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### Research Article

We used embryonic chick pelvic cartilage as a model to study the mechanism by which cyclic AMP increases RNA synthesis. Isolated nuclei were incubated with [<sup>32</sup>P]-8-azidoadenosine 3,5'-monophosphate ([<sup>32</sup>P]N<sup>3</sup>cAMP) with no resultant specific nuclear binding. However, in the presence of cytosol proteins, nuclear binding of [<sup>32</sup>P]N<sup>3</sup>cAMP was demonstrable that was specific, time dependent, and dependent on a heat-labile cytosol factor. The possible biological significance of the nuclear binding of the cyclic AMP-protein complex was identified by incubating isolating nuclei with either cyclic AMP or cytosol cyclic AMP-binding proteins prepared by batch elution DEAE cellulose chromatography (DEAE peak cytosol protein), or both, in the presence of cold nucleotides and [<sup>3</sup>H]uridine 5'-triphosphate. Poly(A) RNA production occurred only in nuclei incubated with cyclic AMP and the DEAE peak cytosol protein preparation. Actinomycin D inhibited the incorporation of [<sup>3</sup>H]uridine 5'-monophosphate into poly(A) RNA. The newly synthesized poly(A) RNA had a sedimentation constant of 23S. Characterization of the cytosol cyclic AMP binding proteins using [<sup>32</sup>P]N<sup>3</sup>-cAMP with photoaffinity labeling three major cAMP-binding complexes (41,000, 51,000, and 55,000 daltons). The 51,000 and 55,000 dalton cyclic AMP binding proteins were further purified by DNA-cellulose chromatography. In the presence of cyclic AMP they stimulated poly(A) RNA synthesis in isolated nuclei. The 51,000-dalton cyclic AMP-binding protein was the predominant one that bound to the nuclei. While cyclic AMP-dependent protein kinase activity [...]

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# Specific Nuclear Binding of Adenosine 3',5'-Monophosphate-binding Protein Complex with Subsequent Poly(A) RNA Synthesis in Embryonic Chick Cartilage

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**ABSTRACT** We used embryonic chick pelvic cartilage as a model to study the mechanism by which cyclic AMP increases RNA synthesis. Isolated nuclei were incubated with [<sup>32</sup>P]-8-azidoadenosine 3',5'-monophosphate ([<sup>32</sup>P]N<sub>3</sub>cAMP) with no resultant specific nuclear binding. However, in the presence of cytosol proteins, nuclear binding of [<sup>32</sup>P]N<sub>3</sub>cAMP was demonstrable that was specific, time dependent, and dependent on a heat-labile cytosol factor. The possible biological significance of the nuclear binding of the cyclic AMP-protein complex was identified by incubating isolated nuclei with either cyclic AMP or cytosol cyclic AMP-binding proteins prepared by batch elution DEAE-cellulose chromatography (DEAE peak cytosol protein), or both, in the presence of cold nucleotides and [<sup>3</sup>H]uridine 5'-triphosphate. Poly(A) RNA production occurred only in nuclei incubated with cyclic AMP and the DEAE peak cytosol protein preparation. Actinomycin D inhibited the incorporation of [<sup>3</sup>H]uridine 5'-monophosphate into poly(A) RNA. The newly synthesized poly(A) RNA had a sedimentation constant of 23S. Characterization of the cytosol cyclic AMP binding proteins using [<sup>32</sup>P]N<sub>3</sub>-cAMP with photoaffinity labeling identified three major cAMP-binding complexes (41,000, 51,000, and 55,000 daltons). The 51,000 and 55,000 dalton cyclic AMP binding proteins were further purified by DNA-cellulose chromatography. In the presence of cyclic AMP they stimulated poly(A) RNA synthesis in isolated nuclei. The 51,000-dalton cyclic AMP-binding protein was the predominant one that bound to the nuclei. While cyclic AMP-dependent protein kinase activity

was present in the cytosol and DEAE peak cytosol proteins, it was not present in the DNA-cellulose-bound, cyclic AMP-binding proteins. We conclude that one possible mechanism by which cyclic AMP increases RNA synthesis is by complexing to a 51,000-dalton cytosol cyclic AMP-binding protein and being subsequently translocated to the nucleus, where it is specifically bound and associated with induction of poly(A) RNA synthesis.

## INTRODUCTION

Gene transcription regulates the processes of growth and differentiation and is dependent on a variety of hormones and metabolic factors. Adenosine 3',5'-monophosphate (cyclic AMP) increases RNA synthesis in numerous tissues including regenerating rat liver (1), ovariectomized rat uterus (2), bovine and porcine thyroid (3, 4), beef adrenal medulla (5), and chick embryonic pelvic cartilage (6, 7).

Our laboratory has previously demonstrated that addition of N<sup>6</sup>-monobutyl cyclic AMP or theophylline to embryonic chick pelvic cartilage in organ culture increases the incorporation of radiolabeled precursors into the total RNA, total protein, and proteoglycan (7). The increased incorporation of radiolabeled uridine into total RNA is the result of a coordinated stimulation of messenger RNA (mRNA), ribosomal RNA (rRNA), and transfer RNA (tRNA) synthesis (7). These studies suggest that cyclic AMP affects gene transcription in this tissue.

The mechanism by which cyclic AMP affects RNA transcription in eukaryotic cells is unclear. It has been postulated that cyclic AMP may act similar to steroid hormones with binding to a cytoplasmic receptor protein, and then the resulting ligand receptor complex enters the nucleus where it binds to the genome. The

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cytoplasmic cyclic AMP receptor protein most studied is cyclic AMP-dependent protein kinase. Jungman et al. (8, 9) have demonstrated that nuclear protein kinase II of porcine ovary, which is cyclic AMP dependent, is identical to the cytosol cyclic AMP-dependent protein kinase and postulated the translocation of cytosol protein kinase to the nucleus. Costa et al. (10), using beef adrenal medulla, and Walsh et al. (11, 12), using rat liver cells, have reported the translocation of the catalytic subunit of cytosol cyclic AMP protein kinase to the nucleus. Little is known of the nature of the proposed cyclic AMP receptor-genome interactions. Since Kuo and Greengard (13) have postulated that all the biochemical and physiological effects of cyclic AMP are mediated through the phosphorylation of specific proteins by cyclic AMP-dependent protein kinase, several investigators have suggested that the increased transcriptional activity is mediated through phosphorylation of nuclear protein, either histone (14) or acidic protein (15, 16) which subsequently leads to opening or unmasking the DNA template (1, 16, 17) or to activating RNA polymerase II (18). However, the phosphorylation of nuclear proteins as the sole means to increase RNA transcription has been questioned. Kleinsmith (16) reported that cyclic AMP enhanced RNA transcription even though it decreased phosphorylation of RNA polymerase and Varrone et al. (19) demonstrated that cyclic AMP increases RNA synthesis in the absence of phosphorylation of nucleoproteins in a cell free system. In addition, many investigators have found that the net chromatin-bound histone kinase activity is cyclic AMP independent (9, 10, 20).

We have employed the embryonic chick cartilage model to investigate potential mechanisms by which cyclic AMP affects gene transcription. Our studies are compatible with the hypothesis that cyclic AMP binds to a specific cytosol receptor protein; that the protein-cyclic AMP complex translocates to the nucleus where it is specifically bound; and that this binding is associated with induction of mRNA synthesis. This specific cytosol receptor protein does not have cyclic AMP-dependent protein kinase activity.

