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DNA Binding by Cyclic Adenosine 3′,5′-Monophosphate Dependent Protein Kinase from Calf Thymus Nuclei†

Edward M. Johnson,* John W. Hadden, Akira Inoue, and Vincent G. Allfrey

ABSTRACT: Cyclic adenosine 3′,5′-monophosphate (cAMP) dependent protein kinase and proteins specifically binding cAMP have been extracted from calf thymus nuclei and analyzed for their abilities to bind to DNA. Approximately 70% of the cAMP-binding activity in the nucleus can be ascribed to a nuclear acidic protein with physical and biochemical characteristics of the regulatory (R) subunit of cAMP-dependent protein kinase. Several peaks of protein kinase activity and of cAMP-binding activity are resolved by affinity chromatography of nuclear acidic proteins on calf thymus DNA covalently linked to aminoethyl Sepharose 4B. When an extensively purified protein kinase is subjected to chromatography on the DNA column in the presence of 10^{-7} *M* cAMP, the R subunit of the kinase is eluted from the column at 0.05 *M* NaCl while the catalytic (C) subunit of the enzyme is eluted at 0.1–0.2 *M* NaCl. When chromatographed in the presence of histones, the R subunit is retained on the column and is eluted at 0.6–0.9 *M* NaCl. In the presence of cAMP, association of the C subunit with DNA is enhanced, as determined by sucrose density gradient centrifugation of DNA–protein kinase complexes. cAMP increases the capacity of the calf thymus cAMP-dependent protein kinase preparation to bind labeled calf thymus DNA, as determined by a technique employing filter retention of DNA–protein complexes. This protein kinase preparation binds calf thymus DNA in preference to salmon DNA, *Escherichia coli* DNA, or yeast RNA. Binding of protein kinases to DNA may be part of a mechanism for localizing cyclic nucleotide stimulated protein phosphorylation at specific sites in the chromatin.

It appears likely, on the basis of several recent studies, that certain of the effects of cyclic adenosine 3′,5′-monophosphate (cAMP)[1] upon growth and differentiation of eukaryotic cells are due to the ability of this cyclic nucleotide to influence transcription. cAMP or its butyryl derivatives have been observed to stimulate incorporation of radioactive precursors into RNA of cells from uterus (Sharma and Talwar, 1970), adrenocortex (Nussdorfer and Mazzocchi, 1972), thyroid (Wilson and Wright, 1970), into RNA of human lymphocytes (Averner et al., 1972; Rosenfeld et al., 1972), and into RNA of nuclei from rat liver cells (Jost and Sahib, 1971; Dokas et al., 1973). cAMP has been implicated in the activation of specific genes in *Drosophila* salivary glands (Leenders et al., 1970; Rensing and Hardeland, 1972). cAMP-induced morphological changes in rat sarcoma (Korinek et al., 1973) and neuroblastoma (Bondy et al., 1974) cells have been linked to effects of the cyclic nucleotide upon mRNA synthesis. cAMP-induced phosphodiesterase synthesis in chicken embryo fibroblasts has been observed to be dependent upon increased transcription (Russel and Pastan, 1974).

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[1] Abbreviations used are: cAMP, adenosine 3′,5′-monophosphate; cGMP, guanosine 3′,5′-monophosphate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N′*-tetraacetate; PK II, a protein kinase fraction from calf thymus.

cAMP regulates transcription at the *lac* and *gal* operons in *Escherichia coli* (Pastan and Perlman, 1970) through a mechanism that involves binding of the cyclic nucleotide to a cAMP receptor protein with a variable affinity for DNA (Zubay et al., 1970; Emmer et al., 1970; Miller et al., 1971; Nissley et al., 1971; Anderson et al., 1971). In the presence of cAMP, the receptor protein binds to the DNA at the operator-promoter region and promotes the initiation of transcription by RNA polymerase (Riggs et al., 1971; Nissley et al., 1972).

In eukaryotic systems effects of cAMP upon transcription may be mediated through binding of the cyclic nucleotide to receptor proteins which act in the cell nucleus. The most extensively characterized receptor proteins for cAMP in mammalian cells are cAMP-dependent protein kinases (Walsh et al., 1968; Langan, 1968; Miyamoto, et al., 1969), and it has been proposed that in higher organisms many, if not all, the biological effects of cAMP are mediated through activation of this class of enzymes (Greengard and Kuo, 1970). cAMP-dependent protein kinases are composed of regulatory (R) and catalytic (C) subunits. cAMP binds to the R subunit, which splits from the kinase holoenzyme, leaving an activated C subunit (Brostrom et al., 1970; Gill and Garren, 1970; Tao et al., 1970; Rubin et al., 1972; Miyamoto et al., 1973). In the present study we identify the R subunit of cAMP-dependent protein kinase as a prominent cAMP receptor protein in the calf thymus nucleus and describe conditions under which the protein kinase holoenzyme and C subunit bind to DNA.

Experimental Procedure

Preparation of Calf Thymus Nuclei and Nuclear Proteins. Fresh calf thymus was defatted, rinsed in cold 0.32 *M* sucrose, finely minced with scissors, and ground manually in a tissue grinder. The ground thymus was suspended in 4 volumes of cold 0.32 *M* sucrose containing 3 m*M* $MgCl_2$ and 0.5 m*M* $CaCl_2$ and homogenized for 1 min in a Waring Blendor at 1200 rpm. The homogenate was filtered through one layer of flannelette and centrifuged at 1000*g* for 10 min. In order to wash the nuclei, the pellet was resuspended in 4 volumes of 0.25 *M* sucrose containing 3 m*M* $MgCl_2$ and 0.05 m*M* $CaCl_2$ and filtered through first one and then two layers of flannelette before recentrifuging at 1000*g* for 10 min. This washing procedure was repeated twice. The nuclei were then resuspended in 8 volumes of 0.14 *M* NaCl containing 3 m*M* $MgCl_2$ and 0.5 m*M* $CaCl_2$ and centrifuged at 1000*g* for 10 min. After repeating the saline wash once more, the washed nuclear pellet was resuspended in 3 volumes of 66% glycerol and stored at −80°. For preparation of nuclear proteins, nuclei were stirred for 2 hr at 4° in 3 volumes of 3 *M* NaCl, 1 m*M* Na_2EDTA, 1 m*M* 2-mercaptoethanol, 30% glycerol, and 0.1 *M* Tris-Cl (pH 8.0). The dissociated nuclei were centrifuged at 100,000*g* for 12 hr and the gelatinous pellet containing most of the DNA was discarded. The supernatant solution, which contained about 60% of the total nuclear protein, was dialyzed for 12 hr against 0.4 *M* NaCl containing 1 m*M* Na_2EDTA, 10% glycerol, and 10 m*M* Tris-Cl (pH 7.4) before recentrifuging at 20,000*g* for 30 min. The nuclear proteins in the supernatant were then subjected to Bio-Rex 70 column chromatography in order to separate the nuclear proteins into acidic and basic protein fractions. Nuclear proteins in the 0.4 *M* NaCl buffer were applied to a Bio-Rex 70 column (Bio-Rad Laboratories; 10 cm × 6.5 cm diameter) prewashed with 2 *M* NaCl and equilibrated with the 0.4 *M* NaCl buffer. Nuclear acidic proteins were collected as the runoff fraction in 0.4 *M* NaCl. After washing with 3 column volumes of the 0.4 *M* NaCl buffer, nuclear basic proteins, including all classes of histones, were eluted from the column with 2.0 *M* NaCl, 1 m*M* Na_2EDTA, 1 m*M* 2-mercaptoethanol, 10% glycerol, and 10 m*M* Tris-Cl (pH 7.4). The nuclear acidic protein fraction was prepared for DNA aminoethyl Sepharose column chromatography by precipitation with 85% $(NH_4)_2SO_4$ followed by dialysis of the redissolved precipitate against a solution of 0.05 *M* NaCl containing 0.1 m*M* Na_2EDTA, 10 m*M* $NaHSO_3$, 1 m*M* 2-mercaptoethanol, 10% glycerol, and 10 m*M* Tris-Cl (pH 7.4). The final nuclear acidic protein preparation comprised about 15% of the total nuclear proteins and had a ratio $OD_{260\ nm}/OD_{280\ nm}$ = 0.55, indicating that the protein preparation was substantially free of DNA.

