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Cyclic nucleotide-binding proteins detected by photoaffinity labeling in nucleus and cytoplasm of bovine liver

(8-azido cyclic AMP/cyclic GMP/nuclear binding proteins)

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ABSTRACT **A photoaffinity labeling method was used to characterize and compare cyclic nucleotide-binding proteins of bovine liver cytosol with binding proteins of the nucleus. After photoaffinity labeling of cytosol with 8-azido cyclic [^{32}P]AMP, autoradiographs of sodium dodecyl sulfate polyacrylamide gel electrophoresis revealed two major labeled proteins of 47,000 and 52,000–55,000 daltons. DEAE-cellulose column-derived fractions suggested that the larger protein was the regulatory subunit of peak II cyclic AMP-dependent protein kinase and the smaller protein was the regulatory subunit of peak I kinase. The smaller protein was largely present as the free regulatory subunit. The two binding proteins differed in their ability to bind cyclic GMP. Binding to both proteins was abolished by excess unlabeled cyclic AMP but not by 5′-AMP. Photoaffinity labeling of a 0.14 M salt extract of nuclei and a nonhistone chromosomal protein preparation revealed two major binding proteins with the same molecular weight and competition profiles as those of the cytosol. Detergent-washed nuclei gave similar results. Several minor binding proteins were observed in both cytosol and nucleus. One protein (36,000 daltons) was unique to the nucleus and had low affinity for 8-azido cyclic AMP. Photoaffinity labeling with cyclic [^{3}H]GMP revealed a cytosol protein, absent from the nucleus, of 31,000 daltons and the ligand was competed for by both cyclic GMP and 5′-GMP. These studies suggest that the major specific cyclic AMP-binding proteins of bovine liver are the type I and type II regulatory subunits of cyclic AMP-dependent protein kinase and are present in both nucleus and cytoplasm.**

The role of cyclic nucleotides within the cell nucleus is poorly defined. Whole cell $^{32}P_i$ uptake studies in liver have indicated that adenosine 3′,5′-cyclic monophosphate (cAMP) stimulates the phosphorylation of specific histones (1) and nonhistone chromosomal proteins (NHCP) (2). cAMP-dependent protein kinase has been detected in the nucleus of several cell types (3–8), and its presence has been confirmed in nuclei isolated in nonaqueous medium (7, 8). Multiple forms of a cAMP-dependent protein kinase in a phosphoprotein-enriched subfraction of beef liver NHCP have also been reported (4) but not confirmed although multiple nuclear cAMP-independent kinases have been repeatedly described (e.g., 9, 10). Other studies (11, 12) utilizing antibody immunofluorescence have indicated that guanosine 3′,5′-cyclic monophosphate (cGMP) and presumably its binding protein(s) are localized to the nucleus of some cells, whereas other reports (13–15) have suggested the translocation of cAMP-dependent protein kinase and its regulatory subunit into the nucleus in response to increased cAMP. The generality of these phenomena is not yet appreciated.

Further study of this problem is highly desirable in view of the evidence linking cAMP to transcriptional regulation in several systems (16). In addition to a phosphorylation mechanism, one should consider the possibility that cyclic nucleotides bound to specific protein receptors regulate transcription by direct interaction with the chromosome, as in the case of prokaryotes (17). In the present studies we have utilized a photoaffinity labeling method (18–21, §) to determine if binding proteins are present in the nucleus. A preliminary report of this work has been presented (22).

METHODS

Preparation of Cytosol and Nuclear Fractions from Beef Liver. The method for isolation of "nuclear sap" and NHCP was modified from that of Kish and Kleinsmith (4). Fresh beef liver was minced and homogenized in a Potter–Elvehjem Teflon homogenizer in 3 vol of ice-cold 0.32 M sucrose/3 mM $MgCl_2$. Cytosol was prepared by passage of the homogenate through cheesecloth and sedimentation for 2 hr at 24,000 rpm in a Beckman 27.1 rotor. The "cytosol" was removed from the middle portion of the centrifuge tube, dialyzed overnight against 5 mM Tris, pH 7.5/1 mM EDTA, and stored at −70°C.

