PRESENCE OF MULTIPLE PROTEIN KINASE ACTIVITIES
IN RAT LIVER NUCLEI

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SUMMARY

Protein kinase activity was found in the acidic nuclear protein fraction extracted from rat liver nuclei. Four distinct kinase activities were separated by phosphocellulose and DEAE-Sephadex chromatography. Additional evidence for separate enzyme activities is based on differences in pH optimum, enzyme stability, and substrate specificity.

The phosphorylation of nuclear proteins has been suggested to play a role in the control of gene readout in mammalian tissues (1, 2, 3). Phosphorylation of histones has been shown to occur in rat liver in response to hormonal agents (4), and a histone phosphokinase has been isolated from calf liver (5). In addition, many of the non-histone (acidic) proteins of the nucleus are phosphoproteins. The rates of phosphorylation of these acidic nuclear proteins have been shown to be increased in cells undergoing gene activation, preceding an elevation in RNA and protein synthesis (2). In some cell systems, phosphorylation of acidic nuclear proteins precedes DNA synthesis and cell proliferation (6).

The characterization, then, of protein kinases which are involved in the phosphorylation of nuclear proteins is important for understanding the control of these processes in mammalian cells. Reports have appeared indicating that there is protein kinase activity in the non-histone nuclear protein fraction (7, 8). The experiments described in the present report suggest that there are at least four distinct kinase activities. These kinase activities were found in the acidic nuclear protein fraction of rat liver nuclei.

METHODS

Male albino rats (Spartan Farms, Lansing, Michigan) weighing
80-100 grams were used in the present study. After sacrifice, the livers
were removed and immediately homogenized in 3 volumes of ice-cold
0. 32 M sucrose - 3 mM $MgCl_2$ by means of a Dounce homogenizer (4 strokes
with a "loose" and 3 with a "tight" pestle). Nuclei were isolated through
2. 4 M sucrose - 1 mM $MgCl_2$ by a modification of the method of Chauveau
et al. (9), and washed three times with 10 volumes of a solution containing
0. 25 M sucrose, 0. 01 M Tris - HCl (pH 7. 9), 5 mM $MgCl_2$ and 5 mM
β-mercaptoethanol. The resulting white pellet contained 60-70% of the
DNA of the total homogenate. The acidic nuclear proteins (ANP) were
extracted by: (1) resuspension of the nuclear pellet (approximately 1. 0 -
1. 6 g of nuclei pooled from 10 - 14 animals) in 10 mls of the above sucrose -
Tris buffer solution made 1 M with respect to NaCl, (2) sonification with a
Bronwill sonifier (probe intensity 32%) for 30 seconds, (3) stirring of the
sonicated mixture for 1 hour at 2^O C, and (4) centrifugation at 25, 000 $\underline{g}$ in
a Sorvall refrigerated centrifuge for 30 minutes. The resulting pellet
was then re-extracted a second time as above. The supernatants from the
two extractions were pooled and dialyzed for 16 hours against a solution
containing 0. 4 M NaCl, 0. 05 M Tris - HCl (pH 7. 5). Nucleohistone was
precipitated during the dialysis procedure. The dialysate was then
centrifuged at 25, 000 $\underline{g}$ for 30 minutes as above, and the resulting clear
supernatant was utilized for the subsequent experiments.