## METHODS

*Isolation of nuclei and cytosol.* Pelvic leaflets from 12 dozen 10–11-d chick embryos were isolated and placed in a pH 7.5 buffer that contains 0.25 M sucrose, 50 mM Tris-HCl, 25 mM KCl, and 5 mM MgCl<sub>2</sub> (STKM).<sup>1</sup> All procedures were performed at 4°C unless otherwise noted. The cartilages were homogenized in 15 ml of STKM with a Polytron PT-10

homogenizer with a dial setting at 5 for 30 s. The homogenate was filtered through eight layers of cheesecloth and the filtrate was centrifuged at 2,000 g for 10 min. The pellet of crude nuclei was resuspended in 20 vol of pH 7.5 buffer containing 2.1 M sucrose, 50 mM Tris-HCl, 25 mM KCl, and 5 mM MgCl<sub>2</sub> and centrifuged at 10,000 g for 20 min. The pellet of purified nuclei was washed with 10 vol. STKM and centrifuged at 1,500 g for 10 min. The isolated nuclei were examined for purity with phase microscopy. Freshly prepared nuclei were used in all experiments. DNA was measured by the method of Burton (21) with calf thymus DNA type I as the standard.

The 2,000-g supernate of the homogenate was centrifuged at 100,000 g for 20 min. The supernate from this separation was the crude cytosol preparation employed. Freshly prepared cytosol was used in the binding experiments. Cytosol was stored at -20°C before use in purification of cyclic AMP-binding proteins. Protein was measured by the method of Lowry et al. (22) with bovine serum albumin as the standard.

*Nuclear binding of [<sup>32</sup>P]-8-azidoadenosine 3',5' cyclic monophosphate ([<sup>32</sup>P]N<sub>3</sub>cAMP).* Isolated nuclei (30 μg DNA) were incubated in 0.4 ml STKM at 22°C with 0.075 μM [<sup>32</sup>P]N<sub>3</sub>cAMP and one of the following: STKM, cytosol (400 μg protein), heat-treated cytosol (90°C for 10 min), and cytosol with 10-μM unlabeled cyclic AMP. In addition, nuclei were incubated with [<sup>32</sup>P]N<sub>3</sub>cAMP and 10 μM unlabeled cyclic AMP. At various times the tubes were centrifuged at 10,000 g and the resultant nuclear pellets were subsequently washed with 1.0 ml STKM and centrifuged again. The nuclear pellets were solubilized in 0.3 ml NCS (Amersham Corp., Arlington Heights, Ill.) and after addition of 5 ml of Spectrofluor (Amersham Corp.) scintillar, radioactivity was determined by liquid scintillation spectrophotometry.

*Nuclei incubation and preparation of phenol extracted RNA and poly(A) RNA.* All glassware was washed in 1.5 N KOH and rinsed in deionized water. Solutions were filtered through 0.45 μm HAWP Millipore filters (Millipore Corp., Bedford, Mass.). Latex gloves were worn during all procedures.

Freshly prepared nuclei (300 μg DNA) were incubated at 22°C for 1 h in a total vol of 0.5 ml STKM containing 0.75 mM ATP, 0.75 mM guanosine 5' triphosphate (GTP), 0.75 mM cytidine 5'-triphosphate (CTP), uridine 5'-triphosphate (UTP) (0.15 mM containing 50 μCi [<sup>3</sup>H]UTP), and one of the following: 10 μM cyclic AMP, DEAE peak cytosol protein (150 μg protein), or 10 μM cyclic AMP plus DEAE peak cytosol protein. The details of the preparation of the DEAE peak cytosol protein are described below. Viability of the incubated isolated nuclei in each of the above fractions was tested by determining the trichloroacetic acid-insoluble [<sup>3</sup>H]uridine counts at 10, 20, 30, and 40 min. In each of the incubations there was a linear increase in [<sup>3</sup>H]uridine incorporation into TCA-precipitable counts. There was no significant difference in total precipitable counts between the incubation mixtures.

The incubations were stopped by addition of 2 ml of 1:1 mixture of solution A (0.5% sodium-N-lauryl sarcosinate), 0.025 M Na<sub>2</sub> EDTA, and 0.025 M NaCl, pH 8.0) and redistilled phenol saturated with solution A (buffered saturated phenol, pH 8.0). The samples were placed in an ice bath for 30 min and then centrifuged at 10,000 g for 15 min. The aqueous phase was collected and incubated at 37°C with Proteinase K (30 μg/ml). Then an equal volume of solution B (buffered saturated phenol/chloroform/isoamyl alcohol 50:50:1, pH 8.0) was added. After centrifugation, the aqueous phase was collected and polyadenylate (final concentration 40 μg/ml) was added as a coprecipitant. This solution was made 0.2 M in NaCl and nucleic acids were precipitated by addition of 2 vol of 95% ethanol (-20°C). After standing overnight at

<sup>1</sup>Abbreviations used in this paper: buffer B, 20 mM Tris-HCl, 5m, EDTA, 5mM NaCl, and 10 μM mercaptoethanol, pH 7.5; dT, deoxythymidic acid; [<sup>32</sup>P]N<sub>3</sub>cAMP, [<sup>32</sup>P]-8-azidoadenosine cyclic monophosphate; SDS, sodium dodecyl sulfate; STKM, a pH 7.5 buffer that contains 0.25 M sucrose, 50 mM Tris-HCl, 25 mM KCl, and 5 mM MgCl<sub>2</sub>.

-20°C, the samples were centrifuged at 10,000 g for 15 min and the pellet was dissolved in 0.2 ml of high salt buffer (0.01 M Tris-HCl, pH 7.6; 1 mM Na<sub>2</sub> EDTA; and 0.5 M KCl). A modification of the method of Aviv and Leder (23) was used to isolate poly(A) RNA. Each sample was applied to an oligo deoxythymidic acid (dT)-cellulose column and eluted with the high salt buffer until the tritiated counts reached base line. The poly(A) RNA was eluted with a low salt buffer of 0.01 M Tris-HCl, 1 mM EDTA, pH 7.6. 1-ml fractions were collected and 100- $\mu$ l aliquots were counted in 5 ml of Aquasol (New England Nuclear, Boston, Mass.). Total mRNA was calculated using specific activity as 2.2 fmol uridine 5'-monophosphate (UMP) incorporated/cpm.

In addition, nuclear incubation experiments, as described above, were performed using DNA-cellulose-bound cytosol proteins prepared as described below. In some nuclear incubation experiments, actinomycin D (30  $\mu$ M) was added to tubes containing either DEAE peak cytosol protein or DNA-cellulose-bound protein and 10  $\mu$ M cyclic AMP.

**Sucrose density gradient analysis of poly(A) RNA.** The S value of the poly(A) RNA material obtained from oligo-dT-cellulose chromatography was determined by sucrose density gradient centrifugation. RNase-free sucrose was prepared in a buffer of 50 mM Tris-HCl, 20 mM MgCl<sub>2</sub>, 300 mM KCl, and 0.5 mM dithiothreitol pH 7.0 at 22°C. A 12-ml linear gradient of 3–15% sucrose was prepared and the peak poly(A) RNA material from the oligo-dT-cellulose column was added to the surface and centrifuged at 100,000 g for 12 h at 4°C. The gradient was unloaded in 0.5 ml fractions with a Buchler Densi-Flow IIc gradient collector (Buchler Instruments Inc., Fort Lee, N. J.). 15 ml of Aquasol was added to each fraction and radioactivity measured. Standards of 16S and 23S *Escherichia coli* ribosomal RNA were monitored at 260 nm on similarly prepared sucrose density gradient.