Nuclei were prepared by dilution of the homogenate with 6 vol of sucrose/$MgCl_2$, passage through cheesecloth, and sedimentation at 1000 × *g* for 7 min. The pellet was dispersed in a Teflon homogenizer and brought to a final sucrose concentration of 2.15 M (determined by refractometry). Additional 2.15 M sucrose/1 mM $MgCl_2$ was added to bring the volume to 2 ml per g of original tissue, and the mixture was sedimented for 65 min at 24,000 rpm in the 27.1 rotor. The nuclear pellets were suspended and washed in 20 vol of 0.01 M Tris·HCl, pH 7.5/0.25 M sucrose/4 mM $MgCl_2$. Where indicated, the washing medium also contained 0.5% sodium deoxycholate and 1% Tween 40 as described by Penman (23) to remove outer nuclear membranes. The nuclear sap and NHCP were then prepared as described (4) and stored at −70°C.

Photoaffinity labeling was carried out with [^{3}H]cAMP and [^{3}H]cGMP (Amersham/Searle) or with 8-azido [^{32}P]cAMP [generously supplied by Boyd Haley, University of Wyoming (19)]. For photoaffinity labeling with the nonderivatized compounds, the cytosol and nuclear sap were dialyzed overnight against 10 mM Tris/1 mM EDTA/5 mM 2-mercaptoethanol/10% (vol/vol) glycerol, pH 7.4. The NHCP were dialyzed against the same buffer containing 400 mM NaCl. RO-20-1724 (1 mM), a phosphodiesterase inhibitor, was added and, after 15 min, [^{3}H]cAMP or [^{3}H]cGMP was added (0.5 μM; 19–26 Ci/mmol). UV irradiation was performed in a cooling chamber at 4°C for 2 hr with a laboratory UV lamp (rated at 700 μW/cm^2 at 15 cm, 254 nm) at a distance of 1 cm. In experiments with the azido compound, samples were dialyzed against 5 mM Tris/1 mM EDTA, pH 7.4. Mixtures containing 10 mM Tris, 5 mM 2-mercaptoethanol, and 8-azido [^{32}P]cAMP

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Abbreviations: cAMP, cyclic AMP; NHCP, nonhistone chromosomal proteins; cGMP, cyclic GMP.

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§ Chambers, D. A. & Stubbs, J. D. (1976) *Proceedings of the 10th International Congress of Biochemistry*, p. 525.

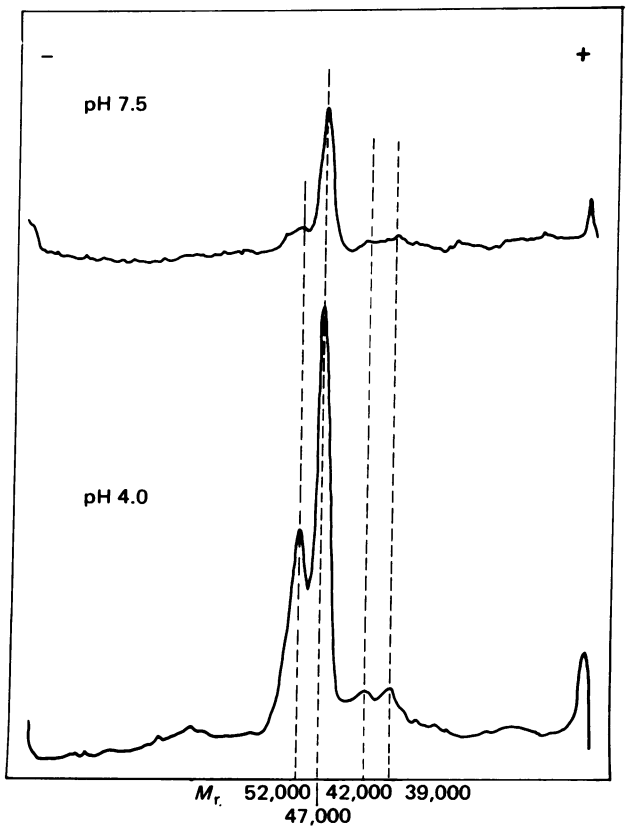


FIG. 1. Densitometry trace of autoradiograms after polyacrylamide gel electrophoresis, showing binding of 8-azido [^{32}P]cAMP to cytosol proteins at pH 7.5 and pH 4.0. Photoaffinity labeling mixtures contained 25 nM 8-azido [^{32}P]cAMP and either 10 mM Tris buffer at pH 7.5 or 10 mM sodium acetate buffer at pH. 4.0. Samples were UV irradiated for 2.5 min at 0°C. Molecular weight standards were serum albumin, ovalbumin, and D-amino acid oxidase.

