution with formamide (Irwin et al., 1975) or low ionic strength buffer at elevated temperature (Bag & Sells, 1979b). When oligo(dT)–cellulose was used to purify particles from polysomally derived and postpolysomal supernatant fractions, after the resin was washed extensively with binding buffer, intact poly(A+) pmRNP particles could be eluted with double-distilled water at 40 °C. Such particles had buoyant densities between 1.35 and 1.38 g/cm^3 in CsCl (Figure 6) and contained approximately 85% protein and 15% RNA (Spirin, 1966).

The translational activity of oligo(dT)–cellulose-purified pmRNP, as well as the RNA extracted from these preparations, was assessed by the incorporation of [^{3}H]arginine into Cl_3CCOOH–tungstate-precipitable proteins in vitro as described previously. Polysomally derived pmRNP particles isolated in the presence of 0.14 M KCl or particles bound in 0.14 M KCl and then washed with 0.5 M KCl buffers were translated as efficiently as the RNA extracted from them. Again, the relative incorporation curves were approximately linear over the range tested (Figure 7). Conversely, free cytoplasmic pmRNP particles isolated in the presence of 0.14 M KCl showed very low activity in directing the incorporation of [^{3}H]arginine (Figure 7). The RNA extracted from these same preparations, however, was fully active. Washing oligo(dT)–cellulose-bound free cytoplasmic particles (which had been isolated in low salt) with 0.5 M KCl removed the inhibitory activity in these preparations, as evidenced by the increased translational activity of these high salt washed pmRNP particles (Figure 7).

In order to obtain a more quantitative estimate of the activity of pmRNP and to determine whether the binding of pmRNP was, in part, ionic strength dependent, crude pmRNP preparations were bound to oligo(dT)–cellulose columns at various concentrations of KCl, and the specific translational activity (counts per minute of ^{3}H incorporated per gram of RNP) of bound and eluted particles was assayed in the cell-free translation system. There was no difference in the binding or specific activity of polysomal pmRNP complexes over the range of salt concentrations tested, suggesting that neither removal of pmRNP proteins nor the increased stability of the poly(A)–oligo(dT) interaction played a major role in the re-

Table II: Translational Activity of Oligo(dT)-Cellulose-Purified pmRNP Particles

source of mRNP	amount of mRNP added (ng)	Cl_3CCOOH–tungstate precipitable (cpm)[a]	sp act. (cpm/μg of RNP)	purity (%)
poly(A+) pmRNA	50	3.0×10^4	6.0×10^5	100
polysomal pmRNP (0.14 M KCl)	50	2.2×10^4	4.4×10^5	74
	200	8.6×10^4	4.3×10^5	
polysomal pmRNP (0.3 M KCl)	50	2.6×10^4	5.2×10^5	85
free cytoplasmic pmRNP (0.14 M KCl)	100	8.0×10^1	8.0×10^2	
	200	1.4×10^2	7.0×10^2	
free cytoplasmic pmRNP (0.3 M KCl)	50	2.2×10^3	4.4×10^4	7
	100	2.8×10^3	2.8×10^4	
	200	5.2×10^3	2.6×10^4	
free cytoplasmic pmRNP (0.6 M KCl)	50	2.1×10^4	4.2×10^5	70
	100	2.4×10^4	2.4×10^5	
	200	6.1×10^4	3.0×10^5	
free cytoplasmic pmRNP (0.9 M KCl)	50	2.7×10^4	5.4×10^5	86
	100	4.8×10^4	4.8×10^5	
	200	5.3×10^4	2.7×10^5	

[a] Average of two determinations.