**Purification of cytosol proteins by DEAE-cellulose chromatography.** To obtain significant quantities of partially purified cyclic AMP binding proteins, cytosol prepared from 300 pelvic cartilage leaflets homogenized in 50 ml of STKM was dialyzed against a pH 7.0, 5 mM potassium phosphate buffer containing 2 mM EDTA at 4°C overnight. The cytosol was then applied to a DEAE-cellulose column (1.6  $\times$  15 cm) and eluted with the 5 mM phosphate buffer until the absorbance at 280 nm returned to base line; then a pH 7.0, 400 mM potassium phosphate buffer containing 2 mM EDTA was used to batch elute the proteins from the DEAE-cellulose. The proteins were eluted in 20 ml (protein concentration 1.35 mg/ml). This fraction was called the DEAE peak cytosol protein.

In addition, a similar quantity of cytosol was prepared, dialyzed, and chromatographed on DEAE-cellulose with a linear gradient elution of 300 ml of 5–400 mM potassium phosphate buffer (pH 7.0) containing 2 mM EDTA. 7-ml fractions were collected. Aliquots from each fraction were assayed for binding of <sup>3</sup>H-labeled cyclic AMP and [<sup>32</sup>P]N<sub>3</sub>cAMP in separate reaction tubes. Duplicate aliquots (300  $\mu$ l) from each fraction were incubated with 0.04  $\mu$ M <sup>3</sup>H-labeled cyclic AMP or 0.02  $\mu$ M [<sup>32</sup>P]N<sub>3</sub>cAMP at 4°C. At 90 min 1 ml of saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added. The pellet was collected by centrifugation at 10,000 g for 10 min and washed with cold 4:1 saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>/H<sub>2</sub>O several times, solubilized in 0.3 ml NCS, and counted in 5 ml of Spectrofluor scintillar.

**Photoaffinity labeling of [<sup>32</sup>P]N<sub>3</sub>cAMP to cytosol and DEAE peak cytosol protein.** Cytosol cyclic AMP binding proteins were bound to [<sup>32</sup>P]N<sub>3</sub>cAMP in a 1.0 ml reaction mixture of STKM pH 7.4 containing 2.5 mg of cytosol protein and 0.5  $\mu$ M [<sup>32</sup>P]N<sub>3</sub>cAMP. The reaction was incubated at

4°C for 90 min in the dark and then irradiated with ultraviolet light (Mineralight model R51 Ultraviolet Products, San Gabriel, Calif.) at 254 nm for 15 min at 4°C. This photoaffinity labeling produces a covalent bond between [<sup>32</sup>P]N<sub>3</sub>cAMP and its binding proteins (24, 25). Similarly DEAE peak cytosol protein (1.2 mg in 500  $\mu$ l) was incubated with 0.5  $\mu$ M [<sup>32</sup>P]N<sub>3</sub>cAMP at 4°C for 90 min, then photoaffinity labeled with ultraviolet light.

**Binding of [<sup>32</sup>P]N<sub>3</sub>cAMP-DEAE peak cytosol protein complex to DNA-cellulose.** DNA-cellulose was prepared by method of Litman (26) using calf thymus DNA. The DNA-cellulose was equilibrated with buffer B (20 mM Tris-HCl, 5 mM EDTA, 5 mM NaCl, and 10  $\mu$ M mercaptoethanol, pH 7.5). The photoaffinity-labeled DEAE peak cytosol protein was separated from unbound [<sup>32</sup>P]N<sub>3</sub>cAMP by Sephadex G-25 chromatography. The void volume (2 ml) of a Sephadex G-25 column (0.9  $\times$  15 cm) previously equilibrated with buffer B was applied to the DNA-cellulose column (0.9  $\times$  5 cm). This column was washed with buffer B until counts had stabilized. Then the [<sup>32</sup>P]N<sub>3</sub>cAMP-protein complex was batch eluted from the DNA-cellulose with buffer B containing 300 mM NaCl. 1-ml fractions were collected and 10- $\mu$ l aliquots were counted in 5 ml Aquasol.

When free [<sup>32</sup>P]N<sub>3</sub>cAMP was applied to the DNA-cellulose column no counts could be eluted with high salt (300 mM NaCl) buffer B. The peak from the DNA-cellulose high salt elution containing [<sup>32</sup>P]N<sub>3</sub>cAMP-binding protein complex was used in nuclear incubation experiments to determine if it stimulated RNA transcription.

**Polyacrylamide gel electrophoresis and autoradiography of cyclic AMP-binding proteins.** Characterization of molecular weights of cyclic AMP-binding proteins were performed using [<sup>32</sup>P]N<sub>3</sub>cAMP as the binding ligand followed by photoaffinity labeling with ultraviolet light under conditions listed above. Sources for the binding proteins were cytosol and DEAE peak cytosol as well as the cyclic AMP-binding proteins having affinity for DNA-cellulose. The labeled proteins were layered on 0.1% sodium dodecyl sulfate (SDS)-10% polyacrylamide slab gels prepared by the method of Ames (27) using the Bio-Rad model 221 vertical slab gel system (Bio-Rad Laboratories, Richmond, Calif.). The samples of protein in 50–100- $\mu$ l aliquots were dissolved in 10  $\mu$ l 10% SDS and 10  $\mu$ l of 0.1 M dithiothreitol and heated at 90°C for 10 min. These solutions were cooled to room temperature and then 20  $\mu$ l of 50% sucrose and 0.02% bromophenol blue added. The slab gel (0.8 mm  $\times$  15 cm  $\times$  27 cm) was electrophoresed at 20 mA for 10–12 h. The gel was stained with 0.1% Coomassie Brilliant Blue in methanol/acetic acid/H<sub>2</sub>O 4:1:5 for 2 h then destained in 40% methanol/10% acetic acid for 8–12 h at room temperature and the gel dried on Bio-Rad model 224 slab dryer. Autoradiography was done with Kodak X-O-Mat G film (Eastman Kodak Co., Rochester, N. Y.) for 3–24 h. Bio-Rad SDS-polyacrylamide gel electrophoresis low molecular weight standards using concentration of 10  $\mu$ g for each protein were used as standards.

**Polyacrylamide gel electrophoresis and autoradiography of the nuclear bound cyclic AMP-binding protein complex.** To identify the cyclic AMP-binding proteins which bound to the nuclei, the following experiment was performed. [<sup>32</sup>P]-N<sub>3</sub>cAMP (1  $\mu$ M) was incubated with each of the following: 0.5 ml STKM, cytosol (1.2 mg) protein, and DEAE (1.2 mg) peak cytosol protein for 90 min at 4°C in the dark. Each mixture was photoaffinity labeled with ultraviolet light. Nuclei (300  $\mu$ g DNA) were added to each sample, incubated at 22°C for 60 min, and then centrifuged at 10,000 g for 10 min at 4°C. The nuclear pellet was vortexed with 2 ml ice cold STKM, and centrifuged again. The nuclear pellet was solubilized with 10  $\mu$ l 10% SDS and 10  $\mu$ l of 0.1 mM

dithiothreitol and heated at 90°C for 10 min. Polyacrylamide gel electrophoresis and autoradiography were performed as described above.