Assays for cAMP-Binding Activity. cAMP-binding proteins were detected by a modification of the procedure of Gilman (1972); 5–100 μg of protein to be tested for cAMP-binding activity were incubated in a solution consisting of 50 m*M* Tris-Cl, 1 m*M* 2-mercaptoethanol, 5×10^{-5} *M* papaverine, 10% glycerol, and 0.1 m*M* Na_2EDTA (pH 7.4) in a total volume of 0.4 ml. After 20 min at 30°, 6 μl of [^{3}H(G)]cAMP (32–35 Ci/mmol; New England Nuclear, Boston, Mass.) together with unlabeled 2′,3′-AMP and 5′-AMP were added to give final concentrations of 2.0×10^{-7} *M* [^{3}H]cAMP and 2.0×10^{-5} *M* of each of the unlabeled adenine nucleotides. After 5 min at 30°, the assay mixture was transferred to an ice bath for an additional 25 min. After addition of 3.0 ml of ice-cold 50 m*M* sodium acetate containing 10% glycerol and 2 m*M* sodium phosphate (pH 4.5), samples were filtered through Millipore HA filters (0.45-μ pore size), prewashed with the pH 4.5 buffer, and the filters were washed once with 3 ml of the pH 4.5 buffer. Filters were dissolved with 1 ml of ethylene glycol monomethyl ether and assayed for ^{3}H radioactivity by scintillation spectrometry in 12 ml of Aquasol (New England Nuclear). This binding assay was developed to detect proteins with a specific affinity for cAMP, and to detect in a reproducible manner the several peaks of cAMP-binding activity resolved by chromatography of chromatin proteins. While addition of unlabeled adenine nucleotides and washing with phosphate buffer significantly reduced overall binding activity, these steps were determined to result in increased selectivity for cAMP binding. In all cases simultaneous control experiments were performed in which all points were assayed as described, but with the addition of 10^{-5} *M* unlabeled cAMP. Values presented represent specific cAMP binding as determined by reduction of binding activity with unlabeled cAMP.

Isolation and Measurement of Activity of cAMP-Dependent Protein Kinase. Fresh calf thymus (100–300 g) was homogenized in 4 volumes of 3 m*M* $MgCl_2$, 0.5 m*M* $CaCl_2$, and 10 m*M* Tris-Cl (pH 7.4) for 2 min in a Waring Blendor. The homogenate was centrifuged first at 10,000*g* for 15 min, the supernatant was centrifuged at 20,000*g* for 30 min, and the final supernatant was filtered through eight layers of cheesecloth. Solid ammonium sulfate was added to the supernatant (32.5 g of ammonium sulfate/100 ml of supernatant), and after 30 min the resulting precipitate was collected by centrifugation at 20,000*g* for 10 min, dissolved in 50 ml of 5 m*M* sodium phosphate–2 m*M* EDTA (pH 6.9), and dialyzed overnight against the same buffer. After centrifuging at low speed to remove insoluble material, the dialysate was applied to a Cellex-D column (Bio-Rad Labo-

ratories; 21.5 cm × 2.6 cm diameter). The column was washed with 5 m*M* sodium phosphate–2 m*M* Na_2EDTA (pH 6.9) and eluted with a sodium phosphate gradient essentially as described by Gilman (1972). Two peaks of cAMP-dependent protein kinase activity were eluted. The second peak of cAMP-dependent protein kinase activity, eluting at about 0.3 *M* sodium phosphate, had the higher specific activity of the two peaks, and fractions containing this peak were pooled, solid ammonium sulfate was added to 85% saturation, and the resulting precipitate was collected by centrifugation at 20,000*g* for 30 min. The ammonium sulfate precipitate was dissolved in 15 ml of 0.03 *M* sodium phosphate (pH 6.9) and dialyzed overnight against the same buffer. Hydroxylapatite (Bio-Gel HTP, Bio-Rad Laboratories) in H_2O suspension was added dropwise to the dialysate to a final ratio of 10 mg/mg of protein, stirred gently, and incubated for 30 min at 0°. The hydroxylapatite was collected by centrifugation for 5 min at 1000*g*, the pellet was washed five times with 5 ml of 0.03 *M* sodium phosphate (pH 6.9), and protein kinase was eluted by extraction of the pellet three times with 4 ml of 0.3 *M* sodium phosphate (pH 6.9). The resulting protein kinase solution (termed PK II) was dialyzed against 50 volumes of H_2O and stored frozen at −80°. This purification procedure resulted in approximately 300-fold increases in specific activities of both protein kinase activity and cAMP-binding activity; 5×10^{-6} *M* cAMP stimulates protein kinase activity of the final preparation 3.2-fold when assayed as described. Sodium dodecyl sulfate disc gel electrophoresis of PK II results in separation of a band of molecular weight of 123,000, two bands of molecular weights of about 50,000, and two bands of molecular weights of about 30,000. In some experiments employing DNA affinity chromatography, a commercial protein kinase preparation (bovine heart, DEAE peak II; Sigma, St. Louis, Mo.) was used and gave results similar to those obtained with the calf thymus kinase preparation.

Protein kinase activity was assayed by incubating aliquots of protein for 5 min at 30° with [γ-^{32}P]ATP (New England Nuclear, Boston, Mass.; 5×10^{-6} *M*, $3\text{–}5 \times 10^3$ cpm/pmol) and 100 μg of histone F1 as substrate in the presence or absence of 5×10^{-6} *M* cAMP in 50 m*M* NaOAC buffer (pH 6.5) with 10 m*M* $Mg(OAC)_2$ and 0.1 m*M* EGTA as described previously (Johnson and Allfrey, 1972). The reaction was stopped by addition of 4 ml of 5% trichloroacetic acid containing 0.25% sodium tungstate and 0.06 *N* H_2SO_4. After addition of 625 μg of bovine serum albumin to act as carrier, the precipitate was collected by centrifugation at low speed and washed three times with the trichloroacetic acid–tungstate solution as described previously (Johnson and Allfrey, 1972). The washed precipitates were dissolved in 0.1*N* NaOH, and assayed for radioactivity by scintillation spectrometry in 6 ml of scintillation fluid consisting of 4 g/l. of Omnifluor (New England Nuclear) dissolved in toluene–ethylene glycol monomethyl ether (1:1).

Affinity Chromatography on DNA Covalently Linked to Aminoethyl Sepharose. DNA-aminoethyl Sepharose was prepared as described previously (Allfrey et al., 1974). Sepharose 4B (Pharmacia, Uppsala, Sweden) was activated with cyanogen bromide and aminoethylated according to the method of Cuatrecasas (1970). Calf thymus DNA (Type I; Sigma Chemical Co., St. Louis, Mo.) dissolved in 0.15 *M* NaCl–0.015 *M* sodium citrate (pH 7.0) was purified by successive extractions with chloroform and phenol, precipitated with ethanol, and dialyzed against 0.04 *M* (2-*N*-morpholino)ethanesulfuric acid buffer at pH 6.0 before addition to the gravity-packed aminoethyl Sepharose 4B at 2 mg of DNA/mg of swollen Sepharose. After drying to remove excess water, coupling was carried out at 50° by addition of a water-soluble carbodiimide (Rickwood, 1972; Astell and Smith, 1972). The reagent used, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate, was added in three aliquots over a 20-hr period to the DNA-Sepharose slurry to give a final amount of 16 g of the carbodiimide/100 mg of DNA. The DNA-Sepharose was allowed to swell for 8 hr at 4° in 0.14 *M* NaCl containing 0.2 *M* sodium bicarbonate (pH 10.0) to remove hydrolyzed adducts of the coupling reagent and DNA bases; 50–80% of the DNA was bound by this procedure, and DNA was present in the final preparation at 1–3 mg of DNA/ml of swollen Sepharose. DNA-Sepharose columns (5 cm × 1.5 cm diameter) were paired and washed with 2 *M* NaCl in 50 m*M* Tris-Cl buffer (pH 7.4) and equilibrated with 0.05 *M* NaCl containing 0.1 m*M* Na_2EDTA, 1 m*M* 2-mercaptoethanol, 10 m*M* sodium bisulfite, 10% glycerol, and 10 m*M* Tris-Cl (pH 7.4). Proteins to be studied (1–5 mg) were applied to the column in the 0.05 *M* NaCl buffer at a flow rate of 1 ml/60 min and eluted from the column with an NaCl gradient in the same buffer at 1 ml/15 min; 1-ml fractions were collected. Less than 0.3% of the DNA bound to the Sepharose was eluted from the column during chromatography. Each DNA-Sepharose column was used only once.

DNA-Aminoethyl Sepharose Column Chromatography of cAMP Dependent Protein Kinase Regulatory (R) and Catalytic (C) Subunits. In the present study cAMP-dependent protein kinase was dissociated into cAMP-binding (R) and kinase catalytic (C) moieties by incubation at 31° for 15 min followed by incubation at 0° for 2 hr in a solution consisting of enzyme preparation (1 mg/ml), 10^{-7} *M* cAMP, 0.1 m*M* EGTA, 10 m*M* magnesium acetate, 1 m*M* 2-mercaptoethanol, 5×10^{-5} *M* papaverine, 10^{-6} *M* ATP, 10% glycerol, and 50 m*M* sodium acetate (pH 6.5) in a volume of 1.0 ml. Aliquots of this mixture were diluted 1:1 with the 50 m*M* NaCl column buffer, and subjected to DNA-Sepharose column chromatography as described in the previous section, except that in this case all column buffers contained 10^{-7} *M* cAMP and that the column had been previously equilibrated with 10^{-7} *M* cAMP. Prior to assaying the column fractions for cAMP binding activity, cyclic nucleotide in the eluate was removed by dialyzing each of the collected fractions against Norit SG Extra charcoal (Baker) in 10 m*M* Tris buffer (pH 7.0) (1 g of charcoal/l.). This charcoal dialysis step removes some of the bound cAMP from the R subunit and allows the detection of cAMP-binding activity by this protein. Only about 50% of the total R subunit cAMP binding activity was recovered by this dialysis step.