The ANP fraction was placed on a phosphocellulose column (Whatman
P-11; 0. 9 x 26 cm) previously equilibrated with 0. 05 M Tris - HCl (pH 7. 5) -
0. 4 M NaCl. Elution was begun with this same buffer. When 100 ml to
150 ml had passed through the column, the buffer in the reservoir was
replaced with 0. 05 M Tris - HCl (pH 8. 1) - 0. 6 M NaCl, and an additional
150 ml was collected. Assay of protein kinase activity in the column
fractions was performed essentially by the method of Takeda et al. (8) in
a reaction mixture (0. 7 ml) containing: 200 μg casein (Hammersten;
Schwarz/Mann), 7 mM magnesium acetate, 0. 2 - 0. 3 M NaCl, 0. 05 M
Tris - HCl (pH 7. 5), 0. 4 μCi ATP - γ - ^{32}P (10 Ci/mmole; New England
Nuclear Corp.), and 0. 4 ml of column fraction. Incubation was carried
out at 30° C for 10 minutes. The reaction was stopped by adding 1. 0 ml
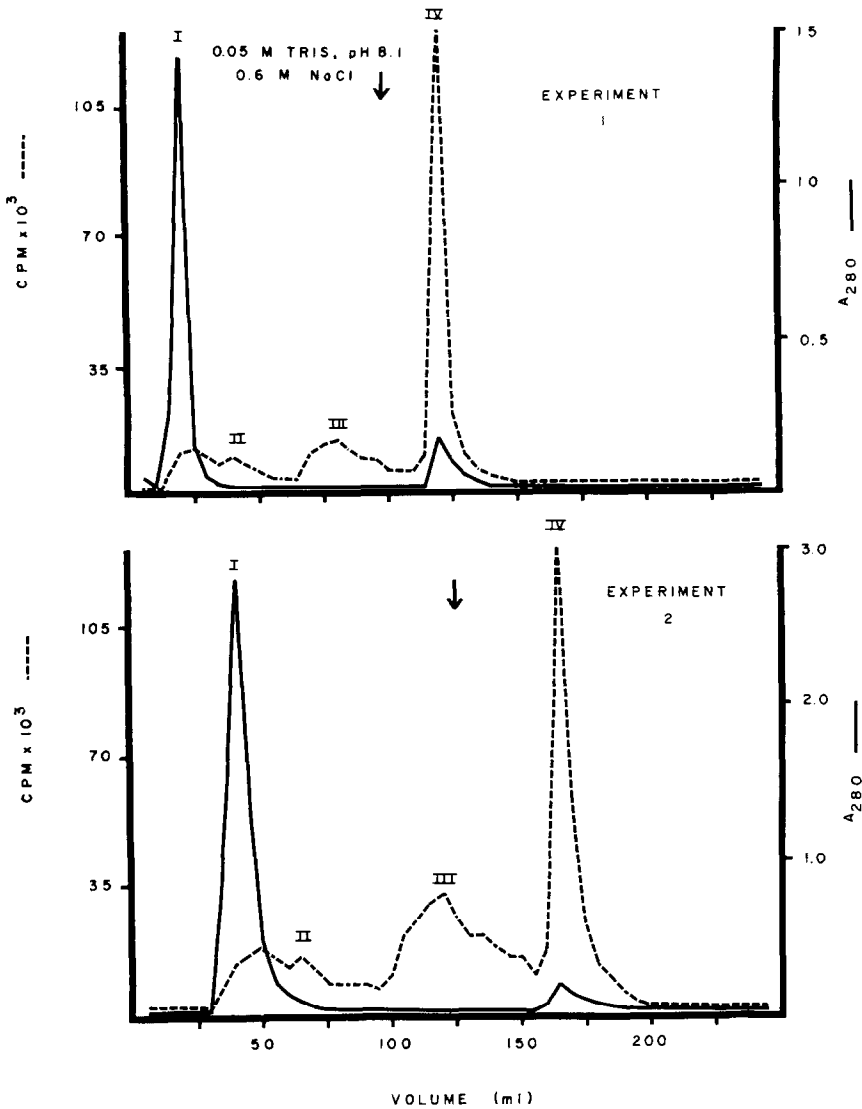
Figure 1.   Separation of protein kinase activities of the ANP extract by
             phosphocellulose column chromatography.   The columns were
             equilibrated with 0. 05 M Tris (pH 7. 5) - 0. 4 M NaCl.   The
             ANP extract was applied (Experiment 1 = 15 mg protein;
             Experiment 2 = 40 mg protein), and 20 or 25 five ml fractions
             were collected.   The buffer was then changed (as indicated by
             the arrows) and 100-150 ml of 0. 05 M Tris (pH 8. 1) - 0. 6 M
             NaCl were collected.   The samples were assayed for kinase
             activity as indicated in the Methods.   The peaks representing
             Fractions I-IV were collected, pooled, and stored at -80° C.

of cold 10% trichloroacetic acid - 0.04 M sodium pyrophosphate. The
samples were then filtered on Millipore filters (0.45 μ pore size), washed
five times with 5% TCA - 0.02 M sodium pyrophosphate, dried, and counted
in a Packard Tri-Carb liquid scintillation spectrometer after the addition
of 10 ml of toluene scintillator. In each case, zero time samples containing
casein were prepared, and these values were subtracted from the experimental
values to give the actual incorporation data. All assays were done in
duplicate or triplicate.