(10–100 nM; 4–11 Ci/mmol) were irradiated for 2.5–4 min at 0°C.

Proteins were precipitated for 2 hr at 0°C with ice-cold ethanol at a final concentration of 90% and dried under reduced pressure. The dried pellets were dissolved by boiling for 5 min in 0.25 M sucrose/0.13 M 2-mercaptoethanol/10 mM sodium phosphate, pH 7.4/0.5% sodium dodecyl sufate. Electrophoresis was by the method of Laemmli (24) using 0.7-mm slab gels of 9.5–10% acrylamide; the gels were dried onto filter paper. In experiments with ^{3}H-labeled cyclic nucleotides, the gel was sliced into 2-mm pieces that were combusted in a Packard oxidizer and assayed for radioactivity. In experiments with 8-azido [^{32}P]cAMP the dried gels were analyzed by autoradiography with XRP-5 Kodak x-ray film exposed for 7–14 days and scanned at 450 nm.

RESULTS

Photoaffinity Labeling of Cytosol with 8-Azido [^{32}P]cAMP. To characterize the cyclic nucleotide-binding proteins in cytosol from bovine liver by photoaffinity labeling, studies were carried out with the nonparticulate cytosol and peaks I and II protein kinases purified from cytosol by DEAE-column chromatography. Specificity of cyclic nucleotide binding to protein was determined by comparing the ability of unlabeled cyclic nucleotide with that of corresponding 5′ nucleotide to compete for the protein binding site. The cytosol was incubated for 10 min at 0°C with 8-azido [^{32}P]cAMP (0.01 μM) and irradiated with UV light for 2.5 min. This treatment resulted in the incorporation of 6.7 fmol/mg of protein into a trichloroacetic acid-insoluble product (after subtraction of unirradiated controls). The incorporation was decreased 65% by addition of 10 μM cAMP, suggesting that a portion of the incorporation could be nonspecific. However, the binding was unaffected by cGMP or 5′-AMP.

Fig. 1 shows the results of sodium dodecyl sulfate/polyacrylamide gel electrophoresis of cytosol after irradiation in the presence of 8-azido [^{32}P]cAMP. The results of irradiation at pH 7.5 and pH 4.0 were compared because studies using the Millipore filter binding assay have indicated enhanced binding at the lower pH (25). The results were qualitatively similar at the two pH values, but the amount of radioactivity was much greater at pH 4. Two major bands were observed, one at 52,000 daltons and the other at 47,000 daltons. In addition, there were minor bands at about >120,000, 42,000, and 39,000 daltons.

Protein kinase purification by DEAE-column chromatography revealed two cAMP-dependent kinases peak, a small peak at 0.04 M NaCl (peak I) and a large peak at 0.19 M NaCl (peak II). [Small amounts of peak I protein kinase have been noted in bovine liver by Corbin and Sugden (26).] The peak fractions were individually pooled and tested for binding to 8-azido [^{32}P]cAMP. Considerable incorporation into an acid-insoluble product was observed with both peak I (147 fmol/mg of protein) and peak II (220 fmol/mg of protein). In contrast to the cytosol, with both purified peaks the activity was almost completely abolished by unlabeled cAMP (94% decrease with peak I and 97% with peak II) and significant competition was noted with cGMP (69% inhibition with peak I and 81% with peak II).

The results of binding of 8-azido [^{32}P]cAMP to the pooled peak I and peak II protein kinase peaks are shown in Fig. 2. With peak I, >90% of the bound radioactivity was in a 47,000-dalton protein. The binding of 8-azido [^{32}P]cAMP was abolished by addition of unlabeled cAMP to the reaction mixtures and was markedly decreased by cGMP; 5′-AMP was without effect. Several minor bands were also observed at 41,000 and >120,000 daltons.

In contrast, peak II protein kinase (Fig. 2 right) exhibited both of the major binding proteins, at 52,000 and at 47,000 daltons, and a minor band at 39,000 daltons. All bands were eliminated by unlabeled cAMP. The 47,000- and 39,000-dalton protein sites were competed for by unlabeled cGMP, whereas the 52,000-dalton site was unaffected. No band was affected by 5′-AMP.