**Protein kinase assay.** Protein kinase activity was determined using the method of Desjardins et al. (28) and Wastila et al. (29). High specific activity  $\gamma^{32}\text{P}$ -labeled ATP was prepared by method of Glenn and Chappell (30). The following fractions were assayed: cytosol, peak DEAE cytosol protein, the DEAE peak protein that did not bind to DNA-cellulose, and the fraction that bound to DNA-cellulose. Radiolabeled cyclic AMP was not used as a marker in isolating any of these fractions. The standard reaction mixture of 150  $\mu\text{l}$  contained 10 mM potassium phosphate pH 7.0, 13 mM magnesium acetate, 0.3 mM EDTA, 1 mM dithiothreitol, 0.1% Triton X-100, 250  $\mu\text{g}$  histone II a, [ $\gamma^{32}\text{P}$ ]ATP (0.3 mM, 100 cpm/pmol), with and without 1  $\mu\text{M}$  cyclic AMP, and 12–50  $\mu\text{g}$  protein fraction. The reaction was initiated by adding the [ $^{32}\text{P}$ ]ATP and was linear at 30°C for 10 min (data not shown). In the standard assay the incubated time was 5 min. The reaction was terminated by spotting duplicate 50- $\mu\text{l}$  aliquots on filter paper squares (31 ET, Whatman Inc., Clifton, N. J.) which were immediately submerged in 10% (wt/vol) trichloroacetic acid, then washed, dried, and counted in 5 ml of Aquasol.

**Materials.** [ $^{32}\text{P}$ ]N<sub>3</sub>cAMP (42 Ci/mol sp act) was obtained from ICN Nutritional Biochemicals (Cleveland, Ohio); and  $^3\text{H}$ -labeled cyclic AMP (25 Ci/mol sp act) and [5,6- $^3\text{H}$ ]UTP (37 Ci/mmol sp act) were obtained from New England Nuclear. DEAE-cellulose (DE32) and cellulose (CFII) were supplied from Whatman Inc., oligo-dT-cellulose (T-3) from Collaborative Research Inc., (Waltham, Mass.), and Sephadex G-25 from Pharmacia Fine Chemicals Inc. (Piscataway, N. J.). Low molecular weight polyacrylamide gel electrophoresis standards, electrophoresis chemicals, and analytical equipment were purchased from Bio-Rad Laboratories. Sodium-N-lauryl sarcosinate was obtained from ICN K & K Laboratories, Inc., (Plainview, N. Y.). Proteinase K (EM Biochemicals) was purchased from Beckman Instruments, Inc. (Fullerton, Calif.). *E. coli* 16S and 23S, were obtained from Miles Laboratories, Inc. (Elkhart, Ind.). Nucleotides (ATP, CTP, GTP, UTP), and cyclic AMP as well as calf thymus DNA type I were purchased from Sigma Chemical Co., (St. Louis, Mo.). Polyadenylate (lot BY1960) was obtained from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). All other chemicals were of reagent grade.

## RESULTS

**Nuclear binding experiments.** When isolated embryonic chick cartilage nuclei are incubated with [ $^{32}\text{P}$ ]N<sub>3</sub>cAMP in the presence of cytosol, there is a time-dependent increase in specific binding of radioactivity (Fig. 1). Addition of a large excess of cyclic AMP (200-fold molar excess) with or without the cytosol decreases the nuclear binding of [ $^{32}\text{P}$ ]N<sub>3</sub>cAMP and defines nonspecific binding. In the absence of cytosol, [ $^{32}\text{P}$ ]N<sub>3</sub>cAMP does not show specific binding to the nuclei. Heat-treated cytosol does not promote specific binding. Maximal specific nuclear binding at 22°C occurs at 1 h. These experiments indicate that significant specific nuclear binding of cyclic AMP occurs only when cytosol is present. Most likely, this represents the complex of cyclic AMP-binding proteins binding to the nucleus.

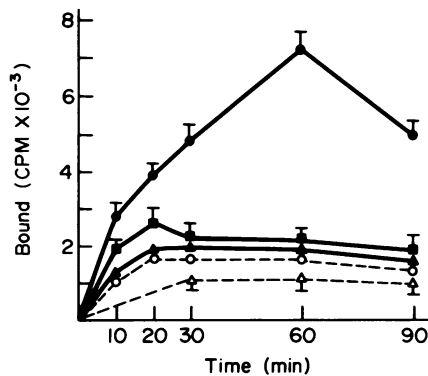
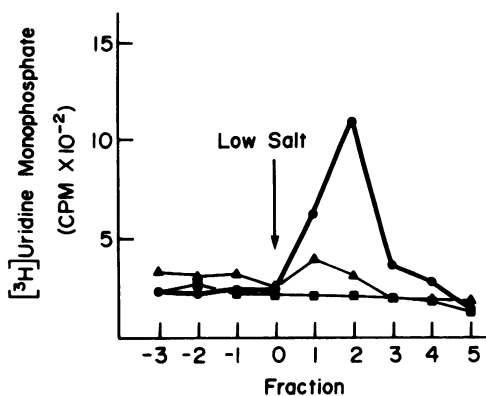


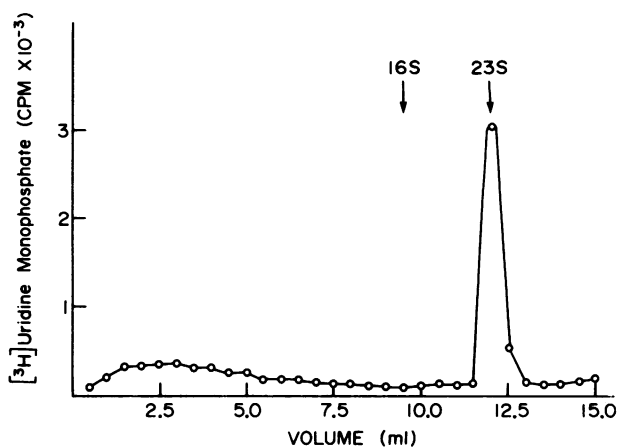
FIGURE 1 Time-course experiment demonstrates cytosol requirements for specific binding of [ $^{32}\text{P}$ ]N<sub>3</sub>cAMP by isolated chick cartilage nuclei. [ $^{32}\text{P}$ ]N<sub>3</sub>cAMP (final concentration, 0.075  $\mu\text{M}$ ) and isolated nuclei (30  $\mu\text{g}$  DNA) were added to 0.4 ml STKM (■) or 400  $\mu\text{g}$  of cytosol protein (●) prepared in STKM, or heat-treated (90°C for 10 min) cytosol (▲). In addition, 10  $\mu\text{M}$  cyclic AMP was added to nuclei alone (△) and to nuclei plus cytosol (○) to determine specific binding. Incubation was at 22°C for the indicated times. The nuclei were sedimented, washed twice with STKM, dissolved in 0.3 ml NCS and counted in 5 ml Spectrofluor as described in Methods. Each point represents the mean of three determinations. Similar results have been reproduced in three time-course experiments.

**Incubation of nuclei and isolation of poly(A) RNA.** To determine if the specific nuclear binding of the cyclic AMP-cytosol binding protein complexes has biological significance, we incubated isolated nuclei in the presence of cyclic AMP, cyclic AMP and DEAE peak cytosol proteins, or DEAE peak cytosol proteins alone and measured poly(A) RNA production. A representative of three such experiments is depicted in Fig. 2. Incorporation of [ $^3\text{H}$ ]UMP into poly(A) RNA is found only when nuclei are incubated with cyclic AMP and DEAE peak cytosol proteins. No significant production of poly(A) RNA occurs when nuclei are incubated with cyclic AMP alone or its binding protein alone. Addition of actinomycin D (30  $\mu\text{M}$ ) to nuclei incubated with cyclic AMP and DEAE peak cytosol proteins abolishes the increased incorporation of [ $^3\text{H}$ ]UMP into poly(A) RNA suggesting that the increase in incorporation represents synthesis. The amounts of UMP incorporated into poly(A) RNA under these various conditions are given in Table I. These experiments show that specific nuclear binding of cyclic AMP-binding protein complexes is associated with synthesis of poly(A) RNA.