DEAE-Sephadex (A-50) column chromatography was also utilized
to resolve the kinase activity of the ANP fraction. The column (0.9 x 22 cm)
was equilibrated with 0.05 M Tris - HCl (pH 7.9), 0.05 M KCl. An aliquot
of the total ANP fraction was adsorbed onto the column. A linear gradient
of KCl in 0.05 M Tris - HCl (pH 7.9) was then applied. Two ml fractions
were collected and assayed for kinase activity as above.

Incorporation of ^{32}P into various ANP fractions was determined
by polyacrylamide gel electrophoresis in sodium dodecyl sulfate buffer as
previously described (3). Following electrophoresis the gels were sliced
into 1 mm transverse sections with a Mickle gel slicer; the slices were
dried, and the radioactivity determined after the addition of toluene
scintillator as above.

RESULTS AND DISCUSSION

Figure 1 illustrates the separation of different kinase activities by
phosphocellulose column chromatography. As reported earlier by Takeda
et al. (8) the majority of the ANP fraction can be separated from a significant
portion of the protein kinase activity by this procedure. This activity
promoted the incorporation of alkali-labile phosphate from ATP - γ - ^{32}P
into protein. Takeda et al. (8) also reported evidence for two distinct
nuclear kinase activities, which were separable on Sephadex G-200. The
presence of the additional peaks I, II and III reported here (Figure 1) may
be due to differences in the extraction procedure and to the fact that
Fractions II and III appear to be labile. Fraction III, for example, lost
about 80% of its original activity when stored at 0° C for 2-3 days.
Fraction III also lost significant activity after 7-10 days of storage at
-80° C. Additional evidence for the presence of four distinct kinase
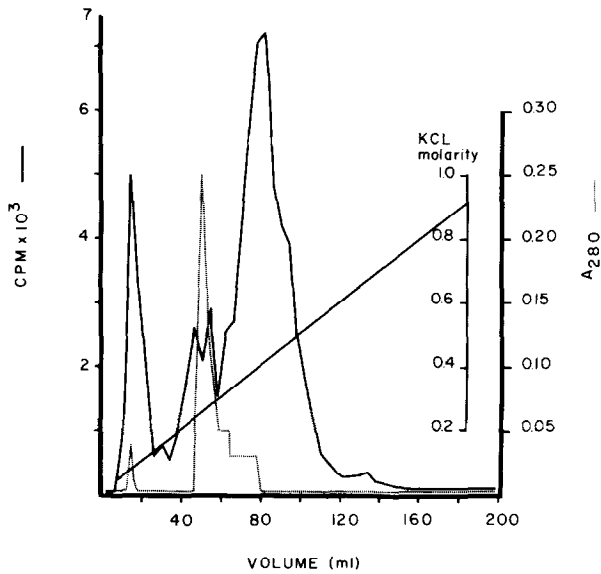
Figure 2.   Separation of protein kinase activities of the ANP extract by
DEAE-Sephadex column chromatography.   The column was
equilibrated with 0. 05 M Tris-HCl (pH 7. 9), 0. 05 M KCl.
The ANP extract (8. 5 mg) was applied, and the elution carried
out by a linear KCl gradient.   Two ml samples were collected
and assayed for kinase activity as indicated in the Methods.

activities was obtained by chromatography of the ANP fraction on DEAE-
Sephadex (Figure 2).

The phosphokinase activity which was associated with the ANP

peak (Fraction I from the phosphocellulose column) was able to utilize

the ANP that co-chromatographed with it as substrate.   The evidence for

this is based on the incorporation of ^{32}P from ATP - γ - ^{32}P into various

ANP species contained in Fraction I.   The components of Fraction I were

separated by SDS-polyacrylamide gel electrophoresis immediately after

incubation of Fraction I in the in vitro kinase assay (Figure 3, dotted line).

Furthermore, the addition of Fraction IV, the most active kinase, to

Fraction I in the incubation mixture resulted in an increased incorporation

of ^{32}P primarily into the higher molecular weight ANP species which are

located at the top of the SDS-polyacrylamide gels (Figure 3, solid line).

