Cyclic AMP-Dependent Histone-Specific Nucleoplasmic Protein Kinase from Rat Liver

By JEFFREY R. NEUMANN,* ANN R. O’MEARA and ROBERT L. HERRMANN†
Department of Biochemistry, Boston University Medical Center, Boston, MA 02118, U.S.A.

(Received 22 July 1977)

A nucleoplasmic histone kinase activity was isolated from livers of adult rats and purified 39-fold compared with whole nuclei by ultracentrifugation of the nuclear extract and Sephadex G-200 gel filtration in the presence of cyclic AMP. Analysis by polyacrylamide-gel electrophoresis as well as by gel filtration indicates a mol. wt. of approx. 60,000 for the catalytic subunit and 130,000–150,000 for the cyclic AMP-binding activity. The purified enzyme displays a 20-fold greater preference for histone fractions 1 and 2b than for any non-histone substrate, including α-casein. Endogenous protein in the preparation is not appreciably phosphorylated. The unfraccionated enzyme is stimulated significantly by cyclic GMP, cyclic IMP and dibutyl cyclic AMP as well as by cyclic AMP. The catalytic reaction requires Mg²⁺ (Kₐ 1.9 mM) and ATP (Kₐ 15.4 μM). Half-maximal activity of the enzyme is observed with histone 2b at 12 μM and histone 1 at a higher substrate concentration. The pH optima are 6.1 and 6.5 with histones 2b and 1 respectively. This nuclear protein kinase appears to be distinct from other nuclear enzymes that have been reported, on the basis of histone specificity, univalent-salt-sensitivity, pH optima and nuclear location. However, the enzyme possesses many properties similar to those of the cytoplasmic kinases, including cyclic AMP-dependence, Mg²⁺ and ATP affinities and pH optima. It differs from cytoplasmic protein kinase type I, the major form in the liver, with respect to bivalent-cation effects and response to the heat-stable protein kinase inhibitor protein isolated from ox heart.

Recent investigations implicate the phosphorylation of histones in the control of both replicative and transcriptional processes. Phosphorylation of histones is observed during both the DNA-synthetic and the mitotic phases of the cell cycle. Lysine-rich histone 1† has been observed to be extensively phosphorylated in developing (Balhorn et al., 1972a) and regenerating (Balhorn et al., 1972b) rat liver and in tumour and tumour-cell-culture systems (Balhorn et al., 1972c). In addition, histone phosphorylation also appears to play a gene-regulatory role. In rat liver stimulated by glucagon or cyclic AMP administration, a specific amino acid residue in histone 1 (serine-37) is phosphorylated (Langan, 1971). Farago et al. (1975) have demonstrated in vivo a similar site-specific (serine-36) modification of histone 2b. Watson & Langan (1973) have shown that the specifically phosphorylated histone 1 is capable of increasing template activity for RNA polymerase in reconstituted chromatin, and Fontana & Lovenberg (1973) have made the same observation on calf thymus chromatin treated with protein kinase. Additional support for this type of function is the finding that site-specific histone phosphorylation may be cyclic AMP-dependent (Langan, 1968; Kish & Kleinsmith, 1974; Sherod et al., 1975). That the specific phosphorylation of histones by kinases may be of physiological significance is reinforced by the demonstration in rat liver of a phosphoprotein phosphatase specific for histones (Meisler & Langan, 1969).

Although isolated cytoplasmic protein kinases, of which the predominant type in rat liver is type I (Corbin et al., 1975), readily phosphorylate histones, it has been demonstrated that the phosphorylation of histones, which is significant for replication or transcription, occurs in the nucleus (Oliver et al., 1972, 1974; Ord & Stocken, 1975). It is thus logical to assume that any gene-regulatory role for histone phosphorylation requires a nuclear kinase capable of phosphorylating interphase histones in response to external signals such as hormones.

Paradoxically, there have been only a few reports of nuclear kinases utilizing histones as substrate.
Those nuclear enzymes that have been described display preference for acidic proteins such as casein, phosvitin or endogenous non-histone acidic proteins. Relatively few investigators have reported cyclic AMP-dependent nuclear enzymes. Thus it remains to be demonstrated that there can be isolated from the nucleus a cyclic AMP-dependent protein kinase that is capable of phosphorylating histones and which may be responsible for the observed modification in vivo of these basic proteins.

A current concept that has received much support is the phenomenon of translocation of protein kinases to nuclei under the influence of a cytoplasmic hormone signal. This has been observed in rat adrenal medulla (Costa et al., 1976), perfused rat liver (Castagna et al., 1975) and calf ovary (Jungmann et al., 1975). These investigators report hormone-induced increases in nuclear kinase activity at the expense of cytoplasmic activity. However, except for the work of Castagna et al. (1975), all of the nuclear activities show preference for acidic proteins as substrate and are not cyclic AMP-dependent. Although Jungmann et al. (1975) observed cyclic AMP-binding activity in nuclei after stimulation, the kinase activity is not able to be stimulated by cyclic AMP.

The present paper details the isolation and partial characterization of a nucleoplasmic cyclic AMP-dependent histone kinase from rat liver. The enzyme was prepared under conditions of low ionic strength; recent reports indicate that such conditions may be essential for the preservation of the integrity of the cyclic AMP-dependent holoenzyme (Corbin et al., 1975; Johnson et al., 1975; Keller et al., 1976). We believe that the high-salt and sonic-disruption methods used by other investigators to extract and/or fractionate nuclear kinases may have resulted in the dissociation of the nucleoplasmic cyclic AMP-dependent activity. A detailed discussion of this proposition is included.

