THE BINDING OF ADENOSINE-3',5'-MONOPHOSPHATE BY MESSENGER RIBONUCLEOPROTEIN-LIKE PARTICLES

Tom G. Obrig, Ross S. Antonoff, K. Siena Kirwin, and James J. Ferguson, Jr.

Department of Biochemistry
School of Medicine
University of Pennsylvania
Philadelphia, Pennsylvania 19174

Received July 10,1975

SUMMARY: Salt-washed ribosomal preparations contain ribonucleoprotein particles which bind adenosine-3',5'-monophosphate. These particles have informosome-like density and contain adenosine-3',5'-monophosphate-binding proteins of 35,000 to 42,000, 52,000 and > 130,000 molecular weight.

Since the first description of mRNA[1]-protein complexes (mRNP[1] particles or informosomes) in eukaryotic cells (1), similar components have been observed in the cytoplasm of numerous cell types (1-8). Reviews of this subject have been published (9,10). Compared to ribosome particles, cytoplasmic mRNPs are characterized by a high protein/RNA ratio and a low bouyant density in CsCl (1.37 to 1.47g/cm^3). They exist either as free particles in a post-ribosomal fraction (1,7) or attached to ribosomes (8,11,12,13). Estimates of molecular weights of proteins in mRNP particles have been made for a number of cell types (8,11,12,13,15,17). These 'm-proteins' (14) are acidic proteins (15) which are phosphorylated in serine hydroxyl residues (16).

While studying hormone action in testicular tissue we observed that cAMP could mimic FSH induction of protein synthesis. In an effort to determine how cAMP might affect this process, cAMP binding in salt-washed ribosomal preparations was examined. We found that cAMP-binding proteins occur in low density informosome-like RNP particles. While we are not yet able to assign a function to these proteins, the data suggest that they may be involved in some post-transcriptional or translational process regulated by cAMP.

METHODS: [^{3}H]cAMP (27.5 Ci/mmol) was obtained from Amersham/Searle. Buffer A is 40 mM triethanolamine, pH 7.4, 70 mM KCl, 3 mM $MgCl_2$.
Preparation of ribosomes and s-100 fraction. Procedures were carried out at 4°C unless stated otherwise. After removal of the tunica, a 150 g lamb testis was homogenized in a Waring blender for 1 min with 150 ml of buffer

[1]Abbreviations: cAMP, adenosine-3',5'-monophosphate; RNP, ribonucleoprotein; mRNP, messenger ribonucleoprotein; mRNA, messenger ribonucleic acid; Cl_3CCOOH trichloroacetic acid; poly (A), polyadenylic acid; UV, ultraviolet.

(35 mM triethanolamine, pH 7.4, 25 mM KCl, 10 mM $MgCl_2$, 250 mM sucrose). Ten percent solutions of sodium deoxycholate (in 50% ethanol) and Triton X-100 (in water) were added to a final concentration of 2% each. The contents were re-homogenized for 15 sec and centrifuged at 1500 x g for 10 min. After filtration of the supernatant solution through cheesecloth, 1 M β-mercaptoethanol was added to 5 mM final concentration. The preparation was centrifuged at 27,000 x g for 30 min and the resulting postmitochondrial supernatant solution was centrifuged at 100,000 x g for 2.0 hr. Resuspension of the high speed pellet in 'salt wash' buffer (35 mM triethanolamine, pH 7.4, 500 mM KCl, 8 mM $MgCl_2$, 5 mM β-mercaptoethanol) was carried out in a glass homogenizer fitted with a teflon pestle. The suspension was stirred gently for 30 min at 4° C, sedimented through a 15% sucrose cushion in buffer A at 100,000 x g for 3 hr and resuspended in buffer A containing 5 mM β-mercaptoethanol. Aliquots were stored at -60°C. Supernatant (s-100) containing soluble cAMP-binding proteins was prepared as described above through the first 100,000 x g centrifugation, except detergents were omitted.