Gel Electrophoresis of cAMP Binding Proteins. Protein kinase preparations containing 50 μg of protein were dissociated into R and C subunits by incubation in 0.2 ml of 50 m*M* sodium acetate–10 m*M* magnesium acetate (pH 7.0) containing 10% sucrose, 10^{-6} *M* ATP, and 10^{-7} *M* cAMP. After 30 min at 0°, this solution was layered onto 7.5% polyacrylamide gels (5 cm × 0.6 cm) containing 0.1 *M* Tris-Cl (pH 8.0) and subjected to electrophoresis at 2 mA/tube for 2.25 hr at 0°. To elute protein, gel slices (2 mm) were incubated in 0.2 ml of 10 m*M* Tris buffer (pH 8.0) for 1 hr at 0°. The gel eluates were then assayed for cAMP-

binding activity as described using 10^{-6} *M* [^{3}H]cAMP. Incubation during the cAMP binding assay was carried out for 5 min at 30° followed by 1 hr at 0° prior to diluting and filtering the samples and washing and assaying the filters for radioactivity as described.

Sucrose Density Gradient Centrifugation of Protein Kinase and DNA. Solutions containing various combinations of protein kinase (333 μg/ml), calf thymus DNA (667 μg/ml), deproteinated as described, and [^{3}H]cAMP (5×10^{-7} *M;* 32 Ci/mmol) were prepared in a buffer consisting of 5×10^{-5} *M* papaverine, 0.1 m*M* Na_2EDTA, 0.1 m*M* dithiothreitol, and 20 m*M* Tris-acetate (pH 6.9). After incubation for 1 hr at 25°, 0.3 ml of each solution to be examined was layered on top of 4.5 ml of a 5–25% sucrose density gradient containing 5×10^{-5} *M* papaverine, 0.1 m*M* Na_2EDTA, 0.1 m*M* dithiothreitol, bovine serum albumin (50 μg/ml), and 20 m*M* Tris-acetate (pH 6.9). Solutions containing labeled cAMP were layered onto gradients also containing 5×10^{-7} *M* [^{3}H]cAMP. Gradients, prepared in tubes for a Beckman SW 50.1 rotor, were centrifuged for 3.5 hr at 4° at 200,000*g*. Fractions (0.3 ml) were eluted from gradients from the top and were diluted with 1.0 ml of 20 m*M* Tris-acetate buffer (pH 6.9) containing 5×10^{-5} *M* papaverine, 0.1 m*M* Na_2EDTA, 0.1 m*M* dithiothreitol, and 10% glycerol. Aliquots of these diluted fractions were taken for measurements of bound cAMP, protein kinase activity, and DNA concentration. Bound [^{3}H]cAMP was measured by filtering 1.0-ml aliquots of diluted fractions directly through Millipore filters, washing, and assaying filters for radioactivity as described for the cAMP binding assays; 0.1 ml of each diluted fraction was assayed for protein kinase activity as described. DNA concentration was measured by determination of OD_{260} of each diluted fraction and subtracting OD_{260} of appropriate control gradient fractions run in the absence of DNA.

Binding of Proteins to [^{3}H]Methyl-DNA. Purified calf thymus DNA was dissolved in 0.15 *M* NaCl containing 0.015 *M* sodium citrate (pH 7.0) and sheared to 500–1500 base pairs in length by sonication. After overnight dialysis against 0.1 *M* sodium phosphate (pH 7.7), 8.25 mg of DNA was labeled with [^{3}H]methyl groups by incubation with vigorous stirring for 4 hr at 25° with 0.30 mCi of [^{3}H]dimethyl sulfate (150 Ci/mol; Amersham-Searle) in 0.1 *M* sodium phosphate buffer (pH 7.7) in a total of 3 ml. The procedure used was based upon a method previously described for labeling of RNA (Smith et al., 1967). The labeled DNA was precipitated with ethanol and was dissolved in 0.15 *M* NaCl containing 0.015 *M* sodium citrate (pH 7.0). The DNA was reprecipitated with ethanol and redissolved in the NaCl-citrate buffer several times to remove unbound radioactivity. It was calculated that 3–10% of the DNA bases are methylated by this procedure. In cases in which double-stranded DNA was used, DNA remained double-stranded after labeling, as determined by hyperchromicity at OD_{260} upon denaturation with 0.1 *N* NaOH. Protein binding to the labeled DNA was measured by a modification of the method used to study binding of the *lac* repressor to bacterial DNA (Riggs et al., 1968; Bourgeois, 1972); 20 μg of labeled DNA (2000–6000 dpm/μg) was incubated for 30 min at 25° with aliquots (1–50 μg) of protein in an assay mixture composed of 5×10^{-5} *M* papaverine, 0.1 m*M* Na_2EDTA, 0.1 m*M* dithiothreitol, 50 μg/ml of bovine serum albumin, 5% dimethyl sulfoxide, and 20 m*M* Tris-acetate (pH 6.9) in the presence or absence of experimental additions in a total volume of 2.0 ml. After incubation, the mixture was filtered through B6 membrane filters (Schleicher and Schuell) which had been pretreated for 10 min with 0.5 *N* NaOH and prewashed extensively with H_2O and assay buffer. Upon filtering, unbound DNA passes through the filter while DNA bound to protein is retained. Filters were washed twice with 2 ml of assay buffer (with bovine serum albumin omitted), dissolved in 1 ml of ethylene glycol monomethyl ether, and assayed for radioactivity by scintillation spectrometry in 12 ml of Aquasol. In experiments to determine specificity of DNA binding of protein kinase, unlabeled polynucleotides used for competition with labeled calf thymus DNA were all obtained from the Sigma Chemical Corp.

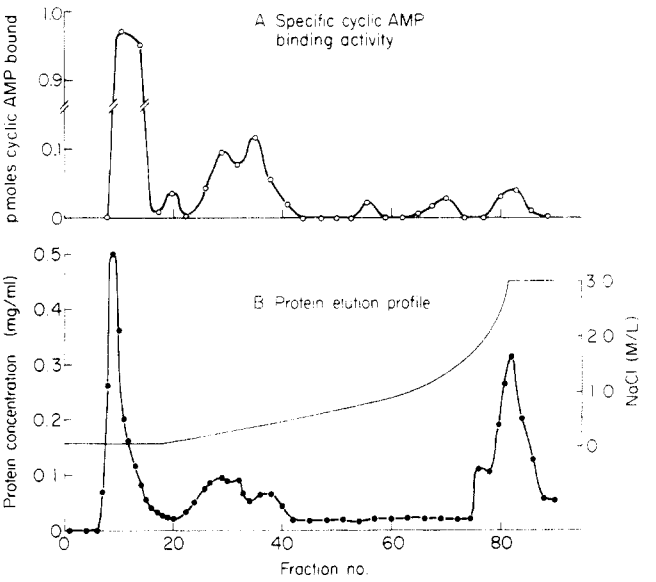

FIGURE 1: Binding of [^{3}H]cAMP by calf thymus nuclear acidic proteins separated by DNA aminoethyl Sepharose column chromatography. Nuclear proteins were extracted from nuclei and subjected to Bio-Rex 70 column chromatography as described under Experimental Procedure. Nuclear acidic proteins used for DNA affinity chromatography were those eluted from the Bio-Rex 70 column with 0.4 *M* NaCl; 5 mg of nuclear acidic protein was chromatographed on a DNA aminoethyl Sepharose column (5 cm × 1.5 cm diameter) containing 10 mg of calf thymus DNA as described under Experimental Procedure; 1-ml fractions were collected; 50 μl of each fraction from the DNA affinity column was taken to assay for ability to bind [^{3}H]cAMP as described under Experimental Procedure.

It was found that under the conditions used neither double-stranded DNA nor denatured DNA are retained on the filter in the absence of DNA-binding proteins. Nonsheared calf thymus DNA also passes through the filter in the absence of binding proteins. In all cases only about 0.2% of the labeled DNA present in the assay mixture in the absence of added binding protein was retained upon filtration. Retention of proteins by the nitrocellulose filter has been described (Bourgeois, 1972). Under the conditions employed for the present study, about 40% of total ^{32}P-labeled nuclear acidic proteins are retained by filtration in the absence of added DNA.

Measurements of DNA and Protein. DNA was determined by the diphenylamine reaction described by Burton (1956). Protein was determined by the method of Lowry et al. (1951).