Experimental

**Materials**

Cyclic AMP, cyclic GMP, calf thymus histones and calf thymus histone subfractions, α-casein mixture, bovine serum albumin (Cohn fraction V), phosvitin, phosphorylase b, phosphorylase kinase, calf thymus DNA and the protein kinase inhibitor from ox heart were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ-32P]ATP [tetakis-(triethylammonium) salt] and cyclic [3H]AMP were purchased from New England Nuclear Corp. (Boston, MA, U.S.A.). Purified α-casein and dephosphorylated α-casein were generously given by Dr. Elizabeth Bingham of the Eastern Regional Research Laboratory, U.S. Department of Agriculture, Philadelphia, PA, U.S.A. Marker proteins for column and electrophoresis calibration were purchased from Boehringer Mannheim (Indianapolis, IN, U.S.A.). Bivalent metals were obtained in chloride form from Fisher Scientific (Medford, MA, U.S.A.), and were of reagent grade.

**Preparation of nuclei**

All operations were performed at 2–4°C unless otherwise specified. Male Sprague–Dawley rats, (2 months old) obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.) and fed ad libitum, were killed by cervical dislocation; liver cell nuclei were prepared by a modification of the method of Blobel & Potter (1966). Livers (100–200 g) were minced finely and washed several times in ice-cold 0.25 M-sucrose/TKM buffer [0.05 M-Tris/HC1 (pH 7.5)/0.025 M-KCl/0.005 M-MgCl2]. The tissue was homogenized by means of 20–25 strokes in a Dounce glass homogenizer with loose-fitting pestle, and then filtered through four layers of cheesecloth. Sufficient 2.3 M-sucrose/TKM buffer was added to adjust the homogenate to 0.34 M in sucrose. Crude nuclei (fraction NI) were obtained by centrifuging the homogenate at 900 g, for 10 min. The pellet, suspended in 0.34 M-sucrose/TKM buffer containing 0.2% Triton X-100, was homogenized in the Dounce homogenizer by 10 strokes of the loose pestle, and centrifuged (900 g for 10 min) to yield fraction NII. Fraction NII was suspended in 0.34 M-sucrose/TKM buffer, combined with 2 vol. of 2.3 M-sucrose/TKM buffer, mixed, and layered over 10 Ml of 2.3 M-sucrose/TKM buffer in each of six cellulose nitrate centrifuge tubes [2.5 cm x 8.8 cm (1 in x 3½ in)]. Centrifugation in a Beckman SW 27 rotor at 88000 g, for 60 min produced a pellet, fraction III, which contained nuclei free of cytoplasmic contamination when examined by phase-contrast microscopy, electron microscopy and by fluorescence microscopy after staining with Acridine Orange.

**Electron microscopy of nuclei**

Nuclei were examined by electron microscopy after sample preparation by the method of Karnovsky (1965).

**Acridine Orange staining of nuclei**

The method of Bartalanffy (1962) as modified by Gurley et al. (1973) was used to stain nuclei for fluorescence microscopic examination.

**Preparation of nuclear enzyme**

The procedure given is based on 300 g of liver.

**Preparation of crude nuclear extract.** Purified nuclei prepared by the method described above were suspended in 105 ml of NaCl/EDTA (0.025 M-NaCl/0.008 M-EDTA, pH 7.5). The nuclear pellets were dispersed with a 'rubber policeman' and stirred in the cold. After 30 min the suspension was centrifuged at 8000 g, for 5 min and the supernatant was 1978
collected (first nuclear extract). The pellet was suspended in an additional 105 ml of NaCl/EDTA by homogenization (five to ten strokes with a loose-fitting Dounce pestle). This homogenate was stirred for 10 min and the extracted nuclei were collected by centrifugation as above. The supernatant (second nuclear extract) was immediately combined with the first supernatant, mixed and the combined extracts clarified of a small amount of precipitate by centrifugation at 8000g for 10 min.

**Ultracentrifugation of crude extract.** The clarified crude extract (210 ml) was immediately divided among six cellulose nitrate tubes (2.5 cm x 8.8 cm) and centrifuged for 5 h at 25000 rev/min (88000g) in the Beckman SW 27 rotor. This procedure produced a small clear gelatinous pellet of chromatin which was discarded. The supernatant (88000g supernatant) was dialyzed exhaustively against 0.01 M-Tris/HCl (pH 7.3) / 0.025 M-KCl / 0.01% NaN₃. After dialysis, the enzyme preparation was concentrated from approx. 210 ml to 10–15 ml by using an Amicon model 65 stirred ultrafiltration cell fitted with a UM-10 membrane (Amicon Corp., Bedford, MA, U.S.A.).

**Gel filtration of 88000g supernatant.** Protein kinase in the 88000g supernatant was chromatographed in descending fashion on a Sephadex G-200 column (2.6 cm x 62 cm) that had been pre-equilibrated with 0.01 M-Tris/HCl (pH 7.3) / 0.025 M-KCl / 0.01% NaN₃. Fractions (3.55 ml) were collected, with alternate fractions sometimes containing 0.5 ml of a 5% (w/v) bovine serum albumin solution to stabilize the kinase activity.

At times the column was equilibrated with the same buffer containing 5 μM-cyclic AMP. When the 88000g supernatant was to be fractionated on a cyclic AMP-equilibrated column, it was first incubated as follows: to 5 ml of the 88000g supernatant was added 0.6 ml of 150 nm-cyclic [³H]AMP (4.8 mCi/mmol). After incubation for 10 min at 37°C, 0.3 ml of 100 μM-cyclic AMP was added with mixing. This mixture was immediately applied to the column and eluted with the eluent buffer containing 5 μM-cyclic AMP. Conditions were otherwise identical with those described above.

Histone kinase activities were identified by assaying individual fractions. Peak kinase fractions were pooled and concentrated approx. 20-fold with the Amicon ultrafiltration device as described above. When the column was operated with cyclic AMP, location of the regulatory subunit (³H-binding activity) was done by mixing 0.5 ml of each fraction with 4.5 ml of Aquasol (New England Nuclear Corp.) and measuring radioactivity in a Packard Tri-Carb liquid-scintillation spectrometer.