These results suggested the possibility that the type I regulatory subunit was the 47,000-dalton binding protein. However, the presence of this binding protein as a major component of the peak II protein kinases along with the 52,000-dalton binding protein was confusing. To clarify these interpretations, an experiment was carried out in which each fraction of the DEAE-cellulose column was assayed for both histone kinase activity and binding with 8-azido [^{32}P]cAMP. The relative amounts of each molecular weight species in each column fraction were determined for the area under the curves in the densitometry tracings. The results (Fig. 3) show that the 52,000–55,000 dalton‖ protein cochromatographed with the peak II protein kinase, which suggests that this protein is the type II regulatory subunit. A slight excess of binding protein relative to kinase activity in the higher fractions could be accounted for by the presence of a small amount of free type II regulatory subunit. The 47,000-dalton binding protein eluted as a large peak between the two kinase activity peaks with a shoulder coincident with the peak I kinase activity. This result is consistent with the interpretation that the 47,000-dalton protein is the type I regulatory subunit and a large excess of free type I regulatory subunit elutes at a higher salt concentration

‖ In the experiment in Fig. 3, the 52,000-dalton protein appeared as a poorly separated doublet of 52,000 and 55,000 daltons and is treated as a single protein.

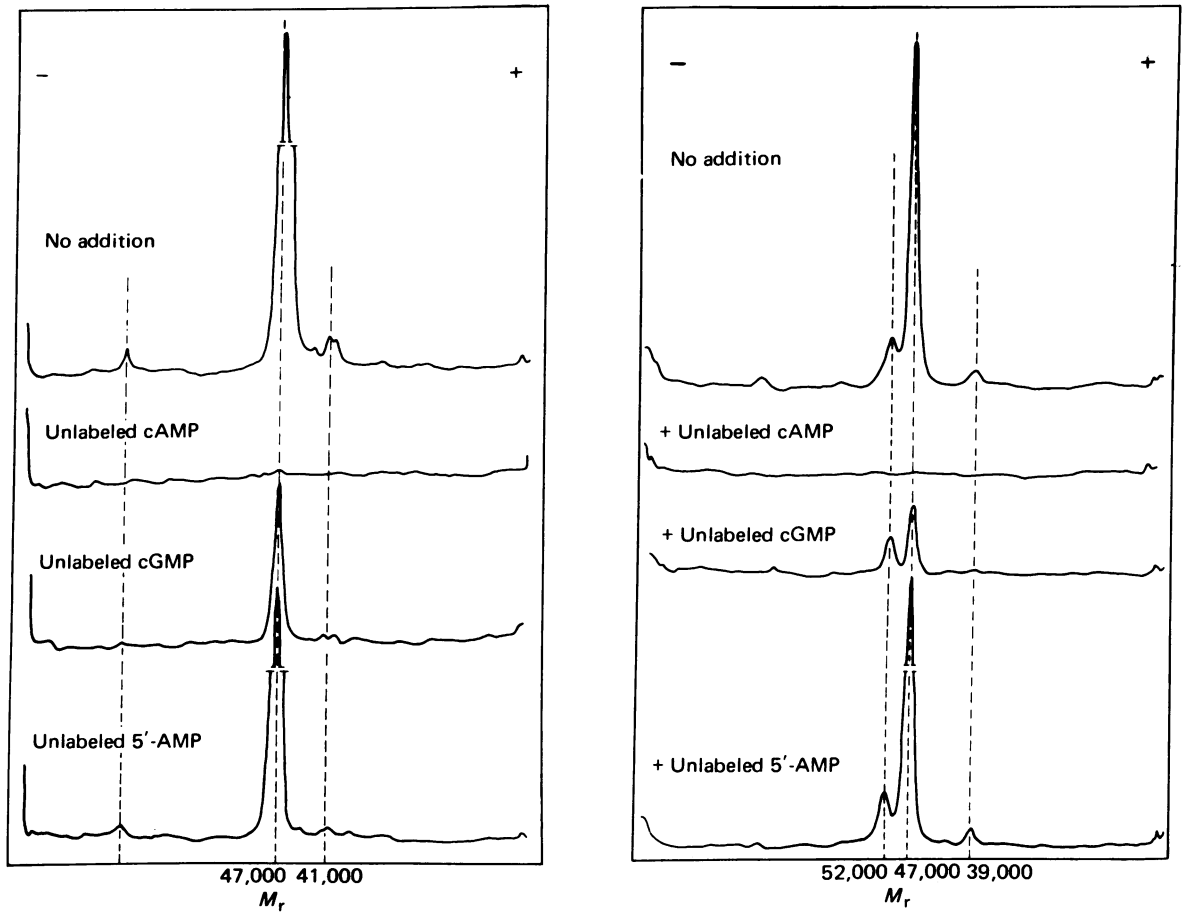


FIG. 2. Binding of 8-azido [^{32}P]cAMP to peak I (*Left*) and peak II (*Right*) cAMP-dependent protein kinases. The kinases were separated on a DEAE-cellulose column (0.19 × 6.0 cm) equilibrated with 5 mM Tris/1 mM EDTA, pH 7.5. Five milliliters of dialyzed cytosol was passed over the column; elution was with 50 ml of 5 mM Tris/1 mM EDTA, pH 7.5, followed by 200 ml of a linear gradient of 0–0.4 M NaCl in the same buffer. The fractions were assayed for protein kinase activity in the presence and absence of 2.0 mM cAMP. The kinase reaction mixtures (100 μl) contained 30 mM 3-(*N*-morpholino)propanesulfonic acid (pH 6.8), 7.5 mM Mg acetate, 100 μM [α-^{32}P]ATP (40 μCi/μmol), 0.1 mM 1-methyl-3-isobutylxanthine, 2.5 mg of mixed histone (fraction IIA, Sigma) per ml, and 25 μl of extract. Two peaks of kinase activity were observed; both were >90% dependent upon cAMP. Peak I had approximately 20% of the acitivity of peak II. The pooled fractions from each peak were precipitated by addition of ammonium sulfate to 70% saturation. The pellets were dissolved in 2 ml (peak I) or 4 ml (peak II) of 10 mM Tris/1 mM EDTA, dialyzed against the same buffer, and stored at −20°C. Photoaffinity labeling mixtures contained 10 nM 8-azido [^{32}P]cAMP, 10 mM Tris (pH 7.5), and unlabeled cAMP, cGMP, or 5′-AMP (each at 10 μM).