Results

cAMP-Binding Proteins of Calf Thymus Nuclei. Calf thymus nuclei contain specific cAMP-binding proteins

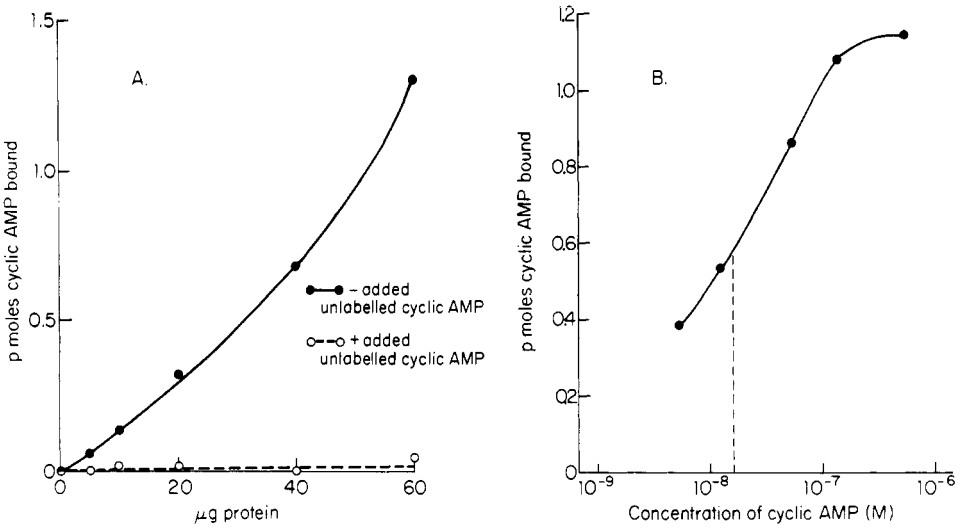

FIGURE 2: cAMP-binding characteristics of the major cAMP-binding peak resolved from calf thymus nuclear acidic proteins by DNA aminoethyl Sepharose column chromatography. Protein assayed was that from pooled fractions 8–15, shown in Figure 1, eluted from the DNA column at 0.05 *M* NaCl; 2×10^{-5} *M* 2′,3′-AMP and 2×10^{-5} *M* 5′-AMP were included in all assay mixtures. Assays for cAMP-binding activity were conducted as described under Experimental Procedure. (A) Ability of unlabeled cAMP to block protein binding of [^{3}H]cAMP. Unlabeled cAMP (10^{-5} *M*) was added to the assay mixture prior to addition of [^{3}H]cAMP (2×10^{-7} *M*). (B) Concentration dependence of binding of [^{3}H]cAMP to 50 μg of binding protein. Half-maximal binding (- - -) was obtained at 2.6×10^{-8} *M* [^{3}H]cAMP.

which are not removed from the nuclei by washing with 0.14 *M* NaCl and which are located among the nuclear acidic proteins. Upon extraction of nuclei with 2 *M* NaCl and subsequent chromatography of nuclear proteins on Bio-Rex 70, it is found that the nuclear acidic proteins, those proteins eluted from the Bio-Rex column with 0.4 *M* NaCl, bind cAMP with a specific activity of 1.1 pmol of cAMP bound/mg of protein. Binding of cAMP is proportional to the amount of protein up to 270 μg of protein in the assay mixture. In contrast, the nuclear basic proteins, those proteins, including the histones, which are eluted from the Bio-Rex column with concentrations of NaCl from 0.4 to 2 *M*, exhibit no cAMP-binding activity under the conditions tested. Proteins in nuclear acidic protein fractions were analyzed for their ability to bind to double-stranded calf thymus DNA covalently linked to aminoethyl Sepharose 4B. Figure 1 shows that several peaks of specific cAMP-binding activity are resolved upon DNA affinity chromatography of the 0.4 *M* NaCl eluate from the Bio-Rex 70 column. It can be seen that about 70% of the total cAMP-binding activity in the acidic protein fraction is detected in the runoff fraction from the DNA column. Other peaks of cAMP-binding activity are eluted from the DNA column at concentrations of NaCl ranging from 0.1 to 3.0 *M*. Upon rechromatography of this DNA column runoff fraction, about 70% of cAMP binding activity is again detected in the runoff position. This result indicates that the bulk of the cAMP binding protein component of the runoff fraction has a low affinity for the DNA column. An alternative explanation is that the cAMP binding protein binds to DNA sites which are present in very small quantities on the column and which are easily saturated. Evidence from several experiments indicates that this major cAMP-binding protein eluted from the DNA column with a low concentration of NaCl (0.05 *M*) is the R subunit of cAMP-dependent protein kinase.

Figure 2 depicts the specificity of binding of cAMP to the protein fraction eluting from the DNA aminoethyl Sepharose column at 0.05 *M* NaCl. Panel A of Figure 2 shows the ability of unlabeled cAMP to compete for protein binding with [^{3}H]cAMP. A 50-fold excess of unlabeled cAMP added with 2×10^{-7} *M* [^{3}H]cAMP virtually eliminates the binding of the labeled cAMP by this protein fraction. Panel B of Figure 2 depicts the binding of this cAMP-binding protein fraction to cAMP at different concentrations of [^{3}H]cAMP. Half-maximal binding of cAMP in the presence of 5′- and 2′,3′-AMP is observed at 2.6×10^{-8} *M* [^{3}H]cAMP. This value is lower than the apparent K_m's for cAMP of protein kinases from several bovine tissues (Greengard and Kuo, 1970). Binding of [^{3}H]cAMP to the nuclear cAMP binding fraction is observed at even lower concentrations in the absence of the 100-fold excess of added 2′,3′- and 5′-AMP which were present in all the experiments presented. These results are consistent with the possibility that this binding protein fraction contains the protein kinase R subunit, as it has been observed that cAMP has a very high affinity for isolated R subunits of cAMP-dependent protein kinase (Brostrom et al., 1971). Figure 3 is a comparison of gel electrophoretic properties of the major cAMP-binding protein from the DNA column and the R subunit of cAMP-dependent protein kinase. It can be seen that the nuclear cAMP binding protein and the R subunit (after preincubation with cAMP) migrate to the same position in the acrylamide gel system employed. These data concerning specificity of cyclic nucleotide binding, half-maximal binding activity and gel electrophoretic properties suggest that the primary nuclear cAMP-binding protein is the R subunit of cAMP-dependent protein kinase.

DNA Affinity Chromatography of Nuclear Protein Kinase Activity. Figure 4 is a comparison of elution profiles of protein kinase activity and cAMP binding activity obtained upon DNA aminoethyl Sepharose chromatography of the nuclear acidic proteins. It can be seen from panel B of Figure 4 that most of the recovered protein kinase activity is independent of activation by cAMP. Stimulation by cAMP is observed with two of the four peaks of nuclear protein kinase activity obtained, that eluting at 0.05 *M* NaCl, and that eluting at about 0.14 *M* NaCl. In both cases this stimulation is about two-fold. Protein kinase activity eluting at 0.05–0.1 *M* NaCl and at 0.15–0.25 *M* NaCl is completely

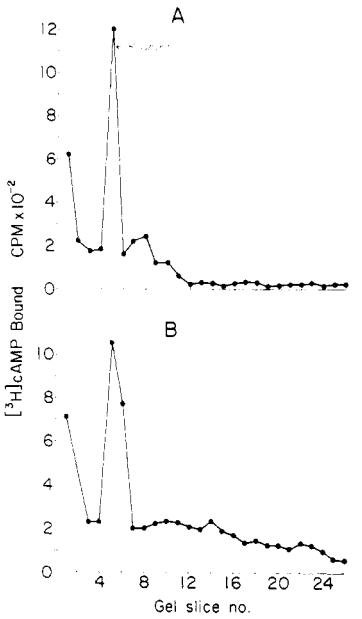

FIGURE 3: Comparison of disc gel electrophoretic migration of the major cAMP-binding protein from calf thymus nuclei with the cAMP-binding (R) protein of cAMP-dependent protein kinase. Prior to electrophoresis, proteins were incubated with 10^{-7} *M* cAMP in the presence of Mg^{2+} and ATP as described under Experimental Procedure. Gel electrophoresis was conducted for 2.25 hr at 2 mA/tube at 0° on 7.5% polyacrylamide gels (5 cm × 0.6 cm) containing 0.1 *M* Tris-HCl (pH 8.0) and cAMP binding activity in gel slices (2 mm) was assayed as described under Experimental Procedure. (A) Gel containing 50 μg of bovine heart protein kinase (DEAE peak II; Sigma Chemical Co., St. Louis, Mo.). (B) Gel containing 50 μg of protein from the DNA aminoethyl Sepharose cAMP-binding fraction eluted at 0.05 *M* NaCl (Figure 1). Note that the cAMP-binding protein from the nuclear acidic protein preparation migrates to the same position as the R subunit of protein kinase.