**Preparation of cytoplasmic enzyme**

Cytoplasmic protein kinase was partially purified from the cytosol of rat liver by the method of Keller et al. (1976). The predominant protein kinase activity (over 75%) was eluted from the DEAE-cellulose column between 0.05 M and 0.10 M NaCl and was designated as protein kinase type I according to the classification system of Corbin et al. (1975). The small type-II peak eluted at 0.25 M NaCl was not used for the present study.

**Rat liver nuclear protein preparation**

Rat liver whole histones were prepared from chromatin by the acid extraction technique of Panyim et al. (1971) and the histone subfractions further purified on Bio-Gel P-100 by the method of Sommer & Chalkley (1974). Total non-histone proteins were prepared by the method of Kish & Kleinsmith (1974) up to and including the Bio-Gel 70 batch-chromatography step.

**Protein kinase assay**

The assay mixture, adapted from that of Kuo & Greengard (1969), contained, in a final volume of 0.2 ml: 15 mM-potassium phosphate buffer, pH 6.9; 2 mM-theophylline; 2.5 mM-NaF; 10 mM-MgCl₂; 50 μM-[³²P]ATP (specific radioactivity 1–2 Ci/mmol); 1 μM-cyclic AMP; variable amounts of both substrate protein and enzyme preparation. The reaction was initiated by the addition of ATP, incubated at 37°C for 10 min, and terminated by the addition of 2 ml of ice-cold 30% (w/v) trichloroacetic acid / 0.25% Na₂WO₄ / 0.2 M NaH₂PO₄, pH 2. After 5 min at 0°C, 1.2 mg of bovine serum albumin was added and, after mixing, an additional 2 ml of the above trichloroacetic acid/tungstate/phosphate solution was added. After standing for 10 min the precipitated protein was collected by centrifugation at 2000g for 10 min. The protein pellets were solubilized in 0.2 ml of 1 M NaOH, diluted with 2 ml of 30% trichloroacetic acid/0.25% Na₂WO₄, pH 2, and precipitated with 0.2 ml of 2.5 M H₂SO₄. The precipitated protein was sedimented as described above and the washing procedure repeated. The final pellet was solubilized in 0.6 ml of 1 M NaOH and counted for radioactivity in Aquasol in a Packard Tri-Carb liquid-scintillation spectrometer.

A unit of kinase activity is defined as that amount of enzyme catalysing the transfer of 1 pmol of phosphate to protein substrate/min.

**Glucose 6-phosphate dehydrogenase assay**

The method utilized was that of DeMoss (1955), by following the increase in As₄₅₀ with time as a function of enzyme concentration.

**Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis**

Enzyme fractions were analysed by the method of
Weber & Osborn (1969) in 5% gels. Protein standards used in calibration were: cytochrome c (mol.wt. 12700), chymotrypsinogen A (mol.wt. 25000), ovalbumin (mol.wt. 45000), bovine serum albumin (mol.wt. 67000) and phosphorylase b (mol.wt. 94000).

Other determinations

Protein was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard. DNA concentrations were determined by the method of Burton (1956), with calf thymus DNA as standard.

Results

Isolation and purification of nuclear protein kinase

Purified nuclei were examined by electron microscopy and fluorescence microscopy of Acridine Orange-stained preparations and shown to be free of particulate cytoplasmic contamination.

To eliminate the possibility of contamination by adsorbed soluble enzymes, marker-enzyme studies were carried out. Table 1 shows the results of one such experiment in which the activities of glucose 6-phosphate dehydrogenase and protein kinase were assayed in parallel throughout the nuclear-purification procedure. No detectable glucose 6-phosphate dehydrogenase activity was observed in the purified nuclear pellet, which contained 13% of the measurable cytosol kinase activity. Comparable experiments indicated that glucose 6-phosphate dehydrogenase contamination to the lower limit of 0.06% of the activity in a crude homogenate would have been detected in this assay system. Moreover, the sharp increase in specific activity of the protein kinase between fractions NII and NIII indicates that the removal of the contaminants in processing fraction NII to NIII resulted in a significant decrease in protein, but no loss of protein kinase activity. Consequently, the protein kinase activities of the final nuclear pellet are true components of the nuclei and not merely contamination by cytoplasmic kinase activities.

Nucleoplasmic proteins were extracted from the purified nuclei with successive NaCl/EDTA washes. Two 30 min washes with 0.025M-NaCl/0.008M-EDTA, pH 7.5, with gentle stirring were sufficient to extract 25-35% of the total nuclear kinase activity. The combined washes (crude nuclear extract) contained considerable material with chromatin-like characteristics, as established by the protein/DNA ratio of 2:1 (w/w) in the crude extract and by the observation that the preparation underwent a hyperchromic shift of 23% with a Tm (midpoint of heat-denaturation profile) of 76.5°C (measured in 0.25 mM-EDTA, pH 7.0). These data are consistent with published observations of melting characteristics for rat liver chromatin (Marushige & Bonner, 1966). That the kinase activity in the crude extract did not represent the chromatin-bound activity reported by other investigators (Lake & Salzman, 1972; Siebert et al., 1971; Takeda et al., 1974) was

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (mg/ml)</th>
<th>Activity (units/ml)</th>
<th>Specific activity (units/mg)</th>
<th>Activity (total units/% of cytosol)</th>
<th>Specific activity (total units/% of cytosol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude homogenate</td>
<td>23.0</td>
<td>560</td>
<td>28000</td>
<td>24</td>
<td>301</td>
</tr>
<tr>
<td>Cytosol</td>
<td>10.0</td>
<td>670</td>
<td>33500</td>
<td>100.0</td>
<td>67</td>
</tr>
<tr>
<td>Fraction NI</td>
<td>9.7</td>
<td>60</td>
<td>3000</td>
<td>9.0</td>
<td>6</td>
</tr>
<tr>
<td>Fraction NII</td>
<td>8.8</td>
<td>20</td>
<td>1000</td>
<td>3.0</td>
<td>2</td>
</tr>
<tr>
<td>Fraction NIII</td>
<td>3.0</td>
<td>(0.7)</td>
<td>(19.2)</td>
<td>(0.06)</td>
<td>—</td>
</tr>
</tbody>
</table>