than the holoenzyme. A 39,000-dalton protein comigrated with the peak of the 47,000-dalton protein. The similar elution profile suggests that this protein may result from proteolytic degradation of the 47,000-dalton protein. These interpretations are supported by recent reports of molecular weights of the type I and type II regulatory subunits from other tissues (ref. 27; J. D. Corbin, personal communication).

Photoaffinity Labeling of Nuclear Fractions with 8-Azido [^{32}P]cAMP. Two nuclear fractions were studied for their ability to bind 8-azido [^{32}P]cAMP: the nuclear sap, and a preparation of NHCP. These fractions bound 34 and 22 fmol of cyclic nucleotide per mg of protein, respectively. The binding protein profiles with these two fractions were qualitatively similar in that four binding proteins were observed (Fig. 4). Two of the binding proteins had the same molecular weights as the two major cytosol binding proteins (i.e., 47,000 and 52,000) and identical nucleotide competition profiles. A third binding protein (42,000 daltons) may be identical with the minor cytosol binding proteins observed at 41,000. The fourth binding protein (36,000 daltons) was unique to the nucleus and appeared to be a low-affinity binding protein because it was unaffected by the presence of unlabeled cAMP.

Results similar to those obtained above were also observed with nuclei treated with a mixture of sodium deoxycholate and Tween 40 and with nuclei prepared in the presence of 1 mM phenylmethylsulfonyl fluoride, a serine protease inhibitor. The nuclear binding protein pattern does not appear to be explicable by the presence of contaminating plasma membranes in the nuclear preparation or by the action of serine proteases.

Binding Studies with [^{3}H]cAMP and [^{3}H]cGMP. Photoaffinity labeling with [^{3}H]cAMP was far less efficient than that with azido derivative in that irradiation for 2 hr resulted in less binding. Prolonged UV treatment resulted in marked protein destruction or modification as evidenced by smearing of bands in the stained gel. Nevertheless, some specific binding, by the criterion of competition with unlabeled cAMP, was observed with cytosol and to a lesser extent with nuclear sap and NHCP. Analysis of the labeled cytosol proteins showed a single major labeled band at 47,000 daltons. The technique lacked the sensitivity to detect the additional bands observed with the azido derivative.