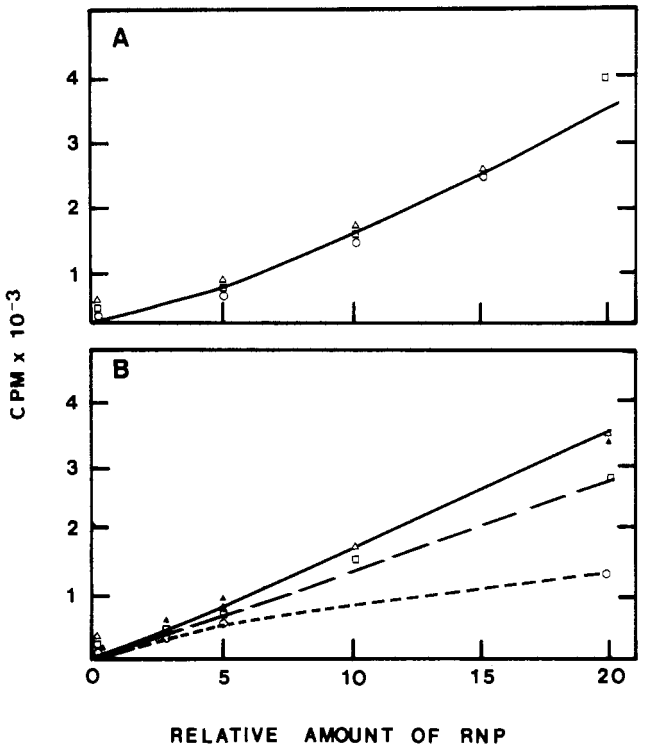

FIGURE 7: Translational activity of oligo(dT)–cellulose-purified poly(A+) pmRNP. Poly(A+) pmRNP was selectively bound and eluted from oligo(dT)–cellulose columns as described under Materials and Methods. Aliquots from eluted material were precipitated with ethanol in the presence of 1–2 μg of carrier tRNA (from calf thymus) and incubated at 30 °C with prepared reticulocyte lysate containing 10 μCi of [^{3}H]arginine. (A) Incorporation of [^{3}H]arginine into Cl_3CCOOH–tungstate-precipitable material in the presence of increasing amounts of phenol-extracted, polysomal-derived pmRNP (△), native, 0.14 M KCl washed polysomal mRNP (○), and 0.5 M KCl washed polysomal pmRNP (□). (B) Incorporation of [^{3}H]arginine into Cl_3CCOOH–tungstate-precipitable material in the presence of increasing amounts of phenol-extracted, postribosomal supernatant pmRNP (▲, △), native, 0.14 M KCl washed postribosomal supernatant pmRNP (○), and 0.5 M KCl washed postribosomal supernatant pmRNP (□).

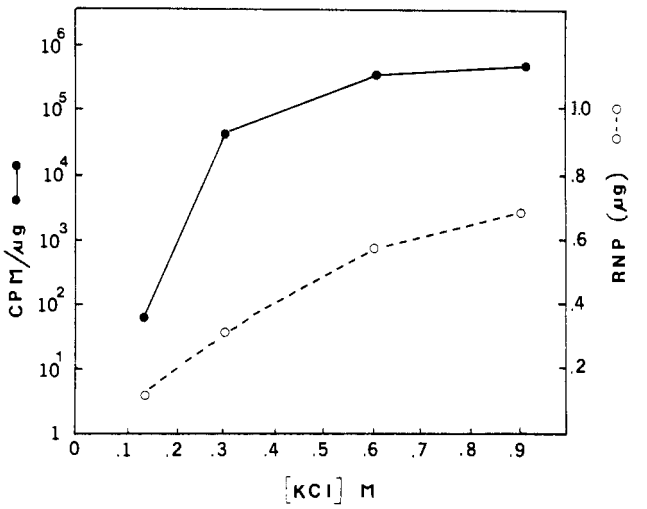

FIGURE 8: Yield and translational activity of free cytoplasmic pmRNP particles bound to oligo(dT)–cellulose at various salt concentrations. The yield of pmRNP per gram of tissue bound to oligo(dT)–cellulose at various salt concentrations (○) was compared to the specific activity (cpm/μg of RNP) of 50 ng of the eluted pmRNP in translational assays (●).

covery or the high translational activity of polysomal pmRNP complexes (Table II).

Parallel experiments on free cytoplasmic pmRNP particles, however, indicated that the ionic strength had a considerable effect on both the binding and translational activity of bound and eluted pmRNP particles (Figure 8 and Table II). A 7-fold increase in the binding of postpolysomal supernatant pmRNP particles to oligo(dT)–cellulose was observed after the concentration of KCl in the binding buffer was raised from 0.14 to 0.9 M (Figure 8). Furthermore, the specific translational activity of particles isolated in buffers containing greater than 0.3 M KCl was increased approximately 1000-fold over that for the same particles isolated in 0.14 M KCl. Clearly, increasing the salt concentration in the binding buffer had two overlapping effects: one which increased the affinity of the complexes for the oligo(dT)–cellulose matrix and a second which activated the bound pmRNP's so that they become translationally competent in the rabbit reticulocyte lysate.