Table 1. Distribution of protein kinase and glucose 6-phosphate dehydrogenase activities in subcellular fractions

The activities of glucose 6-phosphate dehydrogenase and protein kinase were monitored at each purification step in the subcellular fraction and nuclear purification. Briefly, the fractions were derived as follows: crude homogenate is the tissue homogenized in 0.25M-sucrose/TKM buffer and filtered through cheesecloth; cytosol refers to the supernatant derived from centrifugation of the crude homogenate for 45 min at 120000g av, in the Beckman Ti50 rotor; fraction NI is the 1000g pellet of the crude homogenate; fraction NII is the 1000g pellet obtained after homogenization of fraction NI in sucrose/TKM buffer containing 0.2% Triton X-100; fraction NIII is the purified nuclear pellet, the result of centrifugation through 2.3M-sucrose/TKM buffer. The activity of protein kinase in the purified nuclei (fraction NIII) is the activity actually measured in the nuclei recovered, which were 17%, based on the recovery of DNA from the crude homogenate. Although no glucose 6-phosphate dehydrogenase activity was detected in the fraction NIII, the values in parentheses indicate the maximum possible activity present at the lower limit of sensitivity of the method used.
NUCLEOPLASMIC HISTONE-SPECIFIC KINASE indicated by the purification step in which the enzyme was separated from the bulk of the nucleoprotein by ultracentrifugation at 88000g for 5 h. By this procedure more than 90% of the DNA and 70–80% of the protein present in the crude extract were sedimented completely and the protein kinase in the resulting supernatant underwent a 4-fold purification (Table 2). Indeed, the increased activity in the 88000g supernatant is not inconsistent with the removal of an inhibitor; however, this phenomenon was not further investigated.

The kinase activity in the 88000g supernatant was further purified by chromatography on a Sephadex G-200 column. This procedure was carried out in either the presence or the absence of cyclic AMP. When the column was operated in the absence of the cyclic nucleotide, the kinase activity was eluted as two peaks (Fig. 1), one corresponding to the void volume (NKI) and a second activity corresponding to a mol. wt. of 300000–400000 (NKII). We have observed that both activities, especially NKII, are eluted from the column in a very unstable form and with a low yield, unless bovine serum albumin is included in the fraction-collection tubes to stabilize the enzyme as it is eluted from the column. When the latter precaution was observed, a 2.3-fold purification of the NKII activity over that of the 88000g supernatant was attained (Table 2). The NKII activity was stimulated 2-fold by cyclic AMP with whole histones as substrate, and 3.5-fold with histone 1 as the phosphate acceptor. This increased stimulatability in the presence of the preferred substrate has been consistently observed in this laboratory. NKI was judged to be an aggregated form of the enzyme and was not further investigated.

![Fig. 1. Sephadex G-200 column chromatography of 88000g supernatant](image)

The 88000g supernatant (5 ml, 5.25 mg) was chromatographed in descending fashion on a column (2.6 cm × 62 cm) of Sephadex G-200. The flow rate was 10 ml/h; fractions (3.55 ml) were collected and analysed for protein kinase activity by using 500 μg of unfraccionated calf thymus histones as phosphate acceptor protein. Duplicate assays were performed in either the presence (●) or the absence (○) of cyclic AMP. The cyclic AMP stimulation is plotted as the ratio of activity in the presence and absence of cyclic AMP (▲).

### Table 2. Purification of nuclear kinase activity

Protein kinase was purified from nuclei by the procedure described in the Experimental section. The enzyme activity was measured at each stage by the protein kinase assay (see the Experimental section), with 500 μg of unfraccionated calf thymus histone as substrate. All assays were performed in duplicate in the presence of 1 μM-cyclic AMP. Data are the average of three preparations. The average starting material was 174 g of liver.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Yield (%)</th>
<th>Overall Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Volume (ml)</td>
<td>(units/ml)</td>
<td>Protein (mg/ml)</td>
<td>(units/mg)</td>
</tr>
<tr>
<td>Nuclear suspension</td>
<td>59</td>
<td>292.7</td>
<td>17269</td>
<td>3.500</td>
</tr>
<tr>
<td>Crude nuclear extract</td>
<td>117</td>
<td>49.7</td>
<td>5555</td>
<td>0.180</td>
</tr>
<tr>
<td>88000g supernatant</td>
<td>117</td>
<td>54.3</td>
<td>6390</td>
<td>0.049</td>
</tr>
<tr>
<td>Whole nuclear kinase activity after fractionation on Sephadex G-200 (NKII)</td>
<td>34</td>
<td>50.7</td>
<td>1725</td>
<td>0.022</td>
</tr>
<tr>
<td>Catalytic activity of nuclear kinase after fractionation on Sephadex G-200 in the presence of 5 μM-cyclic AMP (NKIII)</td>
<td>36</td>
<td>36.2</td>
<td>1300</td>
<td>0.011</td>
</tr>
</tbody>
</table>
The observed cyclic AMP stimulation of the NKII activity suggested that this enzyme might be a 'classic' cyclic AMP-dependent protein kinase, similar in nature to cytoplasmic enzymes that have been previously well characterized (Brostrom et al., 1970). In an effort to determine whether cyclic AMP acted by dissociating this large enzyme into regulatory and catalytic subunits, the 88000g supernatant was incubated with cyclic [\(^3\)H]AMP and chromatographed on a Sephadex G-200 column that had been equilibrated with unlabelled cyclic AMP, giving the elution profile shown in Fig. 2. Although the aggregated form NKI still appeared, NKII was no longer apparent and a previously unobserved activity NKIII was eluted at a volume corresponding to mol.wt. 40000-45000. A macromolecular form of cyclic [\(^3\)H]AMP occurred at mol.wt. 130000-150000, indicating what may be a cyclic AMP-binding regulatory subunit (R), since the NKIII activity (purified 40-fold compared with nuclei) is not stimulated by cyclic AMP. The regions corresponding to activities NKII and NKIII and subunit R were concentrated and subjected to electrophoresis in sodium dodecyl sulphate/polyacrylamide gels. The resulting analyses (Fig. 3) demonstrated a relatively pure peptide of mol.wt. 60000 in fraction NKIII (arrow, curve c). When the cyclic [\(^3\)H]AMP-binding peak fractions were pooled and electrophoresed (curve b), a peptide of approx. mol.wt. 68000