[^{3}H]cAMP binding to ribosomes and light-induced attachment to ribosomes. Cyclic nucleotide binding to ribosomes and RNPs was measured by the nitrocellulose filter method of Nirenberg and Leder (17). Ribosomes or sucrose gradient fractions were incubated in a total volume of 0.25 ml containing Buffer A, 5 mM β-mercaptoethanol, 1 mM phosphodiesterase inhibitor Ro 20-1724/1 (Lot #1849-105B, Hoffman La Roche, Inc.) in 2.5% ethanol and 10^{-7} M [^{3}H]cAMP (20,000 cpm/pmol). Following incubation at 33° for 20 min, 2 ml of ice cold 40 mM triethanolamine buffer (pH 7.4) was added, the contents were filtered and the filter washed three times with buffer. Addition of KCl or $MgCl_2$ to the wash solution had little effect on binding. Filters were then dried and counted in 5 ml Hydromix scintillation fluid (Yorktown Research). It should be noted that [^{3}H]cAMP is dissociated from its binding protein in 5% Cl_3CCOOH. Covalent attachment of cyclic nucleotides to proteins, demonstrated by stability in 5% Cl_3CCOOH, was achieved by irradiation (254 nm) of the incubation mixture in a quartz cuvette for 3 hrs (18). Efficiency of [^{3}H]cAMP incorporation was 2% to 3% compared to the non-covalent binding of [^{3}H]cAMP. To measure [^{3}H]cAMP incorporation following UV irradiation, 0.1 ml of the mixture was withdrawn, precipitated at 4° C with addition of 0.4 mg bovine serum albumin and one volume of 10% Cl_3CCOOH. The pellets were washed three times with 10% Cl_3CCOOH and the final pellet was dissolved in 0.2 ml formic acid and counted in 5 ml of Hydromix.

CsCl gradient centrifugation. Fractionated and total ribosome preparations were labeled with [^{3}H]cAMP in the presence of UV light as described above. The preparations were dialyzed twice against 100 volumes of buffer A, concentrated, and incubated in 7% formaldehyde for 24 hrs at 4° C. Samples were layered onto 4.5 ml preformed gradients prepared from 1.30 and 1.70 g/cm^3 CsCl solutions (20°) in buffer A. Centrifugation was for 12 hrs at 35,000 rpm in a Beckman SW 39 rotor at 4° C. Fractions (0.15 ml) were collected and monitored for A_{260} with a Gilford gradient analyzer. Each fraction was measured for refractive index and radioactivity insoluble in 5% Cl_3CCOOH. CsCl density was calculated with a formula (19) where n is the refractive index corrected for formaldehyde content and temperature: density = (10.860 n - 13.497) g/cm^3. Protein content was calculated with the formula (9): %protein = (1.85 - density)/0.006. Such data are presented only as approximate values for the reasons noted by McConkey (21).

RESULTS: [^{3}H]cAMP bound readily to preparations of salt-washed ribosomes as measured by the nitrocellulose filter assay. Binding of 10^{-7} M [^{3}H]cAMP was complete after 15 min at 33° C. Approximately 0.093 pmol of cAMP were bound per A_{260} unit of ribosomes. As shown in Table 1, [^{3}H]cAMP binding is cyclic nucleotide-specific, being markedly inhibited by a 100-fold excess

Unlabeled nucleotide (10μM)	Binding		UV light-induced incorporation	
	[^{3}H]cAMP bound (cpm)	Inhibition (%)	[^{3}H]cAMP Incorporation (cpm)	Inhibition (%)
none	9310	----	538	----
3',5'-cAMP	190	98	46	91
3',5'-cGMP	4934	47	242	55
5'-AMP	8844	5	575	0
5'-GMP	9123	2	522	3
5'-ATP	8937	4	527	2
5'-GTP	8751	6	546	0

Table I. Binding and light-induced incroporation of [^{3}H]cAMP into ribosomes: nucleotide competition

Assay contained in 1.0 ml of Buffer A: 1mM phosphodiesterase inhibitor, 20 A_{260} units of salt-washed ribosomes, 10^{-7} M [^{3}H]cAMP and unlabeled nucleotides as indicated. Binding of [^{3}H]cAMP was measured on a 0.25 ml aliquot as described in Methods. The remaining 0.75 ml was irradiated with UV light at 4° C and Cl_3CCOOH insoluble radioactivity was measured. Backgrounds of 210 and 83 cpm have been subtracted from the binding and incorporation values, respectively.

of cAMP and cGMP but not by 5'-AMP, 5'-GMP, ATP or GTP. To determine if cAMP was bound to non-ribosomal RNPs, ribosomal subunits, monosomes or polysomes, unlabeled total ribosomes were centrifuged in a linear sucrose gradient. Fractions were then assayed for their ability to bind [^{3}H]cAMP. The results (Fig. 1) indicate that a major portion of the [^{3}H]cAMP was bound to non-ribosomal particles which were located in the upper one-third of the gradient. It is apparent that cAMP binding is not proportional to the amount of A_{260} material in the gradient fractions. Much of the binding activity was detected in a region lighter than 60S, including large discrete peaks of 54S and 27S, and a 13S shoulder. This 13S fraction could be resolved by re-sedimentation. These reproducible cAMP-binding patterns have also been observed in salt-washed ribosomal preparations from rabbit reticulocytes.