(d) Characterization of the Translational Products. The specificity of in vitro translation was determined by gel electrophoresis and CM-52 column chromatography of ^{3}H-labeled translation products. The sole, acid-soluble translation products in these assays comigrated with the products of reactions containing highly purified protamine mRNA (Figure 9) and coeluted (Figure 10) with the individual marker protamine components C_I, C_{II}, and C_{III} separated by CM-52 chromatography (Gedamu & Dixon, 1976a). Virtually no other labeled products were detected in assays containing putative pmRNP complexes, and there were no apparent

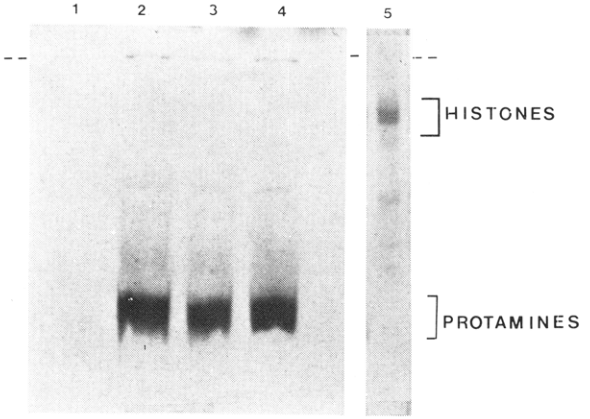

FIGURE 9: Acid–urea–polyacrylamide gel electrophoresis of labeled translation products. [^{3}H]Arginine incorporation into acid-soluble translation products in control (no RNA added) (1) and in the presence of pmRNA (2), 0.14 M KCl washed, polysomal pmRNP (3), 0.5 M KCl washed pmRNP (4), and histone mRNP gradient region RNA (5) was analyzed by acid–urea gel electrophoresis and fluorography as described under Materials and Methods.

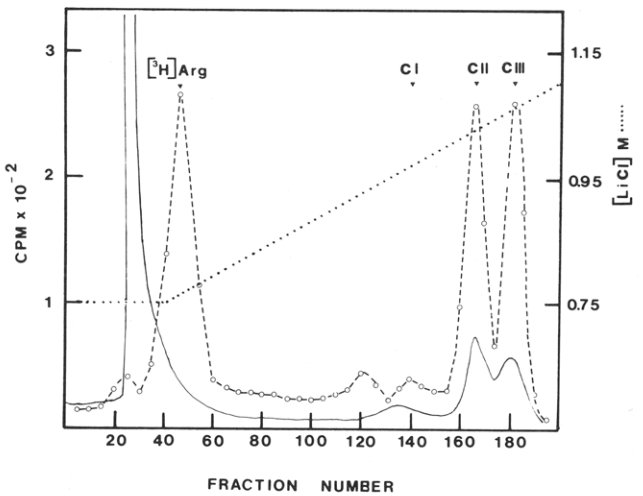

FIGURE 10: Analysis of polypeptides synthesized in the rabbit reticulocyte cell-free system in the presence of oligo(dT)–cellulose-bound pmRNP by CM-52 column chromatography. Acid-soluble ^{3}H-labeled products from translation assays were fractionated by CM-52 column chromatography by elution with increasing concentrations of buffered LiCl. Radioactive samples were mixed with 75 μg of unlabeled total basic testis proteins and eluted fractions assayed for the absorbance at 230 nm (—) and radioactivity by scintillation counting (O). Identification of protamine components was based on the concentration of LiCl at which they were eluted (Gedamu et al., 1979).

differences between the products of translationally active pmRNP particles from polysomal and free cytoplasmic cell compartments. There was, however, evidence of the synthesis of histones in gradient fractions showing high lysine incorporation (Figure 5); however, these fractions sedimented faster than pmRNP's and were not major contaminants of oligo(dT)–cellulose-purified pmRNP complexes. This is consistent with the known lack of 3′ poly(A) sequences in the majority of histone mRNAs (Adesnik & Darnell, 1972; Kedes, 1979).