![Histone kinase activity (units/fraction) vs. Fraction no.](image)

**Fig. 2. Effect of cyclic AMP on the resolution of kinase activity by Sephadex G-200 gel filtration**

A 5 ml portion (6mg) of protein of the 88000g supernatant was mixed with 0.6 ml of 150nm-cyclic [\(^3\)H]AMP (4.8 mCi/mmol). After standing for 10 min at 4°C, 0.3 ml of unlabelled 21 μm-cyclic AMP was added. The mixture (5.9 ml) was chromatographed on Sephadex G-200 as described in the legend to Fig. 1, except that the column was pre-equilibrated with 3 vol of 0.01 m-Tris/HCl (pH 7.5)/0.025 m-KCl/0.02% Na\(_2\)SO\(_4\), containing 5 μm-cyclic AMP. Collecting tubes contained 0.5 ml of 5% (w/v) bovine serum albumin to stabilize the enzyme activity; this accounts for the significant increase in yield of kinase activity over that seen in Fig. 1. After elution, fractions were assayed (as described in the Experimental section) for kinase activity (●) and cyclic [\(^3\)H]AMP-binding activity (○). R, Cyclic AMP-binding regulatory subunit.

![Electrophoretic analysis of enzyme fractions](image)

**Fig. 3. Electrophoretic analysis of enzyme fractions**

Samples of the purified enzyme preparation were electrophoresed in sodium dodecyl sulphate/polyacrylamide gels as described in the Experimental section. All samples were from a single enzyme preparation: (a) NKII (Sephadex G-200 fractions 40-47 from Fig. 1; protein load of 50 μg); (b) cyclic [\(^3\)H]AMP-binding activity (Sephadex G-200 fractions 46-53 from Fig. 2; protein load of 30 μg); (c) NKIII (Sephadex G-200 fractions 59-68 from Fig. 2; protein load of 5 μg). Sample volume on all gels was 0.2 ml and electrophoresis was carried out for 4 h at 8 mA/gel. Gels were stained for 18 h, destained by diffusion and scanned at 550 nm. Molecular-weight calibration was made by using the protein standards described in the Experimental section. See the text for comments on peaks indicated by arrows.
NUCLEOPLASMIC HISTONE-SPECIFIC KINASE

The effects of cyclic nucleotides and protein kinase inhibitor protein on kinase activity were measured under standard assay conditions (see the Experimental section) with 500 μg of unfractionated calf thymus histone as substrate. All assays were performed at nucleotide concentrations of 1 μM. The protein kinase inhibitor, freeze-dried, from ox heart was prepared by Sigma Chemical Co. as described by Gilman (1970). It is noteworthy that protein kinase activity of the free catalytic subunit of the nuclear enzyme (NKIII), when incubated with 50 μg of inhibitor protein, was 50% inhibited with respect to an NKIII control.

Table 3. Effect of cyclic nucleotides and protein kinase inhibitor protein on kinase activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>Nuclear kinase 88000g supernatant</th>
<th>Cytoplasmic kinase type I</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Phosphate incorporated (pmol)</td>
<td>Activity (% of control)</td>
</tr>
<tr>
<td>Control</td>
<td>96.3</td>
<td>100</td>
</tr>
<tr>
<td>Cyclic AMP</td>
<td>230.1</td>
<td>239</td>
</tr>
<tr>
<td>Cyclic GMP</td>
<td>144.3</td>
<td>150</td>
</tr>
<tr>
<td>Cyclic IMP</td>
<td>219.6</td>
<td>228</td>
</tr>
<tr>
<td>Dibutyryl cyclic AMP</td>
<td>116.9</td>
<td>121</td>
</tr>
<tr>
<td>Adenosine</td>
<td>94.9</td>
<td>99</td>
</tr>
<tr>
<td>30 μg of inhibitor</td>
<td>126.2</td>
<td>131</td>
</tr>
<tr>
<td>50 μg of inhibitor</td>
<td>125.7</td>
<td>131</td>
</tr>
<tr>
<td>Cyclic AMP + 10 μg of inhibitor</td>
<td>239.6</td>
<td>249</td>
</tr>
<tr>
<td>Cyclic AMP + 30 μg of inhibitor</td>
<td>251.6</td>
<td>261</td>
</tr>
<tr>
<td>Cyclic AMP + 50 μg of inhibitor</td>
<td>251.6</td>
<td>261</td>
</tr>
<tr>
<td>Cyclic GMP + 10 μg of inhibitor</td>
<td>156.0</td>
<td>162</td>
</tr>
<tr>
<td>Cyclic GMP + 30 μg of inhibitor</td>
<td>175.6</td>
<td>182</td>
</tr>
<tr>
<td>Cyclic GMP + 50 μg of inhibitor</td>
<td>192.1</td>
<td>199</td>
</tr>
</tbody>
</table>

The results obtained with the inhibitor protein are noteworthy. Consistent with its postulated role as a form of regulatory subunit (Langan, 1973), the inhibitor decreased the activity of cytoplasmic type-I kinase and fraction NKIII. However, the 88000g supernatant form of the nucleoplasmic enzyme is slightly stimulated by the inhibitor. In the presence of both inhibitor and cyclic nucleotides the nuclear kinase activity is also slightly increased above that in the presence of the nucleotides alone. The explanation for these phenomena is unknown, but they remain a distinct difference between the nuclear kinase activity and cytoplasmic kinase activity.