Photoaffinity labeling with [^{3}H]cGMP also required a long period of irradiation. Approximately twice as much cGMP was bound to cytosol proteins as cAMP under equivalent conditions. In this instance the gels revealed two major binding proteins in the cytosol, one at 47,000 and the other 31,000 daltons. In the nuclear sap, a single binding protein at 47,000 was observed (Fig. 5). Competition studies with cytosol indicated that the 47,000-dalton protein binding was competed for by cAMP and cGMP but not by 5′-GMP. The 31,000-dalton protein binding

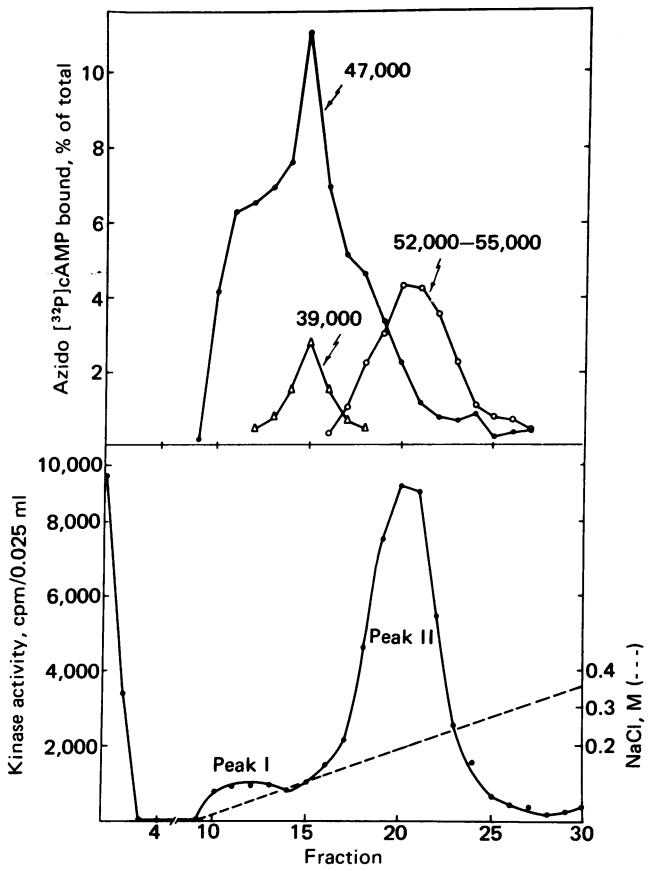


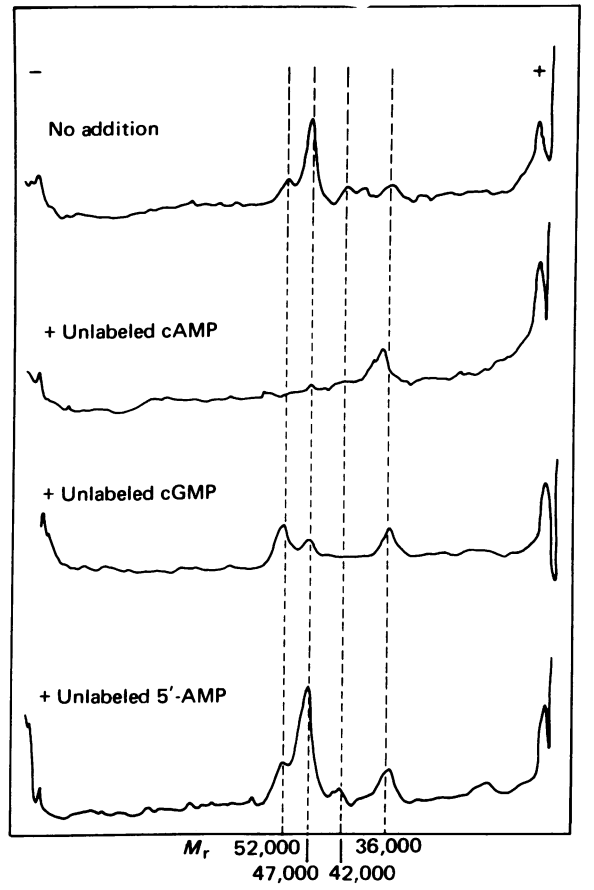
FIG. 3. Binding of 8-azido [^{32}P]cAMP (*Upper*) and cAMP-dependent protein kinase activity (*Lower*) in DEAE-cellulose column fractions. The DEAE-cellulose column preparation and elution were as in Fig. 2. Peak I kinase activity was only about 10% that of peak II. The protein in each 8-ml fraction was precipitated with $(NH_4)_2SO_4$, dissolved in 0.5 ml of 10 mM Tris/1 mM EDTA, pH 7.5, and dialyzed for 4 hr. Each sample was assayed for protein kinase activity as in Fig. 2 or for 8-azido [^{32}P]cAMP binding. The photoaffinity labeling mixture contained 100 nM 8-azido [^{32}P]cAMP and 10 mM Tris at pH 7.5; irradiation was for 4 min. The photoaffinity labeled proteins were processed as in Fig. 1. Three major labeled bands were observed in the DEAE-cellulose fractions, at 47,000, 39,000, and 52,000–55,000 daltons. The radioactivity in each of these bands in each fraction was quantitated from the area under the curve. Total radioactivity was taken as the total area of the three bands from all fractions.