Discussion

Preparative sucrose gradient centrifugation of polysomally derived and free cytoplasmic mRNP complexes in conjunction with the highly sensitive [^{3}H]pcDNA and [^{3}H]poly(U) hybridization assays permitted the localization and preliminary characterization of cytoplasmic poly(A+) pmRNP complexes. Although in the presence of EDTA both polysomally derived and free cytoplasmic pmRNP particles had identical sedimentation rates in sucrose gradients, their translational activity appeared quite different when assayed in the rabbit reticulocyte lysate. Polysomally derived complexes were actively transcribed in this in vitro system while free cytoplasmic complexes were inactive and at higher concentrations inhibited the endogenous levels in control assays. Following deproteinization, however, RNA from these complexes showed high activity in directing the synthesis of [^{3}H]arginine-containing polypeptides. Similar differences in the translational activity of polysomal and free cytoplasmic mRNP in other species have been described and have been postulated to reflect in vivo functional differences between the particles in these two cytoplasmic pools during development (Olsen et al., 1972; Gander et al., 1973; Chen et al., 1976; Civelli et al., 1976; Huynh-Van-Tan & Shapira, 1978; Geoghegan et al., 1979). However, it is apparent from this study that the gradient fractionation technique alone is not sufficient to separate these mRNP complexes from othe cosedimenting RNP complexes, protein aggregates, and translational inhibitors. For example, the analysis of proteins across the entire gradient after fractionation of EDTA-dissociated polysomes revealed considerable overlap in the polypeptides present in regions both containing and lacking putative polysomal pmRNP particles. Even after high-salt washing, putative polysomal pmRNP was still contaminated with cosedimenting 5S RNA–34K-dalton polypeptide complexes. In addition, comparative translational activity analysis has demonstrated that polysomally derived particles were only 10% pure after the first gradient centrifugation (data not shown). Even after two successive gradient purifications, polysomal pmRNP was only approximately 50% pure (Gedamu et al., 1977). The extent of contamination was particularly evident in the case of the free cytoplasmic complexes, which were found to cosediment with many other protein-containing particles. Little can be concluded about the specificity of proteins present in pooled, gradient-fractionated mRNP and the significance of the observed translational activity of these particles in vitro when large amounts of contaminating material are present in these fractions. Clearly, meaningful analysis of the biochemical properties and biological activity of these complexes can only be made with highly purified preparations, obtained by more selective and effective isolation techniques.

However, following purification by binding to oligo(dT)–cellulose, the particles can be characterized further and some conclusions drawn. CsCl density gradient analysis demonstrated, for the first time, that defined mRNP particles did, in fact, exist in both the polysomal and free cytoplasmic subcellular compartment of trout testis cells. The buoyant densities of the two mRNP populations were quite similar and within the range observed for mRNP complexes isolated in isotonic buffers. This suggested that both populations of mRNP were likely to possess similar protein contents at physiological ionic strength.

Particles isolated by this procedure were judged to be at least 75–80% pure by comparative translational analysis. Furthermore, the sole products of translation assays containing these particles were shown by acid–urea–polyacrylamide gel electrophoresis and CM-52 column chromatography to be protamine polypeptides. The profound difference in the translational activity of the two pmRNP populations provided the first direct evidence that a translational control mechanism might be acting through ribonucleoprotein complexes in vivo to regulate temporally the utilization of pmRNA sequences during sperm development.

An indication of the molecular basis of the masking of free cytoplasmic pmRNP was obtained from a study of both the

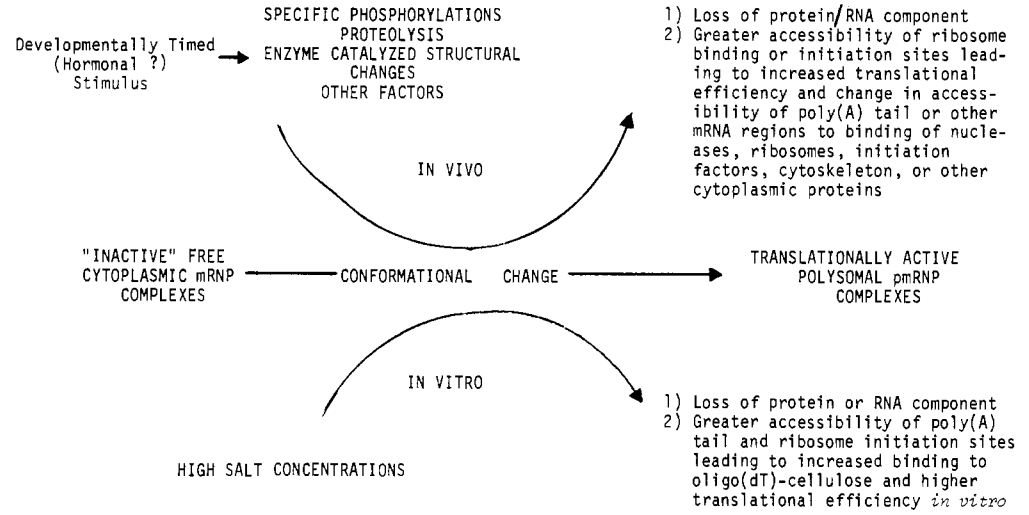

FIGURE 11: General model for the activation of pmRNP in vivo and in vitro.