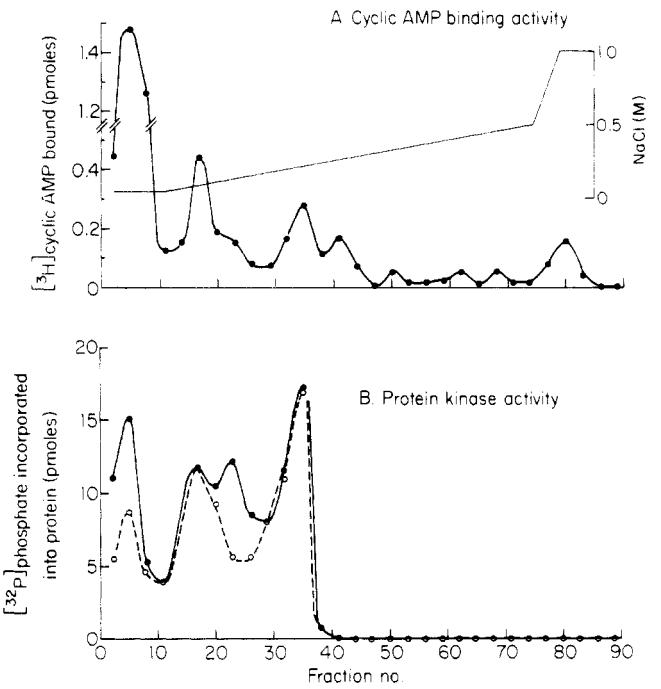

FIGURE 4: Protein kinase activity and cAMP-binding activity in calf thymus nuclear acidic proteins separated by affinity chromatography on DNA aminoethyl Sepharose. Nuclear acidic proteins were prepared and subjected to DNA affinity chromatography as described under Experimental Procedure and in the legend to Figure 1, except that in this case a linear NaCl gradient from 0.05 to 0.5 *M* was used followed by stepwise elution with a 1 *M* NaCl solution; 1-ml fractions were collected; 50 μl of each fraction was taken to assay for cAMP-binding activity; 20 μl was taken to assay for protein kinase activity using histone as substrate. Assays were conducted as described under Experimental Procedure. Panel A depicts cAMP-binding activity (— ● —). Panel B depicts protein kinase activity assayed in the absence (- - ○ - -) or presence (— ● —) of 5×10^{-6} *M* cAMP. Note the differential capacities of nuclear protein kinases and cAMP-binding proteins to bind to the DNA column. Also note that some peaks of cAMP-binding activity do not correspond to peaks of protein kinase activity.

independent of cAMP. Both peaks of cAMP-activated protein kinase correspond to fractions in which cAMP-binding activity was found. Figure 4 shows that all of the protein kinase activity is eluted from the DNA column by 0.3 *M* NaCl. It is notable that several peaks of specific cAMP-binding activity which bind to the DNA column do not correspond to peaks of protein kinase activity.

DNA Binding Properties of R and C Subunits of Isolated Protein Kinase. The affinities for DNA of protein kinase holoenzyme and separated protein kinase R and C components were examined by DNA aminoethyl Sepharose column chromatography. Figure 5 depicts the elution profiles of cAMP-dependent protein kinase chromatographed on the DNA column in the presence or absence of 10^{-7} *M* cAMP. It was found that the R and C components of the protein kinase can be separated by DNA affinity chromatography. Panel A of Figure 5 shows that in the absence of cAMP, protein kinase R and C subunits chromatograph together as protein kinase holoenzyme eluting from the column at about 0.14 *M* NaCl. In the presence of cAMP, the protein kinase components are separated. The C subunit, represented by kinase catalytic activity, is retained on the DNA column, eluting at the same position as the kinase holoenzyme. The R subunit, represented by cAMP binding activity, is detected in the runoff fraction. Similarity of elution of the R protein, seen in Figure 5, to that of the major cAMP binding protein of the nucleus, seen in Figures 1 and 4, is further evidence that the primary nuclear cAMP-binding protein is the R subunit. It is notable that binding of protein kinase to the DNA column in the absence of cAMP (Figure 5, panel A) does not result in separation of kinase R and C subunits. In separate experiments (not depicted) it was observed that incubation of protein kinase with DNA under the conditions employed for chromatography does not affect either kinase catalytic activity or the ability of cAMP to activate the enzyme.

Figure 6 illustrates an experiment designed to determine whether binding of the protein kinase C subunit to the DNA affinity column actually represents binding of the protein to DNA. It can be seen that when DNA is omitted during preparation of the column material, the resulting aminoethyl Sepharose column is incapable of retaining the chromatographed C subunit. Comparison of Figures 5 and 6 with Figure 4 shows that the protein kinase holoenzyme and C subunit are eluted from the DNA column at approximately the same NaCl concentration (0.14 *M*) as a peak of nuclear cAMP-activated protein kinase. This similarity indicates that cAMP-dependent protein kinase of the nucleus has DNA binding properties similar to the extensively purified soluble protein kinase used for the detailed DNA-binding studies presented in Figures 5 and 6.

Sucrose Density Gradient Centrifugation of DNA–Protein Kinase Complexes. Data depicted in Figure 5 suggest that the R protein of protein kinase from calf thymus has a low affinity for calf thymus DNA. Experiments performed using sucrose density gradient centrifugation offer further

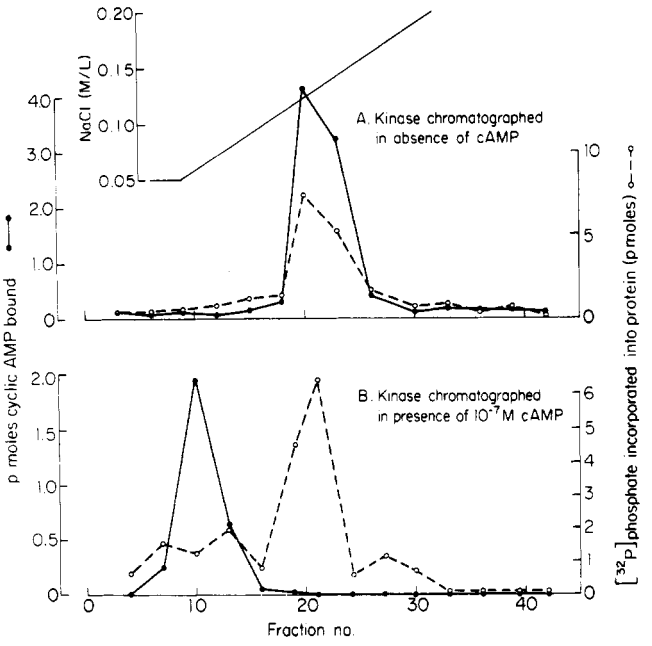

FIGURE 5: DNA aminoethyl Sepharose column chromatography of bovine cAMP-dependent protein kinase; 1.0 mg of protein kinase, purified through DEAE column chromatography as described, was subjected to DNA affinity chromatography on a column (5 cm × 1.5 cm diameter) containing 17.6 mg of calf thymus double-stranded DNA in the presence or absence of 10^{-7} *M* cAMP as described under Experimental Procedure. Column fractions were assayed for cAMP-binding activity (●—●) and for histone kinase activity in the presence of 5 × 10^{-6} *M* cAMP (O - - - O) as described. (A) Protein kinase chromatographed in the absence of cAMP. In some experiments a peak of cAMP-dependent protein kinase eluted at the runoff fraction in addition to the peak eluted at the position shown. (B) Protein kinase chromatographed in the presence of cAMP after preincubation with cAMP as described. Note that kinase R and C subunits differ in their affinities for the DNA column.

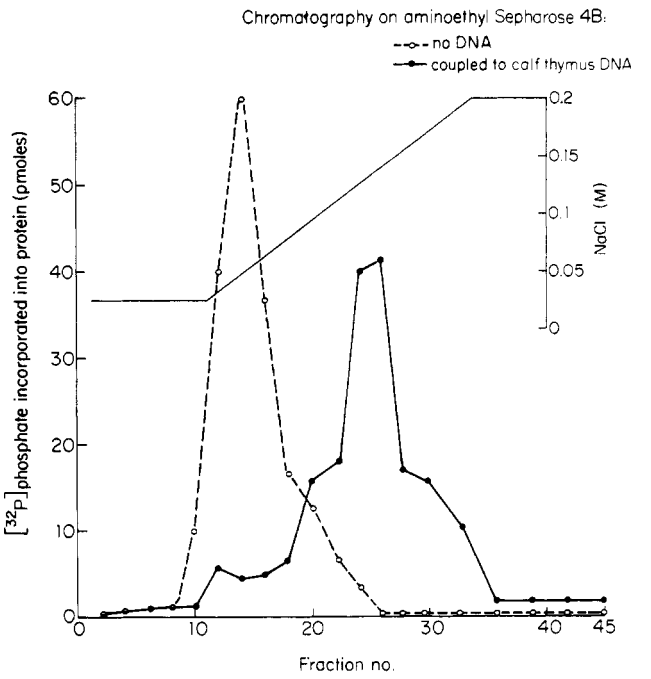

FIGURE 6: Binding of protein kinase C subunit to DNA aminoethyl Sepharose. One lot of aminoethyl Sepharose 4B was divided into two aliquots. One aliquot was coupled to double-stranded calf thymus DNA as described under Experimental Procedure, while the other aliquot was treated in parallel exactly as described except for the omission of the DNA; 1.0 mg of cAMP-dependent protein kinase was chromatographed in the presence of 10^{-7} *M* cAMP on each of two parallel columns (5 cm × 1.5 cm diameter) prepared from these aminoethyl Sepharose aliquots, one containing 17.6 mg of DNA (●—●), and one with DNA omitted (O - - - O). Column fractions were assayed for protein kinase activity using histone as substrate in the presence of 5 × 10^{-6} *M* cAMP as described under Experimental Procedure. Note that the C subunit has affinity only for the column containing DNA.

evidence that, under the conditions studied, the R protein does not bind to a significant proportion of total calf thymus DNA. In these experiments, shown in panel A of Figure 7, protein kinase from calf thymus (PK II) was incubated in the presence or absence of calf thymus DNA prior to centrifugation on a 5–25% sucrose gradient in the presence of 5 × 10^{-7} *M* [^{3}H]cAMP. It can be seen that R protein bound to cAMP migrates to the same position near the top of the density gradient regardless of whether or not DNA is present. No significant portion of the cAMP-binding activity corresponds to the peak position of migration of the DNA. In similar experiments it was confirmed that, under the conditions employed, the protein kinase C subunit binds to the DNA. Panel B of Figure 7 depicts the association of protein kinase catalytic activity with the DNA on sucrose density gradients in either the presence or absence of 5 × 10^{-7} *M* cAMP. It can be seen that while the C subunit binds to DNA under both conditions, about 50% more kinase activity corresponds to the peak position of DNA migration in the presence of cAMP than in the absence of the cyclic nucleotide. The data indicate that while the cAMP-binding subunit of protein kinase does not bind extensively to the DNA, the interaction of cAMP with the holoenzyme results in enhanced capacity of the catalytic subunit to bind DNA.