was competed for by unlabeled 5′-GMP and cGMP but not by cAMP.

Millipore Filter Binding. It was of interest to analyze the same liver fractions by the Millipore filter binding assay of Gilman (25). Specific binding of [^{3}H]cAMP (eliminated by unlabeled cAMP) was observed in the cytosol as well as in the two nuclear fractions. The binding of cAMP in cytosol was enhanced 2- to 4-fold at pH 4.0 compared with pH 7.5. A surprising observation was that binding to both nuclear sap and NHCP was inhibited at pH 4, rather than enhanced. With cGMP, specific binding was observed in the cytosol and nuclear sap but no significant binding was detected in the NHCP. In contrast with the photoaffinity assay, binding of cGMP was only about 5–15% of that of cAMP.

DISCUSSION

Photoaffinity labeling with 8-azido cAMP revealed two major cytosol binding proteins, 47,000 and 52,000–55,000 daltons. These observations are consistent with recent studies with several different mammalian tissues (27) and suggest that these two proteins are the regulatory subunits of type I and type II



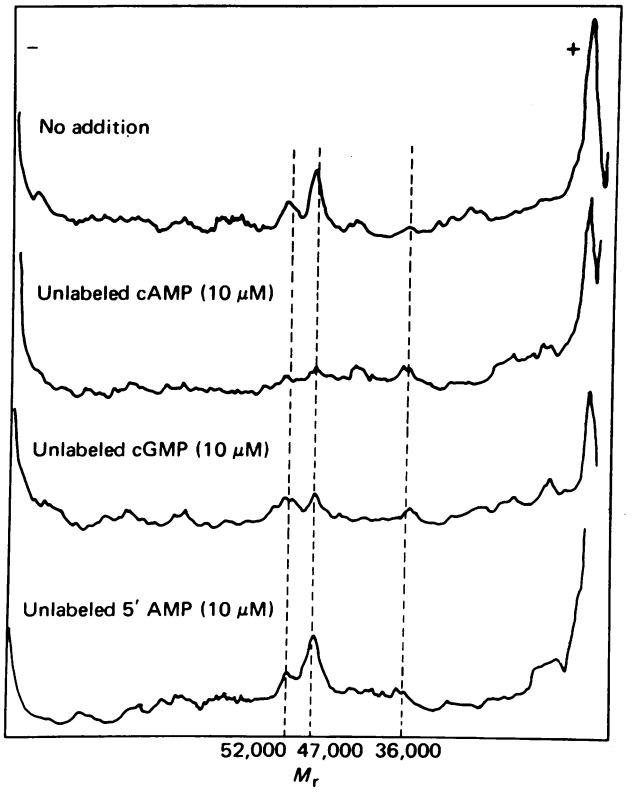


FIG. 4. Binding of 8-azido [^{32}P]cAMP to proteins of the nuclear sap (*Upper*) and to NHCP (*Lower*). Photoaffinity labeling and protein separation were as in Fig. 2.