The bands in this region of the gels are the species of ANP which are also

most avidly phosphorylated in liver tissue in vivo after a 60 min pulse with

inorganic ^{32}P (unpublished observation).   The addition of either Fraction II
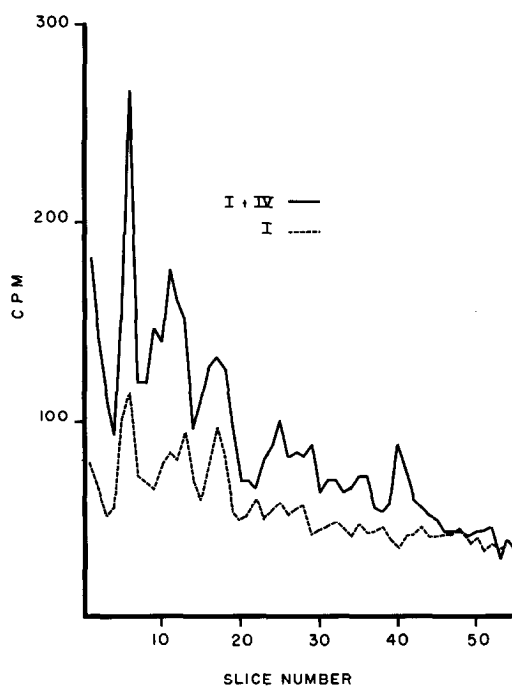
Figure 3.   Incorporation of ^{32}P (from ATP - γ - ^{32}P) into various proteins of the ANP Fractions obtained by phosphocellulose column chromatography.   SDS-polyacrylamide gel electrophoresis was used to separate the proteins after incubation in the kinase assay.   The assay mixtures contained 0. 4 ml Fraction I (200 μg protein) or Fraction I (200 μg) plus 0. 3 ml of Fraction IV (50 μg), but no casein was added.   The incubations were carried out as described in the Methods with the exception that at the end of 10 minutes, the samples were placed in 0.1% SDS, 0. 01 M sodium phosphate (pH 7. 2), 0. 1% β-mercaptoethanol and then dialyzed against two liters of this SDS-phosphate buffer for 16 hours.   Aliquots of the SDS-dialyzed fractions (50-55 μg) were then layered on the gels for electrophoresis.   The gels were sliced and counted for radioactivity as indicated in the Methods.   The cpm obtained from the gels representing Fraction I alone and Fraction I plus IV are plotted on the same scale for comparison.

or Fraction III from the phosphocellulose column to the incubation medium with Fraction I inhibited the incorporation of ^{32}P into the ANP species which were phosphorylated when I was incubated alone (Figure 4).   This suggests that Fraction II and III may be modulators of the total nuclear kinase activity in vivo.   The kinase activity of Fraction I was not dependent on the addition of casein as substrate (Table 1).   This is not surprising since Fraction I contained the bulk of the total ANP fraction, and the proteins of
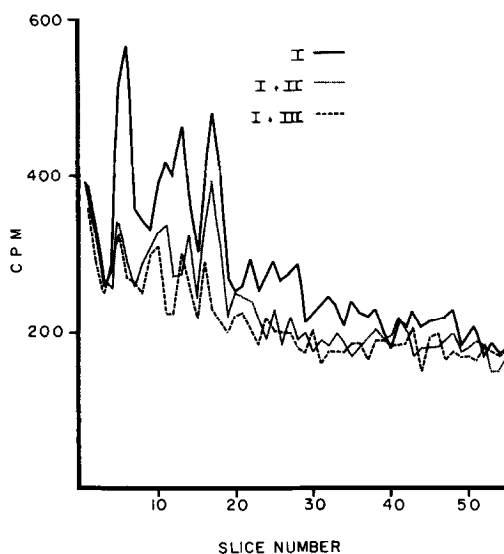
Figure 4.   Incorporation of ^{32}P (from ATP - γ - ^{32}P) into various proteins
of the ANP Fractions obtained by phosphocellulose chromatography.
The assay mixtures contained: 0. 4 ml Fraction I (200 μg protein)
alone; Fraction I (200 μg) plus Fraction II (22 μg); or Fraction I
(200 μg) plus Fraction III (25 μg).   The other parameters are
as indicated in the legend to Figure 3.