**Sucrose density gradient centrifugation of poly(A) RNA.** The poly(A) RNA containing [ $^3\text{H}$ ]UMP that was synthesized during incubation of isolated nuclei with cyclic AMP and the DEAE peak proteins was placed on 3–15% sucrose density gradient and centrifuged at 100,000 g for 12 h at 4°C. The results are shown in Fig. 3. A large single peak of radioactivity that



**FIGURE 2** Oligo-dT-cellulose chromatography of extracted nucleic acids from incubated cartilage nuclei. Isolated nuclei (300  $\mu\text{g}$  DNA) were incubated for 60 min at 22°C in a mixture of 0.5 ml STKM containing 0.75 mM ATP, 0.75 mM CTP, 0.75 mM GTP, and 0.15 mM UTP with 50  $\mu\text{Ci}$  [ $^3\text{H}$ ]UTP and one of the following: 10  $\mu\text{M}$  cAMP ( $\blacktriangle$ ), 150  $\mu\text{g}$  DEAE peak cytosol protein ( $\blacksquare$ ), or 10  $\mu\text{M}$  cyclic AMP and DEAE peak cytosol protein ( $\bullet$ ). The reaction was stopped by addition of phenol-SDS solution and extraction of the nucleic acid performed as described in Methods. The extracted nucleic acid from each sample was placed on an oligo-dT cellulose column in high salt buffer (0.5 M KCl, 0.01 M Tris-HCl, 1 mM  $\text{Na}_2\text{EDTA}$ , pH 7.6) and washed with high salt buffer until stable base-line counts were obtained. Low salt buffer (0.01 M Tris-HCl, 1 mM  $\text{Na}_2\text{EDTA}$ , pH 7.6) eluted the poly(A) RNA. 1-ml fractions were collected and 100- $\mu\text{l}$  aliquots were counted in 5 ml Aquasol.



**FIGURE 3** Sucrose density gradient centrifugation of newly synthesized poly(A) RNA. A 3–15% linear sucrose gradient was prepared in a buffer 50 mM Tris-HCl, 20 mM  $\text{MgCl}_2$ , 300 mM KCl, 0.5 mM dithiothreitol, pH 7.0 at 22°C. The sample containing [ $^3\text{H}$ ]uridine monophosphate incorporated into poly(A) RNA from the oligo-dT-cellulose column was applied to the gradient and centrifuged at 100,000 g for 12 h at 4°C. The gradient was unloaded as described in Methods and was collected in 0.5 ml fractions and counted in 15 ml Aquasol. *E. coli* ribosomal 16S and 23S RNA standards were run on a similar gradient and absorbance was monitored at 260 nm.

migrates with a sedimentation constant of 23S is present.

**Purification of cyclic AMP-binding proteins using [ $^{32}\text{P}$ ]N $_3$ cAMP and  $^3\text{H}$ -labeled cyclic AMP.** The covalent binding of the cyclic AMP radioligand analog [ $^{32}\text{P}$ ]N $_3$ cAMP provides an effective marker for the purification of cyclic AMP binding proteins. 8-N $_3$ cAMP has been shown to have nearly identical binding and activation properties as cyclic AMP (24, 25). We first determined that [ $^{32}\text{P}$ ]N $_3$ cAMP was a useful marker

**TABLE I**  
[ $^3\text{H}$ ]UMP Incorporation into Poly(A) RNA in Three Incubation Experiments with Isolated Nuclei

	Experiment 1	Experiment 2	Experiment 3
Cyclic AMP (10 $\mu\text{M}$ )	8.5	0	0
DEAE peak protein (150 $\mu\text{g}$ )	0	0	0
Cyclic AMP (10 $\mu\text{M}$ ) + DEAE peak protein	509	271	382

The total [ $^3\text{H}$ ]uridine was calculated by addition of the cpm above base line for each fraction during low salt elution (Fig. 2) and correcting for the counting aliquot. Total UMP incorporated into poly(A) RNA was calculated using 2.1 fmol of UMP/cpm. Data are expressed as fmol/ $\mu\text{g}$  DNA.

to characterize cyclic AMP binding proteins in the embryonic chick pelvic cartilage. Cytosol was applied to DEAE-cellulose and a linear salt gradient was used to elute the proteins. Binding of  $^3\text{H}$ -labeled cyclic AMP and [ $^{32}\text{P}$ ]N $_3$ cAMP is depicted in Fig. 4. An identical elution profile is obtained with both radioligands. Three peaks of binding activity are noted corresponding to conductivities of 2.5, 4.5, and 7.5 mmho. These experiments strongly suggest that [ $^{32}\text{P}$ ]N $_3$ cAMP and  $^3\text{H}$ -labeled cyclic AMP bind to identical cyclic AMP binding proteins in the embryonic chick pelvic cartilage and validate [ $^{32}\text{P}$ ]N $_3$ cAMP as a useful probe in cyclic AMP binding to cytosol proteins in this tissue.

**Binding of [ $^{32}\text{P}$ ]N $_3$ cAMP DEAE peak protein complex to DNA-cellulose.** After photoaffinity labeling DEAE peak cytosol proteins with [ $^{32}\text{P}$ ]N $_3$ cAMP and chromatographing the reaction mixture on a Sephadex G-25 column, we chromatographed the [ $^{32}\text{P}$ ]N $_3$ cAMP DEAE peak protein complex (void volume) on DNA-cellulose (Fig. 5). The quantity of [ $^{32}\text{P}$ ]N $_3$ cAMP protein complex that binds to DNA-cellulose varies between 10 and 15% depending on the DEAE protein preparation.

The high salt eluate (100  $\mu\text{g}$  protein) and cyclic AMP were incubated with isolated nuclei as described in Methods to determine if poly(A) RNA synthesis would occur. Under these conditions,  $825 \pm 30$  fmol of UMP/ $\mu\text{g}$  DNA were incorporated into poly(A) RNA. Actinomycin D completely inhibited the incorporation of

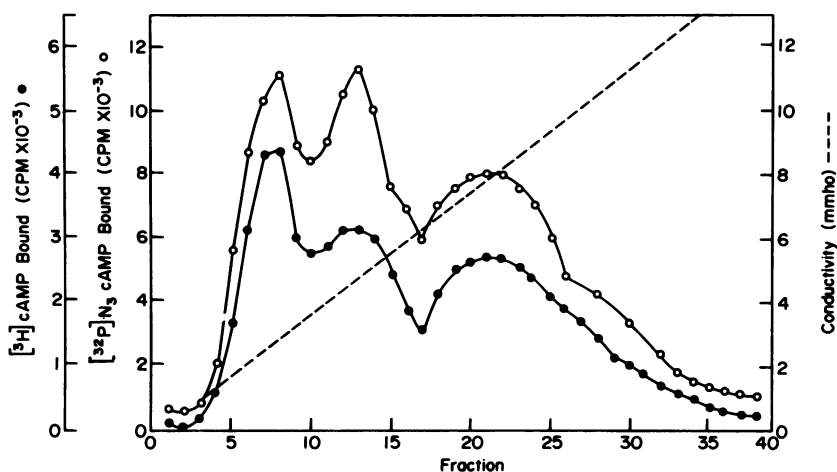


FIGURE 4 DEAE chromatography of cyclic AMP binding proteins in cytosol. Cytosol (75 mg) previously dialyzed in a pH 7.0 buffer containing 5 mM potassium phosphate and 2 mM EDTA was applied to DEAE column and a linear gradient of 300 ml of 5–400 mM potassium phosphate containing 2 mM EDTA used to elute 7-ml fractions. Each fraction was assayed for binding of [<sup>3</sup>H]cAMP 0.04 μM (○) and [<sup>32</sup>P]N<sub>3</sub>cAMP 0.02 μM (●) as described in Methods. The binding profiles are identical. Three major peaks of binding activity are noted at 2.5, 4.5, and 7.5 mmho conductivity (—).