To determine the density, and thus the protein/RNA ratios of the cAMP binding particles, salt-washed ribosomes were covalently labeled with [^{3}H]cAMP and banded in CsCl gradients. (The specificity of covalent labeling, presented in Table 1 is similar to that of [^{3}H]cAMP binding to ribosomes.) Under these conditions, free protein would have banded at the top

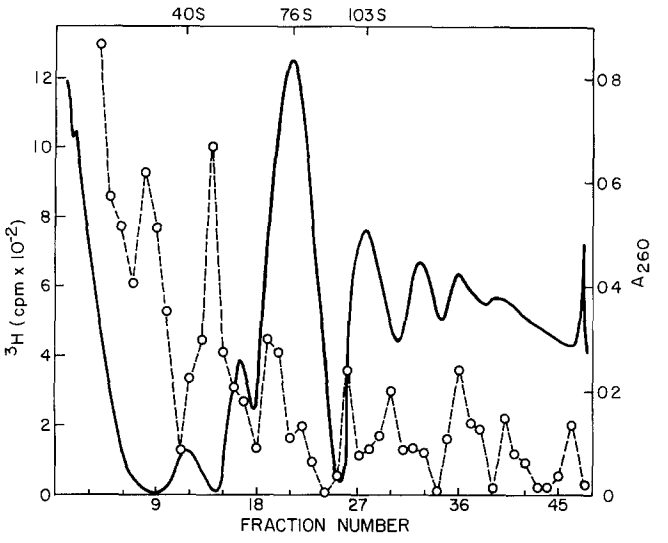

Fig. 1 Cyclic AMP binding to ribosomal fractions from a linear sucrose gradient. Salt-washed ribosomes (12 A_{260} units) were layered onto a 15-45% sucrose gradient in buffer A and centrifuged for 1.5 hr at 49,000 rpm in a Beckman SW 50.1 rotor. Fractions were assayed for [^{3}H]cAMP binding (Methods). A background of 100 cpm has been subtracted. A_{260} (——); cpm (o-------o).

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of the gradient while free RNA would have sedimented to the bottom. The results, illustrated in Fig. 2, show that 75% of the Cl_3CCOOH-insoluble radioactivity banded at densities between 1.376 and 1.462 g/cm^3. Included in this region were informosome-like particles with densities of 1.392, 1.408 and 1.443 g/cm^3 and approximate protein contents of 76%, 74% and 67%, respectively. The major A_{260} peak in Fig. 2 had the density characteristics of a ribosomal RNP particle (7,13,20) though it contained relatively little [^{3}H]cAMP. [^{3}H]cAMP-binding protein, free of RNA, was not detected.

CsCl gradient analysis was also used to determine the density of isolated free cytoplasmic 13S and 27S particles which bound cAMP. All of the [^{3}H]cAMP covalently bound to both fractions banded between 1.361 and 1.472 g/cm^3. Neither of the CsCl gradients contained a [^{3}H]cAMP-labeled particle of ribosomal density. Molecular weights of cAMP-binding proteins from 13S and 27S fractions were estimated by gel electrophoresis. Analysis of both the 13S (Fig. 3a) and the 27S fractions (Fig. 3b) revealed the presence of binding proteins of 35,000 - 42,000 and 52,000 mol wt. A 100,000 x g supernatant (s-100) fraction, in comparison (Fig. 3c), contained a pattern

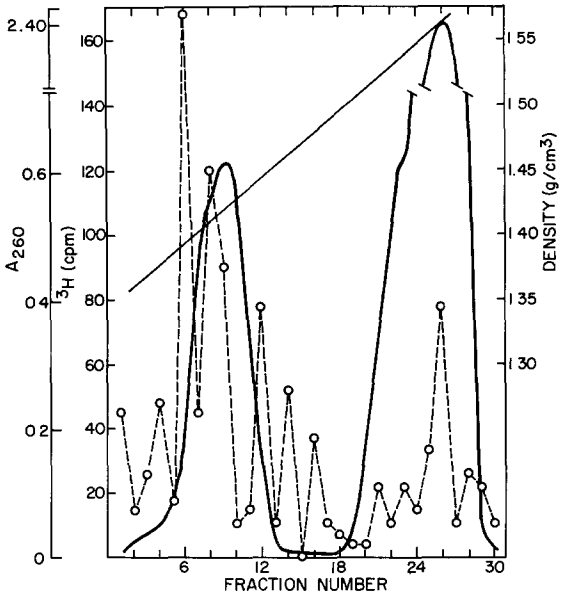

Fig 2 CsCl gradient separation of RNP particles labeled with [^{3}H]cAMP. Salt-washed ribosomes, UV-labeled with [^{3}H]cAMP (Methods) were banded in CsCl gradients. Cl_3C COOH-insoluble radioactivity and refractive indices were determined on collected fractions. 95% of applied radioactivity was recovered. Background of 10 cpm has been subtracted. A_{260} (▬▬); cpm (------); density (———).