Substrate specificity

To evaluate more effectively the role of cyclic AMP-dependent kinases in histone phosphorylation, and potentially in gene regulation, we sought to isolate a nuclear activity that might (a) respond to cyclic AMP and (b) phosphorylate histones preferentially. Such activities have been demonstrated in cytosol (Langan, 1968; Kumon et al., 1972), but no such enzymes have been well characterized in nuclei. Table 4 demonstrates that fraction NKIII fulfils the latter role. Although the enzyme(s) in the 88000g supernatant phosphorylated an array of proteins, including non-histones and the natural phosphoproteins casein and phosphitin, fraction NKIII displayed preference for histones, particularly histone 2b. Moreover, as the specific activity for

Effect of cyclic nucleotides

The 88000g supernatant and, as a control, cytoplasmic protein kinase type I, also prepared from rat liver, were investigated for their response to various cyclic nucleotides and to the heat-stable protein kinase inhibitor. The results are presented in Table 3: cyclic GMP and dibutyryl cyclic AMP, as well as cyclic AMP, stimulated both enzymes. Although stimulation of the nucleoplasmic kinase activity with both cyclic AMP and cyclic GMP was observed, cyclic AMP is most likely to be the physiological activator; for the nuclear protein kinase the apparent K_m for activation (15 nM) is one-seventieth of the apparent K_m for cyclic GMP (1.1 μM). It has been demonstrated that cyclic AMP occurs naturally in the micromolar range (Simon et al., 1973), whereas the concentrations at which cyclic GMP occurs, 1–100 nM (Goldberg et al., 1973; Gill & Kanstein, 1975), would be insufficient to activate the enzymes reported in the present work. Similarly, the physiological concentrations of cyclic IMP are much lower than the observed K_m.

(arrow, curve b) was greatly enriched compared with the same region in the electrophoretic profile of fraction NKiII (arrow, curve a). On the basis of the latter observation it is postulated that subunit R, as it was eluted under the non-denaturing conditions of gel filtration, was a dimer of a 68000-mol.wt. cyclic AMP-binding protein.
histone phosphorylation increased with purification, the ability of the enzyme to utilize proteins other than histones underwent a concomitant decrease.

**Enzyme stability**

Nuclear protein kinases isolated by other investigators have often been described as unstable (Ruddon & Anderson, 1972; Dastugue et al., 1974). This was also the case with the enzyme reported here. The enzyme was completely inactivated by a 10min exposure to temperatures above 40°C. It had a half-life of approx. 14 days at 4°C, the best condition found for storage. Storage at −20°C or −80°C resulted in significant loss of activity, despite the presence of such common stabilizing agents as glycerol, β-mercaptoethanol, dithiothreitol or reduced glutathione. Since phenylmethanesulphonyl fluoride, a potent inhibitor of proteolytic enzymes, was found to inhibit severely the nuclear protein kinase activity also, the possibility of a proteinase activity being co-purified with the kinase and accounting for the instability cannot be ruled out. A promising preliminary observation is that the enzyme appears stable to at least one cycle of freezing and thawing in the presence of 1% bovine serum albumin.

Together with the observation of improved yield after gel filtration in the presence of bovine serum albumin, these data point to a simple concentration effect on stability. The half-life of the enzyme at 4°C was also lengthened by the addition of 0.5% bovine serum albumin.

Attempts at ion-exchange chromatography of the enzyme (either 88000g supernatant or the NKIII form) were hindered by the apparently deleterious effects of univalent salts, including Na+, K+, ammonium and acetate ions. This phenomenon was slightly more pronounced with the 88000g supernatant activity, which additionally lost all cyclic AMP-dependence after DEAE-cellulose chromatography (results not shown). This does not occur with the cytoplasmic kinase, as shown by the fact that protein kinase type I was isolated from a DEAE-cellulose column and still exhibited the cyclic AMP-dependence demonstrated in Table 3. Again, the nuclear and cytoplasmic activities appear to be different.

**Kinetics**

Some kinetic parameters of the NKIII enzyme have been investigated. Fig. 4 shows that the pH

---

Table 4. **Substrate preference of kinase activity in 88000g supernatant and in NKIII** (free catalytic subunit after Sephadex G-200 fractionation in the presence of cyclic AMP)

The activities of the partially purified nuclear protein kinase (88000g supernatant) and the free catalytic subunit (NKIII) were tested with various substrate proteins. The standard protein kinase assay (see the Experimental section) was used and all assays were performed in duplicate.