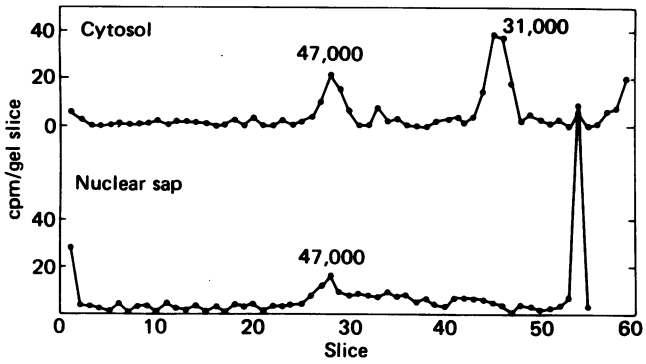


FIG. 5. Binding of [^{3}H]cGMP to proteins of cytosol and nuclear sap. The data for cytosol and nuclear sap were obtained from different slab gel runs.

protein kinases. The two proteins differed in their affinities for cGMP. Photoaffinity binding of azido cAMP to the 47,000-dalton protein was competed for by cGMP, although not as effectively as by unlabeled cAMP, whereas binding to the 52,000-dalton protein was unaffected by the presence of unlabeled cGMP. Assuming that the 47,000-dalton protein is the type I subunit, it is present largely in a free state on the DEAE-cellulose columns. This result could not be explained by dissociation of the type I holenzyme during isolation because an excess of free catalytic subunit was not observed in the column effluent. This large excess of free type I regulatory subunit may be of physiological significance.

In addition to the two major cytosol binding proteins, several other binding proteins were observed in small amounts. The lower molecular weight minor proteins could arise from proteolysis. Such degradation has been described by Sugden and Corbin (26). That these may be degradation products of the type I regulatory subunit is supported by the similarity, in binding competition with unlabeled cGMP and cochromatography on DEAE-cellulose, of one of the minor proteins with the 47,000-dalton binding protein. The high molecular weight (>120,000) minor binding protein is of interest because it cannot result from proteolysis.

Photoaffinity labeling of cytosol with [^{3}H]cGMP revealed two binding proteins. One of these is the 47,000-dalton cAMP binding protein described above. The second protein, 31,000 daltons, is not detected in the Millipore assay. The significance of this protein relative to cGMP action is unclear because unlabeled 5′-GMP competed with [^{3}H]cGMP for binding at least as well as unlabeled cGMP. The absence of this protein in the nucleus argues that the nuclei preparations were free of cytoplasmic contamination.

The photoaffinity labeling studies with the nuclear fractions revealed the presence of two major 8-azido cAMP-binding proteins. These proteins had the same molecular weights and competition profiles as the cytosol proteins, suggesting that the major nuclear binding proteins are the same or closely related to the cytosol binding proteins. Several minor nuclear binding proteins were noted. One protein may be identical to a minor cytosol binding protein, but another appears unique to the nucleus. This latter protein is of questionable significance because of the low affinity of binding, as indicated by the absence of competition with cAMP. These studies suggest that significant levels of binding proteins, with the same molecular weight as cytosol binding proteins, are present in the nuclear sap and NHCP fractions. In contrast, no unique and specific cGMP binding proteins were detected in the nucleus or cytoplasm but this observation can only be confirmed when photoreactive derivatives as active as azido cAMP become available.

It is of interest that, despite the apparent similarity of the same major cAMP binding proteins in cytosol and nucleus when assayed by the photoaffinity method, comparisons of nucleus and cytosol binding by Millipore filter assay showed differences with respect to the effect of pH. Whereas cytosol binding was markedly enhanced at pH 4, nuclear binding was inhibited. This observation could be explained by the presence in the nucleus of an additional binding protein that does not bind at pH 4 and does not bind to 8-azido cAMP. Alternatively, a binding protein might be modified prior to or after it enters the nucleus. Such a modification could be involved in regulation of transport of these proteins into the nucleus.

In conclusion, these results suggest the presence of both type I and type II regulatory subunits of cAMP-dependent protein kinase within the nucleus. The role and mechanism of action of these binding proteins in the nucleus remain to be elucidated.

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