DNA Affinity Chromatography of Protein Kinase in the Presence of Histone. The ability of histones to dissociate R and C subunits of cAMP-dependent protein kinase has been described previously (Miyamoto et al., 1973). In order to determine the effects of histones on the DNA-binding properties of separated R and C subunits, experiments were performed in which bovine protein kinase was incubated with whole calf thymus histone prior to chromatography on DNA aminoethyl Sepharose. Figure 8 depicts the results of DNA affinity chromatography of protein kinase chromatographed in the absence or presence of histone. It can be seen that histones reduce the amount of cAMP-binding activity associated with protein kinase (eluting at about 0.14 *M* NaCl) while inducing the appearance of cAMP-binding peaks eluting at higher concentrations of NaCl (0.5–1.0 *M*). No significant cAMP-binding activity is eluted at 0.05 *M* NaCl. Histones alone were not observed to specifically bind cAMP. Thus it is likely that the cAMP-binding proteins which elute at high NaCl concentrations (Figure 8, bottom panel) are a product of dissociation of the protein kinase. Note that recovery of cAMP binding activity from the column is the same in the absence or presence of histone. These data indicate that in the presence of histone the R subunit of cAMP-dependent protein kinase has a high affinity for the DNA column. A possible explanation for these results is that R subunit, which is an acidic protein (Miyamoto et al., 1973), binds to the histones which are in turn bound to the DNA. The possibility that the R protein binds directly to DNA in the presence of histones cannot be ruled out.

Binding of cAMP-Dependent Protein Kinase to Calf Thymus [^{3}H]Methyl DNA. The filter retention method of measuring binding of protein to labeled DNA, described under Experimental Procedure, was used to study DNA

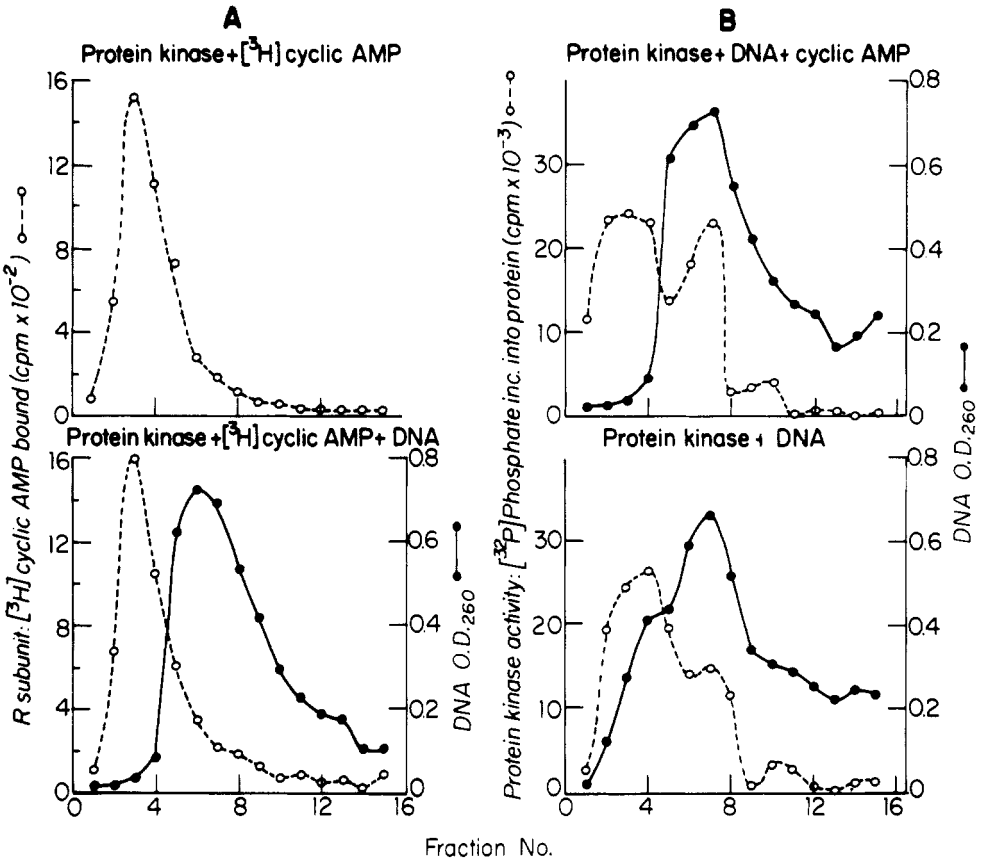

FIGURE 7: Sucrose density gradient centrifugation of cAMP-dependent protein kinase and DNA. Mixtures to be studied were layered on top of 4.5 ml of 5–25% sucrose density gradients and centrifuged for 3.5 hr at 200,000*g* as described under Experimental Procedure. (A) Comparison of sedimentation of the R subunit of calf thymus protein kinase (100 μg) in the presence or absence of calf thymus DNA (200 μg) and in the presence of 5×10^{-7} *M* [^{3}H]cAMP. Gradient fractions (0.3 ml, eluted from the top) were assayed for bound [^{3}H]cAMP as described under Experimental Procedure. Note that DNA has little effect on the position of migration of cAMP-binding protein. (B) Comparison of sedimentation of catalytic activity of cAMP-dependent protein kinase (100 μg) in the presence of native calf thymus DNA (200 μg) and in the presence or absence of 5×10^{-7} *M* cAMP. Gradient fractions (0.3 ml, eluted from the top) were assayed for histone kinase activity in the presence of 5×10^{-6} *M* cAMP as described under Experimental Procedure. Note that a portion of the kinase catalytic activity migrates to the peak position of migration of the DNA, and that kinase activity migrating with the DNA is increased in the presence of cAMP. Protein kinase centrifuged in the absence of DNA (data not shown) yields a sharp peak of activity at tube 3 and a minor peak at tube 6. cAMP only slightly affects the sedimentation pattern of protein kinase in the absence of DNA.

Table I: Binding of a Calf Thymus cAMP-Dependent Protein Kinase Preparation to [^{3}H] Methyl Calf Thymus DNA.

Additions[a]	[^{3}H] Methyl DNA Retained on Filter[b]
Control: PK II omitted	60
Control + 10^{-6} *M* cAMP	60
Control + 10^{-6} *M* ATP	62
PK II (25 μg)	3810
PK II + 10^{-6} *M* cAMP	4920
PK II + 10^{-6} *M* ATP	5100
PK II + 10^{-6} *M* cAMP + 10^{-6} *M* ATP	6180

[a] Sheared calf thymus DNA was denatured prior to labeling by heating for 2 min at 100° followed by rapidly cooling at 0°. DNA was labeled with [^{3}H] methyl groups as described in Experimental Procedure and used at a specific activity of 3950 dpm/μg of DNA. All tubes received 20 μg of labeled DNA. [b] DNA binding was measured by the filter retention method described under Experimental Procedure. Values presented are averages of duplicate points obtained in a representative experiment.

binding to protein kinase since this method provides quantitative measurements of DNA bound by very small quantities of protein. It was found that binding of extensively purified calf thymus kinase (PK II) to calf thymus DNA proceeds optimally at low ionic strength (0–0.04 *M* NaCl) and at slightly acidic pH. Binding is best at pH 6.85, but significant binding can be detected from pH 6.0 to pH 7.4. These conditions may reflect the ability of the kinase preparation to adhere to the nitrocellulose filter as well as its ability to bind to DNA. Table I describes the effects of cAMP and ATP, two nucleotides known to bind to cAMP-dependent protein kinase, upon the ability of PK II to bind labeled calf thymus DNA. 10^{-6} *M* cAMP and 10^{-6} *M* ATP each stimulate the binding of PK II to the DNA by about 30%, and the effects of these agents, at the concentrations used, are approximately additive. It can be seen that cAMP enhances DNA binding by PK II by the same amount in either the presence or absence of added ATP. Figure 9 shows the effect of 10^{-6} *M* cAMP upon DNA binding by different concentrations of PK II. At low concentrations of PK II, cAMP stimulates DNA binding more than twofold. Stimulation of DNA binding by cAMP was observed at both pH 6.0 and pH 6.9. The effect of cAMP was significantly diminished at pH 7.4. The addition of 10 m*M* $Mg(OAc)_2$ to the incubation mixture increases DNA binding by the kinase preparation about twofold, but does not affect the percentage of stimulation of binding induced by cAMP or ATP. Stimulation of protein binding to DNA by cAMP was also observed in DNA-binding studies employing a com-