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>88000g supernatant</th>
<th>NKIII</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity relative to that of calf thymus histone (%)</td>
<td>Activity relative to that of calf thymus histone (%)</td>
</tr>
<tr>
<td>None (endogenous substrate)</td>
<td>238 36</td>
<td>25 1</td>
</tr>
<tr>
<td>Calf thymus whole histone (2.5 mg/ml)</td>
<td>657 100</td>
<td>2316 100</td>
</tr>
<tr>
<td>Calf thymus histone 1 (2.5 mg/ml)</td>
<td>1341 204</td>
<td>6203 268</td>
</tr>
<tr>
<td>Calf thymus histone 2b (2.5 mg/ml)</td>
<td>522 79</td>
<td>4633 200</td>
</tr>
<tr>
<td>Calf thymus histone 2a (1.0 mg/ml)</td>
<td>440 67</td>
<td>886 38</td>
</tr>
<tr>
<td>Calf thymus histone 3 (2.5 mg/ml)</td>
<td>918 140</td>
<td>2620 113</td>
</tr>
<tr>
<td>Rat liver whole histone (2.5 mg/ml)</td>
<td>685 104</td>
<td>1456 63</td>
</tr>
<tr>
<td>Rat liver histone 1 (2.5 mg/ml)</td>
<td>—</td>
<td>3899 168</td>
</tr>
<tr>
<td>Rat liver histone 2b (2.5 mg/ml)</td>
<td>—</td>
<td>11949 516</td>
</tr>
<tr>
<td>Rat liver non-histones (0.05 mg/ml)</td>
<td>330 50</td>
<td>114* 5</td>
</tr>
<tr>
<td>Sigma α-casein mixture (2.5 mg/ml)</td>
<td>1624 247</td>
<td>76 3</td>
</tr>
<tr>
<td>Purified α-casein (2.5 mg/ml)</td>
<td>1140 174</td>
<td>76 3</td>
</tr>
<tr>
<td>Dephosphorylated α-casein (2.5 mg/ml)</td>
<td>0 0</td>
<td>532 23</td>
</tr>
<tr>
<td>Phosvitin (2.5 mg/ml)</td>
<td>232 35</td>
<td>25 1</td>
</tr>
<tr>
<td>Phosphorylase b (2.5 mg/ml)</td>
<td>365 56</td>
<td>— —</td>
</tr>
<tr>
<td>Phosphorylase kinase (2.5 mg/ml)</td>
<td>374 57</td>
<td>— —</td>
</tr>
</tbody>
</table>

* In this experiment the non-histone concentration was 0.1 mg/ml. The non-histone chromosomal proteins were prepared as described by Kish & Kleinsmith (1974) through the Bio-Rex 70 batch-chromatography step.
NUCLEOPLASMIC HISTONE-SPECIFIC KINASE

The apparent $K_m$ values for ATP and Mg$^{2+}$ were 15.4 $\mu$M and 1.9 $\mu$M respectively (Figs. 5 and 6), each parameter being determined at saturating concentration of the other reactants. Essentially the same values were calculated whether the substrate used was histone 2b or 1. The preference of the enzyme for rat liver histone 2b over 1 (Table 4) is reflected in substrate-saturation profiles for these two proteins (Fig. 7). The apparent $K_m$ for histone 2b is 12 $\mu$M [calculations of $K_m$ for histone 1 assume a mol.wt. of 20000 and for histone 2b a mol.wt. of 13800 (DeLange & Smith, 1971)]; the apparent $K_m$ for histone 1 is not clear. If the non-linear data of the reciprocal plot could be interpreted as substrate inhibition and if only the data at low substrate concentrations were considered, the apparent $K_m$ for histone 1 is 92 $\mu$M. However, if the data are indicative of some positive co-operative effects, the apparent $K_m$ calculated from the higher substrate concentrations at which the enzyme is activated is 19 $\mu$M. The lack of data at the very high substrate concentrations obscures a choice of interpretation; however, in either case the apparent $K_m$ for histone 1 is higher than that for histone 2b, thereby substantiating the data on substrate specificity presented in Table 4.

**Effect of bivalent metals**

Several bivalent metals were tested at 10$\mu$M, the optimum concentration for Mg$^{2+}$ in the kinase (NKIII) reaction. The results are presented in Table 5. For the nuclear kinase (NKIII) only Fe$^{2+}$ was highly effective in the reaction; the only other metal ion that could even partially restore activity was Pb$^{2+}$. The data also indicate that the purification step from the 88000g supernatant to fraction NKIII seems to result in specific purification of the Fe$^{2+}$-stimulated
Fig. 6. Effect of varying Mg²⁺ concentration
Conditions were identical with those described in the legend to Fig. 5. The ATP concentration was 50μM.

Fig. 7. Effect of varying the histone substrate concentration
Standard assay conditions were used. Enzyme used was NKIII. Protein substrate used was rat liver histone 1 (a) or 2b (b). No endogenous phosphorylation was observed in the absence of added histone.
NUCLEOPLASMIC HISTONE-SPECIFIC KINASE

133

activity. The results are notable in the light of observations by Desjardins et al. (1972) that only Mn^{2+} could replace Mg^{2+} when the enzyme used was the rat liver nuclear kinase isolated by those investigators. It represents another significant difference between the enzyme reported in the present study and the nuclear kinase activities described elsewhere. There is some precedent for the stimulatory effect of Fe^{2+} in the work of Kleinsmith & Allfrey (1969), who observed that this cation could best replace Mg^{2+} in the endogenous phosphorylation of a nuclear phosphoprotein fraction from calf thymus.

The cytoplasmic kinase type I was also tested in this assay and found to be stimulated only slightly by either Mn^{2+} or Fe^{2+}. This appears to be another clear indication that the enzymes are distinct.

Discussion

In the present work we report the isolation of a protein kinase from rat liver nuclei that both phosphorylates histones preferentially and responds to cyclic AMP. The enzyme, a soluble nucleoplasmic protein, has a higher specificity for histones 1 and 2b as substrates than for other substrates tested. This enzyme, after elution from Sephadex G-200 as a cyclic AMP-dependent activity, is 28-fold purified compared with the original nuclear suspension. It is 39-fold purified when eluted as the catalytic subunit (NKIII) in the presence of cyclic AMP.

Although most of the protein kinases that have been investigated are cytoplasmic in origin, there have been numerous reports of nuclear protein kinases. The histone kinase activity reported herein has several unique properties that appear to distinguish it from previously described nuclear kinases. For example, the enzyme described here is distinct from the rat liver nuclear enzymes studied by Desjardins et al. (1972) and Takeda et al. (1974) on the basis of utilization of substrate protein and the significant stimulation of those enzymes by univalent salts. They are probably involved in the phosphorylation of non-histone chromosomal proteins.