TABLE 1

Dependence on Addition of Exogenous Substrate
(Casein) for Protein Kinase Activity

Assays were carried out as described in the Methods; 0. 4 ml
aliquots of each Fraction from the phosphocellulose column were
assayed in the presence or absence of casein (200 μg).   The
amount of protein added in each case was: Fraction I, 200 μg;
Fraction II, 30 μg; Fraction III, 20 μg; and Fraction IV, 70 μg.

Fraction	Counts per minute	Counts per minute
	+ casein	- casein
I	14, 892	11, 316
II	4, 079	263
III	1, 012	268
IV	11, 046	3, 497

TABLE 2

Response of the Various Phosphocellulose
Fractions to 3',5' - cyclic AMP

Assays were performed as described in the Methods.   The con-
centration of cyclic AMP employed was 10^{-5} M.   Casein (200 μg)
was used as substrate in all incubations.   Assays were done in
triplicate.

Fraction	Counts per minute	Counts per minute
	+ cAMP	- cAMP
I	9,947	11,579
II	6,084	4,894
III	3,278	3,206
IV	23,927	25,513

TABLE 3

Determination of Effects of Varying pH on Kinase
Activity of the Phosphocellulose Fractions

Assays were performed as described in the Methods.
Casein was utilized as substrate; pH was varied from 6.9 to
8.0 by appropriate mixtures of Tris-HCl buffers.   Assays
were done in triplicate.

Fraction	Counts per minute			
	pH 6.9	7.2	7.5	8.0
I	7,300	7,470	8,934	10,576
II	3,823	3,573	3,794	2,976
III	901	2,001	1,330	1,209
IV	774	6,205	7,465	14,096

this fraction are substrates for the kinase activity.   The residual activity

observed in Fraction IV in the absence of casein was apparently due to

the presence of protein in this fraction which was able to act as substrate.

Enzyme activity was not significantly affected by the addition of cyclic AMP to the fractions (Table 2). This is in contrast to the histone kinase described by Langan (5) which does respond to cyclic AMP. In addition, the kinase associated with the ANP fraction does not utilize histone as substrate (8).

The kinase fractions had different pH optima (Table 3). Somewhat greater enzyme activity was noted at pH 8.0 for Fraction I. Fraction II had higher activity at pH 6.9. Fraction III appeared to have higher activity at pH 7.2. Fraction IV had significantly greater activity at pH 8.0 than at pH 6.9.

A 20-fold purification of the most active protein kinase (Fraction IV) above that contained in the total ANP fraction was achieved by a single pass of the ANP through phosphocellulose. However, Fraction IV still contained a complex number of polypeptides as evidenced by the banding
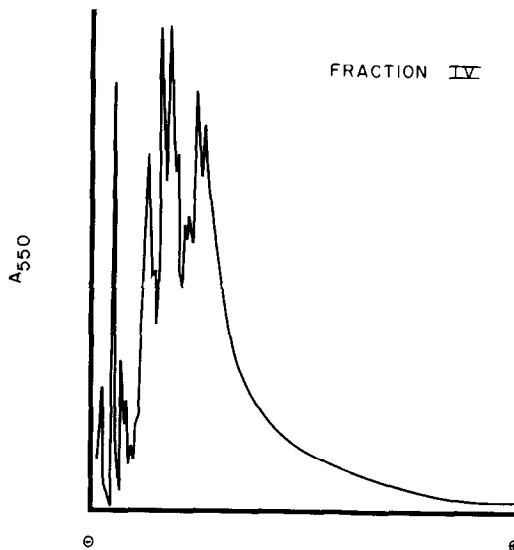

Figure 5.   SDS-polyacrylamide gel electrophoresis of Fraction IV obtained by phosphocellulose chromatography. Twenty μg of protein was layered on the gels. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue, destained, and scanned for absorbancy at 550 nm in a Gilford recording spectrophotometer with a linear transport attachment.

pattern on SDS-polyacrylamide gels (Figure 5).   Further purification of this activity is currently being attempted.

The results reported in this paper confirm that protein kinase activity is present in the acidic nuclear protein fraction of liver tissue (7, 8) and also indicate that there are multiple species of these activities present in the acidic nuclear protein extracts.   It will be of interest to characterize further the nuclear protein kinases and to determine what factors can regulate their activity.

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