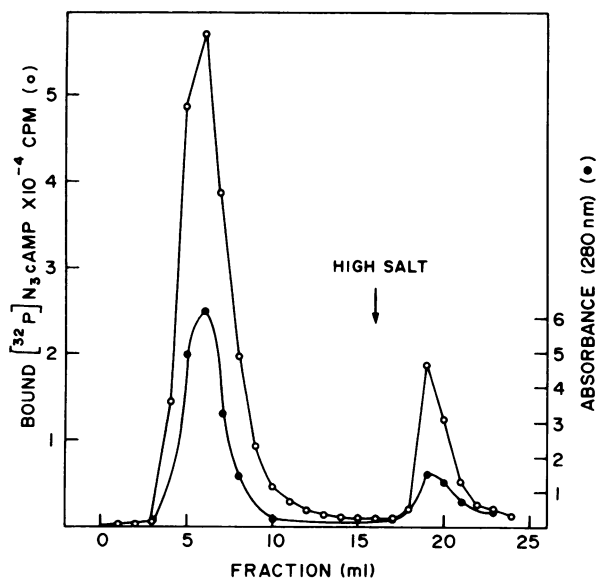


FIGURE 5 DNA-cellulose chromatography of photoaffinity-bound [<sup>32</sup>P]N<sub>3</sub>cAMP to DEAE peak cytosol protein. DEAE peak cytosol protein (1.2 mg protein in 500 μl) was incubated with 0.5 μM [<sup>32</sup>P]N<sub>3</sub>cAMP at 4°C for 90 min in the dark then photolabeled with UV light for 15 min. The mixture was placed on Sephadex G-25 column, equilibrated with buffer B and the void volume of 2 ml was taken and applied to a DNA-cellulose column (0.9 × 5.0 cm). The DNA-cellulose was prepared by method of Litman (26) and previously washed extensively with buffer B. The column was first eluted with buffer B until the counts of [<sup>32</sup>P]N<sub>3</sub>cAMP (○) and A at 280 nm (●) had returned to base line. High salt buffer B (300 mM NaCl) was used to elute [<sup>32</sup>P]N<sub>3</sub>cAMP protein complexes from the DNA-cellulose. 100-μl aliquots of 1-ml fractions were counted in 5 ml Aquasol.

[<sup>3</sup>H]UMP into poly(A) RNA stimulated by the high salt eluate plus cyclic AMP. The low salt eluate plus cyclic AMP produced no [<sup>3</sup>H]UMP incorporation when incubated with isolated nuclei.

Thus, the cyclic AMP-binding protein that has affinity for calf thymus DNA has the ability, when combined with cyclic AMP, to induce poly(A) RNA synthesis in isolated cartilage nuclei.

*Polyacrylamide gel electrophoresis and autoradiography of cyclic AMP-binding proteins.* Three [<sup>32</sup>P]N<sub>3</sub>cAMP photoaffinity labeled protein fractions (cytosol proteins, DEAE peak cytosol proteins, and DNA-cellulose bound proteins) were electrophoresed on 10% polyacrylamide gels. The autoradiograph of the polyacrylamide gel containing [<sup>32</sup>P]N<sub>3</sub>cAMP labeled cytosol proteins is represented in Fig. 6. Three major bands of radioactivity are present at 41,000, 51,000, and 55,000 daltons. The three major bands are invariably present on any cytosol or DEAE peak cytosol preparation. The intensity of binding of [<sup>32</sup>P]N<sub>3</sub>cAMP in each band varies from preparation to preparation with the 41,000 dalton having the most variable intensity. The 41,000 dalton protein probably represents a degradative product of either the 51,000 or 55,000 dalton proteins.

The autoradiograph of the polyacrylamide gel containing [<sup>32</sup>P]N<sub>3</sub>cAMP-DEAE peak cytosol protein complex which binds to DNA-cellulose is represented in Fig. 7. Two major bands of radioactivity corresponding to 51,000 and 55,000 daltons are present in the high salt eluate from DNA-cellulose (Fig. 7B). Since fewer bound [<sup>32</sup>P]N<sub>3</sub>cAMP counts were applied, longer

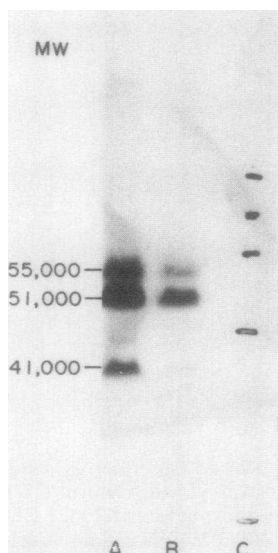


FIGURE 6 Autoradiograph of polyacrylamide gel containing photoaffinity labeled cyclic AMP binding proteins from cytosol. Cytosol (100  $\mu\text{g}$ ) was incubated with 0.1  $\mu\text{M}$  [ $^{32}\text{P}$ ]- $\text{N}_3\text{cAMP}$  for 90 min at 4°C in the dark followed by photoaffinity labeling and electrophoresed on 10% polyacrylamide slab gel as described in Methods. (A) cytosol proteins (B) cytosol proteins incubated with 10  $\mu\text{M}$  unlabeled cyclic AMP (C) molecular weight standards taken from Coomassie Blue stain marked in black pencil. Three major binding proteins are seen at 41,000, 51,000, and 55,000 dalton.

exposure of the X ray film was necessary to show proteins that adhered to DNA-cellulose. By comparison [ $^{32}\text{P}$ ] $\text{N}_3\text{cAMP}$  bound to DEAE-peak cytosol protein is represented in Fig. 7C. Thus, two major cyclic AMP binding proteins in the embryonic chick pelvic cartilage cytosol have affinity for DNA-cellulose.

**Autoradiography of nuclear bound cyclic AMP-binding protein complex on polyacrylamide gel electrophoresis.** To demonstrate which cyclic AMP-binding protein complex specifically binds to the nucleus, cytosol proteins and DEAE peak cytosol proteins were photoaffinity labeled and incubated with isolated nuclei. The nuclei were pelleted, washed, and applied to a 10% SDS-polyacrylamide gel and electrophoresed. Fig. 8 is the graph from the densitometer readings of the autoradiogram of nuclear bound [ $^{32}\text{P}$ ] $\text{N}_3\text{cAMP}$ -protein complex. In Fig. 8A no binding of [ $^{32}\text{P}$ ]- $\text{N}_3\text{cAMP}$  to the nucleus in absence of cytosol can be demonstrated. In Fig. 8B cytosol contains a single major protein with cyclic AMP binding activity that specifically binds to the nucleus. This protein corresponds to the 51,000-dalton cyclic AMP binding protein. The DEAE peak cytosol proteins contain the same 51,000-dalton, nuclear specific cyclic AMP-binding protein as the cytosol (Fig. 8C). When cyclic AMP 10  $\mu\text{M}$  was incubated with 1  $\mu\text{M}$  [ $^{32}\text{P}$ ] $\text{N}_3\text{cAMP}$ , there