The nuclear histone kinase activity reported by Lake & Salzman (1972) appears to be different from the enzyme of the present paper in a number of properties. The enzyme reported by Lake & Salzman (1972) exhibits a significantly higher pH optimum (8.5–9.0). The pH optimum of the kinase reaction may reflect numerous events, including ionization of substrate protein and ATP as well as amino acid residues of the enzyme; however, the fact that the enzymes of both preparations were assayed with histone I as substrate and still displayed a large difference in pH optimum strongly supports the separate identity of each enzyme. In addition, and perhaps more importantly, the enzyme isolated by Lake & Salzman (1972) is chromatin-bound, exhibits no stimulation by cyclic AMP and has a significantly higher K_m for ATP. The nuclear activities reported by Dastugue et al. (1974), Farago et al. (1975) and Ord & Stocken (1975) could be comparable with the enzyme reported herein; however, they have not been sufficiently characterized to permit evaluation of their properties for comparison with the activity of this report. Johnson et al. (1975) have isolated nuclear protein kinases from calf thymus, two of which can be stimulated about 2-fold by cyclic AMP. However, the possible similarity of those kinases to that of the present report is difficult to assess, because the only parameter measured in the earlier study was binding affinity for DNA.

It is noteworthy that the histone kinase activity described in the present paper, although apparently distinct from nuclear enzymes described elsewhere, possesses many properties similar to those of the cytoplasmic kinases; these include cyclic AMP-dependence, affinity for Mg^{2+} and ATP and pH optimum (Langan, 1968; Miyamoto et al., 1969; Kumon et al., 1972; Nesterova et al., 1975). In fact, the data suggest that the cytoplasmic enzyme of the liver and the nuclear histone-phosphorylating enzyme of the present paper could be identical. This proposition is not unreasonable, since nuclear proteins must be transported to that compartment from their cytoplasmic sites of synthesis, and this translocation of the protein kinases may be a biologically regulated process. Several groups have, in fact, noted this type of phenomenon (Jungmann et al., 1975; Castagna

Table 5. Effect of bivalent metal ions on kinase activity

<table>
<thead>
<tr>
<th>Metal ion (10 mM)</th>
<th>Cytoplasmic protein kinase type I</th>
<th>Nuclear 88,000g supernatant</th>
<th>NKIII</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3</td>
<td>15</td>
<td>6</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Ca^{2+}</td>
<td>0.3</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Cd^{2+}</td>
<td>None detected</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Fe^{3+}</td>
<td>6</td>
<td>47</td>
<td>118</td>
</tr>
<tr>
<td>Mn^{2+}</td>
<td>9</td>
<td>16</td>
<td>7</td>
</tr>
<tr>
<td>Pb^{2+}</td>
<td>3</td>
<td>15</td>
<td>19</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>None detected</td>
<td>None detected</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Vol. 171
et al., 1975; Costa et al., 1976). Each of these reports seems to indicate an increase in nucleus-associated kinase activity and cyclic AMP-binding activity after hormone treatment at the expense of cytoplasmic kinase activity, although they find no cyclic AMP-dependent kinase activity. The enzyme of the present paper, however, has at least three significantly different characteristics from the cytoplasmic enzyme, namely, its stimulation by Fe2+, its response to the heat-stable inhibitor protein, and its loss of cyclic AMP-dependence after ion-exchange chromatography. Hence it is unlikely that the cytoplasmic and nuclear liver enzymes are identical, unless the nuclear form is altered in some way during transport.

The fact that the nuclear enzyme reported here is nucleoplasmic in nature may be significant in explaining why it has not been previously reported. In other laboratories the methodology generally involves extraction of nuclei with 0.14M- or 0.35M-NaCl; these washes are subsequently discarded before solubilization of the chromatin. Such procedures may lead to erroneous concepts of the physiological make-up of nuclei in terms of their macromolecular composition. For example, RNA polymerase II is known to be a nucleoplasmic enzyme (Dahmus, 1976). Umansky et al. (1976) have found kinase activity in nucleoplasm (though they have not characterized it) as well as 10% of the nuclear phosphoproteins. They report a completely different electrophoretic spectrum of peptides in nuclear sap from that in total non-histones, and they find appreciable DNA-binding activity in the nucleoplasm.

The lack of cyclic AMP-dependence observed in nuclear kinase preparations reported by other workers may in many cases be due to the high-salt or sonic-disruption conditions used during isolation, as suggested by others (Johnson et al., 1975; Keller et al., 1976). Indeed, Corbin et al. (1975) report that one of the major distinctions between type-II kinases and type-I kinases (which predominate in rat liver) is the sensitivity of the subunits of the type-I enzymes to both salt- and histone-induced dissociation. It is also plausible that translocated kinase subunits found by others in chromatin may serve different functions once in the nucleus, e.g. the catalytic subunit probably phosphorylates specific non-histones and the cyclic AMP-binding subunit may serve a wholly unrelated gene-regulatory role much like the cyclic AMP-receptor protein of Escherichia coli (Pastan & Perlman, 1970).

In conclusion, we report here a nucleoplasmic protein kinase that is distinct from the nuclear kinase activities previously reported and that also appears to differ from the major cytoplasmic kinase from the same tissue. We postulate that this enzyme may be involved in gene transcription by its ability to weaken DNA–histone interactions through modification by phosphorylation. The results of other investigations (Hewish & Burgoyne, 1973; Kornberg & Thomas, 1974; Griffith, 1975; Weintraub et al., 1975) have reinforced the importance of the interaction of histones with DNA in maintaining the structure of chromatin. In the present concept of chromatin as a series of nucleosomes, the histones play a highly specific structural role (Weintraub et al., 1976); moreover, histone I exerts a structural restriction on DNA that is different from that of the remaining histones. According to these current concepts, alteration in the strength of interaction of histone I (or any of the histones) with the rest of the complex could result in the structural reorganization of the nucleosomes into a complex with greatly altered accessibility to the enzymes of transcription.

This investigation was supported by grant no. CA-15683, awarded by the National Cancer Institute, grant no. AG-00478, awarded by the National Institute on Aging, and by American Cancer Society grant IN-97.

References
Griffith, J. D. (1975) Science 187, 1202–1203

1978