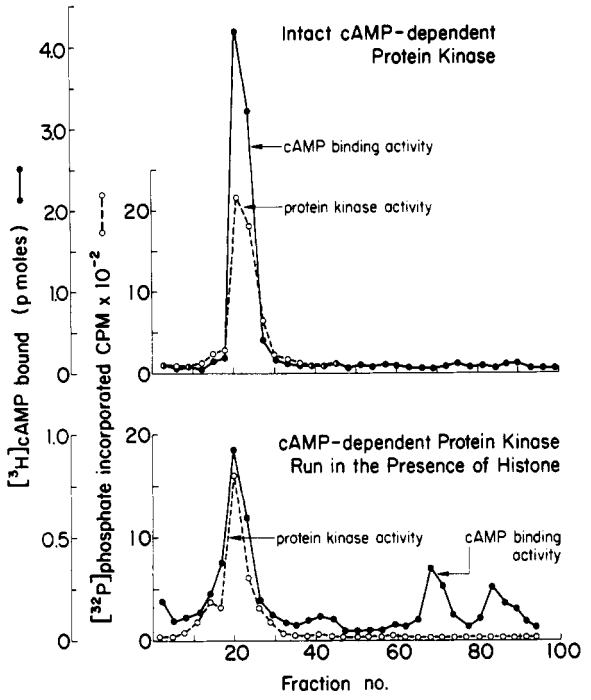

FIGURE 8: cAMP-dependent protein kinase chromatographed on DNA aminoethyl Sepharose in the absence or presence of histone. (Top panel) Chromatography in the absence of histone. Protein kinase (1.0 mg) was chromatographed as described under Experimental Procedure on a column (10 cm × 1.5 cm diameter) containing 25 mg of native calf thymus DNA. Elution was with a linear gradient from 0.05 to 1.0 *M* NaCl. Column fractions (2 ml) were assayed for cAMP-binding and protein kinase activities as described under Experimental Procedure. Histone kinase activity and cAMP binding activity elute at 0.1–0.2 *M* NaCl. (Bottom panel) Chromatography after preincubation of protein kinase (1.0 mg) with whole calf thymus histone (1.0 mg) for 10 min at 37° in 0.1 *M* sodium acetate buffer (pH 6.5) containing 10 m*M* $MgCl_2$, followed by overnight dialysis against 0.05 *M* NaCl, 0.1 m*M* Na_2EDTA, 1 m*M* 2-mercaptoethanol, 10 m*M* sodium bisulfite, 10% glycerol, and 10 m*M* Tris-Cl (pH 7.4). Chromatography was carried out, and cAMP-binding and histone kinase activities were determined as described for the top panel. Note that in the presence of histone cAMP-binding activity of protein kinase eluting at 0.1–0.2 *M* NaCl is reduced, while cAMP-binding activity dissociated from kinase activity is eluted at higher (0.6–0.9 *M*) NaCl concentrations.

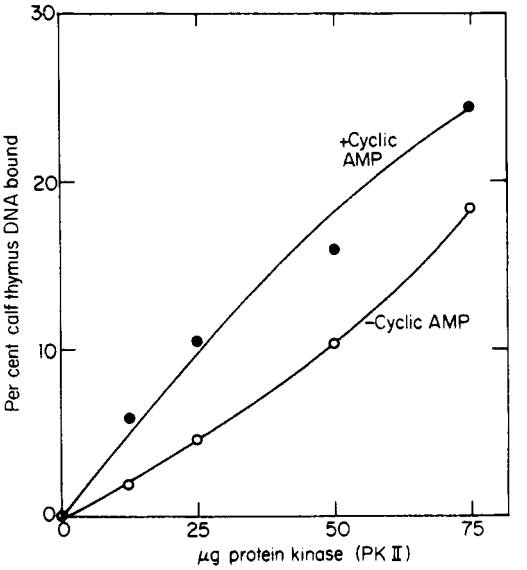

FIGURE 9: Effect of cAMP on binding of [^{3}H]methyl calf thymus DNA by a calf thymus cAMP-dependent protein kinase preparation; 25 μg of labeled denatured calf thymus DNA (3950 dpm/μg of DNA) were incubated with various amounts of calf thymus PK II in the absence or presence of 10^{-6} *M* cAMP and in the presence of 5 m*M* $Mg(OAc)_2$ and 10^{-6} *M* ATP, and protein-bound DNA was measured by the filter retention technique described under Experimental Procedure.

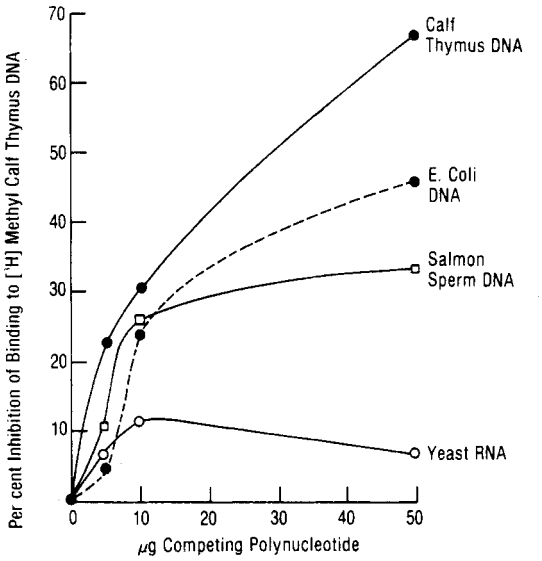

FIGURE 10: Comparison of the abilities of various unlabeled polynucleotides to compete with [^{3}H]methyl calf thymus DNA for binding to a calf thymus cAMP-dependent protein kinase preparation. Varying amounts of unlabeled polynucleotides were preincubated with 20 μg of PK II in the usual assay mixture in the presence of 10^{-6} *M* ATP and 10^{-6} *M* cAMP for 10 min at 25° before addition of 25 μg of [^{3}H]methyl calf thymus DNA (3950 dpm/μg of DNA). After an additional 30 min at 25°, samples were filtered, and filters were washed and assayed for radioactivity as described under Experimental Procedure. Values presented are averages of duplicate points from a representative experiment. The competing polynucleotides were used without shearing or denaturation. Note that unlabeled calf thymus DNA is the most effective of the polynucleotides used at inhibiting binding of the protein kinase preparation to labeled calf thymus DNA.

mercially prepared bovine heart protein kinase preparation. Stimulation of binding of PK II to DNA by cAMP, shown in Figure 9, is in agreement with results presented in Figure 7, in which it was observed that cAMP enhances binding of the C subunit of protein kinase to DNA. In separate experiments, not shown, it was observed that the ability of cAMP to stimulate DNA binding of the protein kinase preparation was maximal at about 10^{-7} *M* and half-maximal at about 5×10^{-9} *M* cAMP.

Figure 10 is a comparison of the abilities of various unlabeled polynucleotides to inhibit the binding of PK II to labeled calf thymus DNA. It can be seen that calf thymus DNA is the most effective of the polynucleotides tested for ability to compete with labeled calf thymus DNA for binding to the protein kinase preparation; 50 μg of added unlabeled calf thymus DNA decreased binding to the labeled DNA by 66%. Salmon DNA and *E. coli* DNA were considerably less effective than celf thymus DNA in their ability to compete with the labeled DNA. Maximum observed inhibition by each of these DNA types was less than 50%. Yeast RNA has almost no effect on the binding of labeled calf DNA by PK II. This result indicates that the calf thymus protein kinase preparation exhibits specificity in its preference for binding calf thymus DNA over the other types of DNA and RNA tested.

Discussion

Results of this study identify cAMP-dependent protein kinase as a major receptor for cAMP in the calf thymus nu-

cleus and indicate that binding of this enzyme to DNA may constitute an important aspect of its mechanism of action. cAMP-dependent protein kinase is present in calf thymus nuclei that have been washed repeatedly with 0.25 *M* sucrose and with a 0.14 *M* NaCl solution containing divalent cations. Less than 10% of the cAMP-dependent protein kinase activity originally present in crude thymus homogenates is found in nuclei isolated as described and washed two times with 0.14 *M* NaCl. However, when nuclei are isolated by nonaqueous procedures, which preserve in the nuclei many soluble components, cAMP-dependent protein kinase activity is present in the nuclei at about the same specific activity as in whole thymus homogenates (E. M. Johnson and V. G. Allfrey, unpublished observations). When thymus nuclei are dissociated with 2 *M* NaCl and the extracted proteins subjected to Bio-Rex 70 column chromatography, cAMP-dependent protein kinase is detected in the nuclear acidic protein fraction eluting at 0.4 *M* NaCl. The acidic proteins of the nucleus include proteins which have been implicated in the positive control of transcription from chromatin (Gilmour and Paul, 1969; Spelsberg et al., 1971; Stein et al., 1972; Kostraba and Wang, 1972) or from free DNA (Teng et al., 1971) *in vitro*.