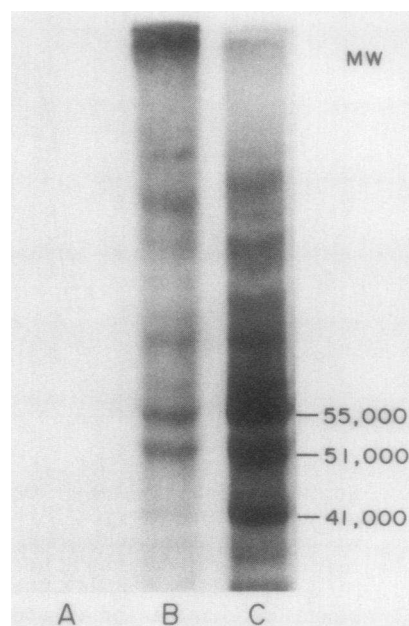


FIGURE 7 Autoradiograph of polyacrylamide gel containing DNA-cellulose bound [ $^{32}\text{P}$ ] $\text{N}_3\text{cAMP}$ -protein complex(es). DEAE peak cytosol protein was photoaffinity labeled with [ $^{32}\text{P}$ ] $\text{N}_3\text{cAMP}$  and applied to a DNA-cellulose column as described in Fig. 5. The DNA column was washed with buffer B then the bound [ $^{32}\text{P}$ ] $\text{N}_3\text{cAMP}$ -protein complex(es) eluted with high salt buffer B containing 300 mM NaCl. The fractions containing high salt eluate were dialyzed overnight against water and lyophilized. 3  $\mu\text{g}$  of bound complex was prepared and electrophoresed on 10% SDS-polyacrylamide gel as described in Methods. (A) Molecular weight standards which were not radiolabeled, (B) DNA-bound [ $^{32}\text{P}$ ] $\text{N}_3\text{cAMP}$ -binding protein complexes, and (C) photoaffinity labeled DEAE cytosol protein (25  $\mu\text{g}$ ) are shown. There are two major bands of 51,000 and 55,000 cyclic AMP-binding proteins present in the high salt eluate from DNA-cellulose. The film was overexposed to the gel since fewer counts per min were placed in B than C. With overexposure many bands of radioactivity are seen in DEAE peak cytosol protein but the major bands are at 41,000, 51,000, and 55,000 dalton.

was good displacement of the radiolabeled protein at a cyclic AMP concentration 10-fold greater than the tracer (Fig. 8D). [ $^{32}\text{P}$ ] $\text{N}_3\text{cAMP}$  bound to DEAE peak cytosol proteins served as the control for this gel (Fig. 8E). Thus, specific nuclear binding of a 51,000-dalton cyclic AMP-cytosol protein complex occurs in the embryonic chick cartilage.

**Protein kinase activity.** Cyclic AMP-dependent protein kinase activity was demonstrated in the cytosol, DEAE peak fraction, and the fraction of DEAE peak proteins that did not bind to DNA-cellulose (Table II). The protein fraction that bound to DNA-cellulose did not have cyclic AMP-dependent protein kinase activity. High concentrations of NaCl (300 mM) are required to elute the protein fraction from DNA-cellulose and could potentially interfere with the meas-



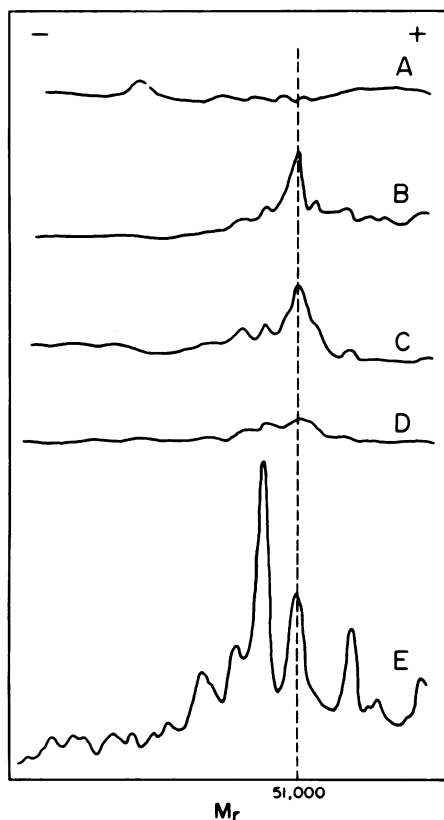


FIGURE 8 Densitometer readings of autoradiograph of polyacrylamide gel containing nuclear bound cyclic AMP-binding protein complex(es). [ $^{32}\text{P}$ ]N $_3$ cAMP (1  $\mu\text{M}$ ) was incubated at 4°C for 90 min in one of the following: 0.5 ml STKM (A); 1.2 mg cytosol protein (B); 1.2 mg DEAE peak cytosol protein (C); cytosol proteins plus 10  $\mu\text{M}$  unlabeled cyclic AMP (D); and then photoaffinity labeled as described in Methods. Nuclei (300  $\mu\text{g}$  DNA) from embryonic chick pelvic cartilage were added to each sample, incubated at 22°C for 60 min, centrifuged 10,000  $g$  for 10 min, washed, and centrifuged twice, solubilized in 10  $\mu\text{l}$  10% SDS and 10  $\mu\text{l}$  0.1 M dithiothreitol and heated at 90°C for 10 min. The material was applied to 10% polyacrylamide gel and electrophoresed followed by autoradiography with a prolonged exposure. DEAE peak cytosol protein bound to [ $^{32}\text{P}$ ]N $_3$ cAMP served as the standard for this gel (E). There is no bound [ $^{32}\text{P}$ ]N $_3$ cAMP in the absence of cytosol or DEAE peak protein. With addition of 10-fold excess of unlabeled cyclic AMP (D), there is good displacement of nuclear bound [ $^{32}\text{P}$ ]N $_3$ cAMP. The 51,000 dalton cyclic AMP-binding protein is the only one that appreciably binds to the nucleus.

urement of cyclic AMP-dependent protein kinase activity (31). Consequently, this fraction was dialyzed against 5 mm potassium phosphate buffer and re-assayed. Again, no cyclic AMP-dependent protein kinase activity was detected.

## DISCUSSION

We studied binding of cyclic AMP, using the radioligand [ $^{32}\text{P}$ ]N $_3$ cAMP, to isolated nuclei from embry-

TABLE II  
Protein Kinase Activity in Protein Fractions\*

	Control	Cyclic AMP (1 $\mu\text{M}$ )	% Increase of [ $^{32}\text{P}$ ] incorporation by cyclic AMP
Cytosol proteins	173	273	57
DEAE peak cytosol proteins	411	556	35
DNA-cellulose low salt elute proteins	413	539	30
DNA-cellulose bound proteins (high salt eluate)	346	344	0

\* Mean of three determinations expressed as counts per minute [ $^{32}\text{P}$ ] incorporated/5 min per  $\mu\text{g}$  protein.

onic chick pelvic cartilage. Specific binding of [ $^{32}\text{P}$ ]N $_3$ cAMP to nuclei occurred only in the presence of cartilage cytosol. The evidence that this binding is probably the result of cyclic AMP forming a complex with a cytosol cyclic AMP-binding protein with subsequent translocation of the complex to the nucleus where it is specifically bound is as follows: (a) specific nuclear binding occurred only when cytosol proteins were present; (b) heat-treated cytosol did not promote specific binding; (c) excess unlabeled cyclic AMP added to the system inhibited the radioligand binding; and (d) only one major cytosol cyclic AMP-binding protein (51,000 dalton) could be identified in the isolated nuclei after incubation with the radioligand and cytosol. Similar binding of cyclic AMP protein complex(es) to the nuclear genome in other tissues has been reported (32, 33).