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of cAMP-binding protein which was qualitatively similar to that seen in Fig. 3a and 3b. It should be noted that most of the [^{3}H]cAMP-binding activity in Fig. 3a and 3b did not migrate with the bulk protein. Minor amounts of other labeled proteins were detected; a 100,000 mol wt. species in the 13S fraction (Fig. 3a) and a >130,000 mol wt. species in the 27S fraction (Fig. 3b).

DISCUSSION: There is conflicting evidence regarding the specificity of interaction between protein and RNA in cytoplasmic RNPs (2,7,9,13,15, 22,23). We believe our data favor the concept of specificity of association and offer the following in support: (a) cAMP-binding proteins remained tightly bound to RNP particles during washing with 0.5 M KCl, (b) these binding proteins were not quantitatively associated with the major ribosomal subunits, monomers or polysomes, (c) cAMP-binding protein was preferentially bound to particles of informosome-like density, (d) preliminary results indicate that purified cAMP-binding proteins from testes do not associate with E. coli ribosomes, ribosomal RNA or transfer RNA.

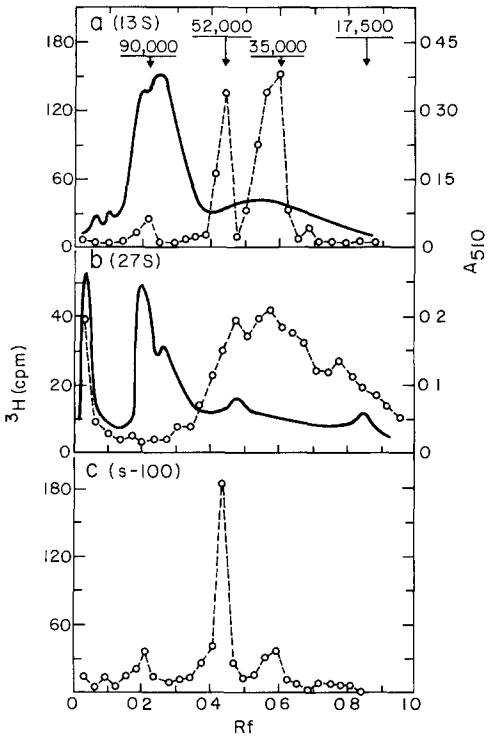

Fig. 3 Sodium dodecylsulfate gel electrophoresis of cAMP-binding proteins from 13S and 27S particles. 13S (a) and 27S (b) fractions from preparative sucrose gradients of salt-washed ribosomes were covalently labeled with [^{3}H]cAMP as described in Methods and subjected to electrophoresis in 7% acrylamide gels according to Weber and Osborn (24). A 100,000 x g supernatant (c) was treated identically. Gels were run at 8mA per tube for 5 hr., stained with Commassie blue, destained, scanned at 510nm and sliced. The 1.5mm slices were incubated at 45° C for 12 hr. in 10 ml of a solution consisting of 4g Omniflor, 30 ml. Protosol (New England Nuclear Corp.) and toluene to 1 liter, and counted. Molecular weights were calculated from protein standards. A background of 20 cpm has been subtracted. A_{510} (———); cpm (o------o).

The cAMP receptors described in this communication are located in both cytosol and in salt-washed mRNPs, have mol wts. of 35,000 and 52,000, and bind to poly (A)-Sepharose (unpublished results). There appears to be a striking similarity between these cAMP-binding proteins and the protein synthesis initiation factor IF-MP. Hellerman and Shafritz (26) recently reported that IF-MP is present in both cytosol and mRNPs and also binds to poly (A). Experiments regarding this possible relationship are now in progress.

ACKNOWLEDGEMENT

The authors are indebted to Miss Anne Strimpler for excellent technical assistance. We thank Dr. John Clark and Dr. Joel Flaks for their interest and comments. Appreciation is extended to Dr. W.E. Scott of Hoffman LaRoche, Inc. for providing the phosphodiesterase inhibitor. This work was supported by National Institutes of Health grant HD 05507.

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