When the nuclear acidic protein fraction is subjected to chromatography on DNA aminoethyl Sepharose, several peaks of cAMP binding activity are resolved (Figures 1 and 4). It is notable that peaks of cAMP-binding activity are eluted from the column at high concentrations of NaCl. It is at present not known whether these peaks represent different cAMP-binding proteins or whether they represent differing affinities of the same protein for separate DNA regions. Recent studies indicate that cAMP-binding proteins with an affinity for the DNA column can be released from the column by treatment with deoxyribonuclease (Johnson et al., 1975a). Two peaks of cAMP-binding activity have been detected in a rat liver nuclear acidic protein preparation subjected to phosphocellulose column chromatography (Rikans and Ruddon, 1973). Evidence has previously been presented that cyclic nucleotide binding proteins eluted from the DNA aminoethyl Sepharose column with concentrations of NaCl >0.6 *M* include components which bind cGMP preferentially over cAMP (Johnson et al., 1975b; Allfrey et al., 1975). Data presented in Figures 2 and 3 indicate that the cAMP binding protein with highest activity in the nucleus possesses physical and chemical properties similar to those of the R subunit of cAMP-dependent protein kinase. This cAMP-binding protein elutes from the DNA column at 0.05 *M* NaCl (Figure 1), as does the R subunit of an extensively purified protein kinase (PK II) eluted in the presence of 10^{-7} *M* cAMP (Figure 5). The presence of protein kinase activity in the runoff fraction from the DNA column (Figure 4) hindered experiments done to determine whether the cAMP-binding protein in this fraction inhibits catalytic activity of isolated C subunits. It is of interest that this major peak of cAMP-binding activity, eluting at 0.05 *M* NaCl, corresponds to a protein kinase peak with which only slight activation by cAMP is obtained. If, as is likely, cAMP-binding activity in this fraction represents binding of the R subunit of cAMP-dependent protein kinase, it is possible that some R subunits in this fraction are not associated with protein kinase subunits. Since it has been observed that histones can dissociate cAMP-dependent protein kinase into R and C subunits (Miyamoto et al., 1973), the possibility must be considered that free R subunits capable of binding cAMP exist in the cell nucleus. It is also possible that our treatment of chromatin with 2 *M* NaCl could dissociate R and C subunits.

Protein kinases of the nucleus are heterogeneous in their ability to bind to the DNA aminoethyl Sepharose column. Four peaks of protein kinase activity are resolved by DNA affinity chromatography, including two peaks which possess cAMP-dependent kinase activity (Figure 4). Two prominent peaks of protein kinase activity, both of which have affinity for the DNA column, are completely independent of activation by cAMP (Figure 4). Heterogeneity of nuclear protein kinases has been noted previously (Takeda et al., 1971; Rikans and Ruddon, 1973; Kish and Kleinsmith, 1974). One peak of nuclear protein kinase activity elutes from the DNA column at 0.14 *M* NaCl, at the same position as does the more highly purified protein kinase holoenzyme (Figure 5).

The results of this study indicate that the C subunit of cAMP-dependent protein kinase from calf thymus binds extensively to calf thymus DNA while the R subunit does not. Binding of the C subunit to DNA can be observed upon DNA affinity chromatography (Figures 5 and 6) and upon sucrose density gradient centrifugation of protein kinase and DNA (Figure 7) in the presence of cAMP. Neither of these two procedures reveals significant binding of the R protein-cAMP complex to the DNA. In interpreting this result, limitations of the sensitivity of both the DNA affinity chromatography and sucrose density gradient centrifugation techniques must be considered. Neither of these techniques would allow detection of binding of protein to DNA regions which constitute only a small proportion of the total thymus DNA. cAMP enchances binding of the protein kinase to DNA (Figures 7 and 9, Table I). It is likely that this enhanced binding is a result of dissociation of R and C subunits, allowing, in some manner, a more favorable interaction of the C subunit with the DNA. The possibility must be considered that interactions of the kinase with DNA are influenced by other proteins present in the kinase preparations studied.

Data presented indicate that when the R protein is chromatographed in the presence of histone, it is retained on the DNA aminoethyl Sepharose column, eluting at a high concentration of NaCl (Figure 8). The ability of the R protein to bind to nucleohistone may be an important aspect of protein kinase activity in the nucleus. It is known that histones can induce partial dissociation and activation of protein kinase (Miyamoto et al., 1973; Figure 8 of this study) and that different histones are specific in their ability to induce differential changes in phosphorylation of non-histone proteins in isolated rat liver nuclei (Johnson et al., 1973). It is possible that histones bound at discrete sites along the DNA can activate protein kinase activity at these sites by binding to the R subunit of the enzyme.

The catalytic subunit of cAMP-dependent protein kinase binds to calf thymus DNA extensively and in such a manner that approximately physiological NaCl concentrations disrupt the binding, as determined by elution of the kinase from the DNA affinity column. Several gene regulatory proteins from bacteria have been shown to possess, in addition to their higher affinity for specific DNA regions, a weak affinity for DNA in general (Bourgeois, 1972). For example, the *lac* repressor binds weakly to all DNA and binds to the *lac* operator regions with an equilibrium dissociation constant of 1×10^{-13} *M* at <0.05 *M* ionic strength, pH 7.4 (Riggs et al., 1970). Increased ionic strength greatly reduces the affinity of several bacterial DNA binding pro-

teins for DNA (Bourgeois, 1972). Recent studies employing labeled mammalian DNA indicate that increasing concentrations of NaCl decrease total binding of certain non-histone chromatin proteins to DNA while enhancing the specificity with which these proteins bind DNAs from different animal species (Sevall et al., 1975).

Studies on binding of calf thymus PK II to labeled calf thymus DNA, if considered in the absence of supporting data, would not be adequate evidence of binding of protein kinase to DNA due to the possibility that protein contaminants of this preparation could significantly bind DNA. However, the observation that effects of cAMP upon DNA binding using this technique (Table I; Figure 9) are of approximately the same magnitude as those observed using the sucrose density gradient technique (Figure 7) suggests that much of the binding of PK II to labeled DNA actually represents binding of the kinase to the DNA. The possibility must be considered that phosphorylation of proteins in the PK II preparation plays a role in the binding of this preparation to the DNA. It should be noted that the stimulatory effects of cAMP and ATP upon DNA binding (Table I) occur in the presence of EDTA and in the absence of added Mg^{2+}. These are conditions unfavorable for protein phosphorylation.

Data presented in Figure 10 indicate that the PK II fraction from calf thymus binds preferentially to calf thymus DNA over several other polynucleotides and suggest that sequence specificity may be an aspect of DNA binding by cAMP-dependent protein kinase. Further investigation will illuminate the roles of the individual components of this protein kinase preparation in the specificity of DNA binding observed.

The physiological significance of DNA binding by protein kinase remains to be determined. The C subunit, as a basic protein, would be expected to bind preferentially to anionic substances. Results presented here suggest that there is some specificity in binding of the kinase to polynucleotides. Additional experiments will determine whether this specificity serves a regulatory function. Work from a number of laboratories indicates that phosphorylation of chromatin proteins may play a role in the regulation of transcription (cf. Allfrey et al., 1974, for review). Phosphorylation of specific nuclear acidic proteins occurs at times of elevated RNA synthesis during the cell cycle of synchronized HeLa cells (Karn et al., 1973) and in lymphocytes stimulated to divide (Kleinsmith et al., 1966; Johnson et al., 1974). Cyclic nucleotides may regulate phosphorylation of certain chromatin proteins. In rat liver dibutyryl cAMP stimulates in vivo phosphorylation of histone F1 (Langan, 1969) as well as of specific non-histone chromatin proteins (Johnson and Allfrey, 1972). cAMP has been observed to directly modify nuclear protein kinase activity (Rikans and Ruddon, 1973; Kish and Kleinsmith, 1974). cGMP has been implicated in the stimulation of phosphorylation of nuclear acidic proteins which occurs early after mitogen stimulation of horse lymphocytes (Johnson and Hadden, 1975). Binding of cyclic nucleotide dependent protein kinases to DNA may be part of a mechanism by which cyclic nucleotides can selectively regulate protein phosphorylation at specific sites in the chromatin. It has been observed that cAMP stimulates protein kinase catalyzed phosphorylation of RNA polymerase II preparations from rat liver (Martelo, 1973) and calf ovary (Jungmann et al., 1974) and thereby enhances RNA polymerase II activity. It is tempting to speculate that protein phosphorylation necessary for transcription occurs at discrete sites in the chromatin determined by binding of active protein kinase catalytic subunits to the DNA.

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