binding of these complexes to oligo(dT)–cellulose at various salt concentrations and the translational activity of the bound and eluted particles. These data indicated that two processes were probably taking place. The increased ionic strength in the binding buffer resulted in an increase in the binding of these particles to the oligo(dT)–cellulose. Superimposed on this effect was the activation phenomenon by which particles isolated in salt concentrations of 0.3 M KCl or greater became translationally competent in the rabbit reticulocyte cell-free system.

The major effect of ionic strength on the subsequent translational efficiency of cytoplasmic mRNP complexes in vitro may explain some of the conflicting data in the literature on the biological activity of free cytoplasmic mRNP particles obtained by different laboratories employing various isolation conditions. Similar conclusions were reached recently by Liautard & Egly (1980), from studies on the translational activity of crude cytoplasmic mRNP complexes isolated in 0.14 M KCl (native RNP) which were translationally inactive in vitro, while the same mRNP preparations exposed to 0.5 M KCl solutions (activated RNP) directed the synthesis of labeled amino acids into acid-precipitable products. Activation of putative, "stored" mRNP was also demonstrated by Raff and co-workers (Jenkins et al., 1978) in studies in which sea urchin free cytoplasmic mRNP complexes were activated in vitro by exposure to 0.35 M NaCl. Messenger RNP particles isolated in 0.35 M KCl (physiological concentration for sea urchins), however, were totally inactive in the translation assays.

It is difficult at this stage to propose a precise mechanism for the activation of cytoplasmic pmRNP complexes by high-salt treatment due, in part, to the incomplete detailed biochemical information on the protein and RNA components of free cytoplasmic pmRNP complexes. The rather low levels of free cytoplasmic pmRNP binding to the oligo(dT)–cellulose precluded a thorough analysis of the protein components in bound complexes and the analysis of those polypeptides removed from the pmRNP by 0.3 M KCl. The data were also complicated due to the uncertainty whether complexes that did bind to the column truly reflect the nature of the majority of pmRNP particles in the free cytoplasmic cell compartment or only represent partially unfolded or denatured mRNP complexes.

These data, however, are consistent with the hypothesis that conformational changes probably brought about by loss of protein or other RNP components by exposure to high salt (but not EDTA) are the major factors in the in vitro activation process (Figure 11). Similar mechanisms have been postulated to operate in other developmental systems; however, translational inactivation by specific RNA or RNP components has only been conclusively demonstrated in embryonic chick muscle cells (Kennedy et al., 1978; Bag et al., 1980), connective tissue (Zeichner & Breitkreutz, 1978), and *Artemia* (Lee-Huang et al., 1977). It is conceivable that a small translation control RNA molecule not unlike those described in the above systems could be involved in the storage of pmRNA sequences in an inactive state in the trout testis in vivo. However, the similarity in sedimentation and buoyant density of polysomal and free cytoplasmic pmRNP particles in the presence of EDTA strongly argues for a more subtle change in the conformation involving, instead, loss of one or more proteins or rearrangement of mRNP protein interactions. Increased binding of postpolysomal supernatant pmRNP to oligo(dT)–cellulose probably reflects this conformational change and may represent an unmasking of poly(A) tracts in these cytoplasmic complexes.

Regardless of the nature of high salt induced activation of stored pmRNP in vitro, activation of pmRNP complexes in vivo is likely to be catalyzed by quite different processes than those discussed above since major changes in ionic strength during testis development are quite unlikely. Several possibilities for activation processes are indicated in Figure 11. For example, a cAMP-independent endogenous kinase activity has been found associated with free mRNP particles from chick muscle cells (Bag & Sells, 1979b; Bag et al., 1980), and specific in vivo phosphorylation of mRNP proteins has been recently documented (Gander et al., 1973; Auerbach & Pedersen, 1975; Mueller et al., 1977). Specific activation of a protein kinase could be a major means of changing the interaction of mRNP proteins, and hence the conformation of mRNP particles in vivo. Now that highly purified pmRNP particles can be obtained from both polysomal and postpolysomal supernatant fractions, a number of more detailed studies on the structure, activity, and metabolism of these particles in vivo can be initiated.

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