The possible biological significance of the cyclic AMP protein complex binding to the nucleus was tested by carrying out a series of nuclear incubation experiments. As shown in Fig. 2 and Table I, isolated nuclei incorporated little or no [ $^3\text{H}$ ]UMP into poly(A) RNA in the absence of cytosol cyclic AMP-binding proteins prepared by batch elution from DEAE-cellulose. The addition of the cytosol cyclic AMP-binding proteins and cyclic AMP resulted in a significant incorporation of [ $^3\text{H}$ ]UMP into poly(A) RNA, suggesting that poly(A) RNA synthesis is stimulated. Several concerns arise in relation to the validity of the *in vitro* nuclear incubation studies. The absence of significant basal poly(A) RNA synthesis raises the possibility that the nuclei are dead and that the incorporation of [ $^3\text{H}$ ]UMP is the result of some artefact. In a series of experiments (data not shown) we have shown that [ $^3\text{H}$ ]uridine incorporation into acid precipitable material increases linearly during the 1-h nuclear incubation. While little specific information could be obtained from the analysis of the acid precipitable material, the linear increase of the

radiolabeled uridine with time suggests that the nuclei are viable. Addition of actinomycin D to the nuclear incubations completely blocked the cyclic AMP-cyclic AMP-binding protein complex stimulation of [<sup>3</sup>H]-UMP incorporation into the poly(A) RNA suggesting that the incorporation was the result of synthesis. Though unlikely the present data do not exclude the possibility that the cyclic AMP-binding protein complex might affect chain elongation or polyadenylation rather than initiate new poly(A) RNA synthesis. We have no explanation for the lack of basal poly(A) RNA synthesis by the isolated cartilage nuclei.

Relatively little information is available about transcription systems involving other isolated nuclei. Marzluff et al. (34) have shown that nuclei isolated from mouse myeloma cells are capable of synthesizing RNA at 25°C for 1 h. The phenol-extracted products were heterogenous ranging in size from 4 to 45S. About 15% of the larger RNA (>22S) were polyadenylated as assessed by affinity chromatography on oligo-dT-cellulose. Wu and Zubay (35) studied transcription using isolated nuclei from Krebs II ascites tumor cells and found that cytoplasmic factors were necessary for net RNA synthesis (assessed as acid precipitable radioactivity). Much variability exists in the characteristics of RNA products in different nuclear incubation systems.

The poly(A) RNA synthesized in our nuclear incubation studies was characterized by a nondenaturing sucrose gradient centrifugation and was found to consist of material with a sedimentation constant of 23S. The singular nature of the peak (Fig. 3) is predictable on the basis of previous studies from our laboratory (7). When embryonic chick pelvic leaflets are incubated in an *in vitro* organ culture with N<sup>6</sup>-monobutyl cyclic AMP and [<sup>3</sup>H]uridine, the radiolabeled mRNA that is isolated is predominately a single peak (>90%). On Sepharose 4B chromatography this single peak has an apparent sedimentation constant close to 23S. Unlabeled mRNA isolated from cartilage has a much greater proportion of lesser molecular weight mRNA suggesting that newly synthesized mRNA is predominately 23S and that much of it is later processed to smaller mRNA. The newly synthesized poly(A) RNA that we analyzed by sucrose gradient centrifugation might include both heteronuclear RNA (36) and mRNA, which have poly(A) regions of >20 adenosine residues. The phenol-extracted material that did not adhere to oligo-dT-cellulose is small (4–9S) and very heterogenous (data not shown), and may well represent incomplete synthetic products or degradative fragments. Identification of our poly(A) RNA as true mRNA requires demonstration of translation in a cell-free system. The amounts of materials available at the present time preclude this characterization.

Since the binding of [<sup>32</sup>P]N<sub>3</sub>cAMP to the isolated

nuclei was dependent on the presence of cytosol proteins, we attempted to partially characterize the cyclic AMP-binding proteins in the cytosol and in the incubated nuclei using the photoaffinity ligand [<sup>32</sup>P]N<sub>3</sub>cAMP. We determined that this binding ligand had identical binding properties to <sup>3</sup>H-labeled cyclic AMP in this tissue. DEAE chromatography of the cytosol followed by binding of [<sup>32</sup>P]N<sub>3</sub>cAMP or <sup>3</sup>H-labeled cyclic AMP shows identical elution profiles (Fig. 4). Cytosol was then photoaffinity labeled with [<sup>32</sup>P]N<sub>3</sub>cAMP and polyacrylamide gel electrophoresis performed. Three major peaks of binding activity on autoradiography were identified at 41,000, 51,000, and 55,000 dalton (Fig. 6). Batch elution of cytosol proteins from DEAE-cellulose (DEAE peak cytosol protein) followed by photoaffinity labeling gave the same pattern on autoradiography.

Since cyclic AMP and its binding protein(s) from a variety of sources (37, 38) have been reported to bind to DNA or DNA-cellulose, we determined whether the cyclic AMP-binding protein complex from the cytosol of the embryonic chick pelvic cartilage would bind to DNA. Between 10 and 15% of the [<sup>32</sup>P]N<sub>3</sub>cAMP-binding protein complex in the cytosol binds to calf thymus DNA (Fig. 5). This cyclic AMP-protein fraction that adheres to DNA-cellulose also induces poly(A) RNA synthesis in isolated chick nuclei. On polyacrylamide gel electrophoresis this same fraction has two major bands of radioactivity, 51,000 and 55,000 dalton (Fig. 7). In addition, the protein fraction that binds to DNA-cellulose has no cyclic AMP-dependent protein kinase activity (Table II). Similar observations of cyclic AMP-independent protein kinase activity has been made in preparations containing cyclic AMP binding proteins that have affinity for whole nuclei or chromatin (32, 33).

We did not examine total nuclear protein kinase activity in this study. In our studies the cyclic AMP-binding protein complex that bound to DNA and stimulated poly(A) RNA synthesis did not have cyclic AMP-dependent protein kinase activity. We conclude that phosphorylation of nuclear proteins may occur but it is not necessary for cyclic AMP stimulation of poly(A) RNA transcription. Similar observations have been made in prokaryotes (40, 41) and in a mammalian cell-free system (19).

The cyclic AMP-binding protein(s) complex that binds to the isolated chick cartilage nuclei has a mol wt of 51,000 (Fig. 8). The exact nature of this protein was not determined in this investigation. There is indirect evidence from our study that this protein is the regulatory (R) subunit of cytosol cyclic AMP-dependent protein kinase. Others have described in detail that the cyclic AMP binding activity in the nucleus can be ascribed to an acidic protein with physical and biochemical characteristics of the regulatory

(R) subunit of cyclic AMP-dependent protein kinase (33, 38, 41).

In conclusion, cyclic AMP stimulates RNA synthesis in the embryonic chick pelvic cartilage. We have investigated the possible mechanisms by which cyclic AMP induces these effects. Our data are compatible with the hypothesis that this process is mediated through cyclic AMP binding to a cytosol protein (51,000 dalton) with transfer to the nucleus. This cyclic AMP-binding protein complex binds to the nucleus. This binding is associated with production of mRNA. Phosphorylation of nuclear proteins may occur, but is not necessary for mRNA synthesis. Further studies directed at purifying and characterizing the specific cyclic AMP binding proteins, characterizing the nuclear transcription products by hybridization techniques and identification of cell-free translation products, and determination of nuclear protein kinase activities are necessary to strengthen this hypothesis. This cyclic AMP regulation of gene transcription in embryonic cartilage is a potential mechanism for the control of growth and differentiation.

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