PARTIAL FRACTIONATION AND CHARACTERIZATION
OF NUCLEAR PROTEIN KINASES IN HeLa S₃ CELLS

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Five fractions of phosphoprotein kinase activity were separated by phosphocellulose chromatography of nuclear non-histone phosphoproteins isolated from exponentially-growing HeLa S₃ cells. Each fraction contained a heterogeneous group of proteins and possibly more than one protein kinase. These fractions were characterized with respect to specific activity and optimal concentrations of Mg^{2+} and Mn^{2+}. The ability of each kinase fraction to catalyze the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]ATP$ into various molecular weight classes of heat-inactivated non-histone nuclear phosphoproteins (as substrate) was examined by electrophoretically resolving the proteins in SDS-polyacrylamide gels. Each kinase fraction induced a distinctive pattern of protein phosphorylation. Substrate preferences (non-histone phosphoprotein or histone) were determined for each fraction by examining the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]ATP$ into both TCA-precipitable material and into specific molecular weight classes of proteins. The activities of two of the fractions to phosphorylate non-histone nuclear phosphoprotein were stimulated by the presence of histones. Studies were undertaken to assess the effects of cAMP or cGMP on the activity of each kinase fraction. No significant sensitivity to the cyclic nucleotides was observed for any of the kinase fractions.

The underlying mechanisms involved in the control of gene expression in eukaryotic systems still remain obscure, although several lines of evidence suggest control of gene readout may in part be attributable to components of the non-histone nuclear proteins [1,2]. The nuclear proteins are highly phosphorylated and numerous correlations exist between changes in the metabolism of phosphoproteins and modifications in gene expression in a broad spectrum of biological situations [3—11]. Such modifications in phosphoprotein metabolism include changes in the extent of phosphorylation, alterations in the phosphorylation of specific polypeptides and variations in the synthesis and turnover of phosphoproteins. Results from chromatin reconstitution experiments suggest an important role for non-histone chromosomal protein phosphorylation in the control of transcription [12,13].

Phosphorylation and dephosphorylation of nuclear proteins are reactions

enzymatically catalyzed by nuclear protein kinases and phosphatases [4,7,11,14—24]. Therefore, understanding the properties of these enzymes should enhance our understanding of the mechanisms by which phosphorylation of chromosomal proteins may modulate gene function. In this paper we report initial results from studies directed towards characterization of nuclear protein kinase in extronentially-growing HeLa S_3 cells.

MATERIALS AND METHODS

Cells. HeLa S_3 cells were grown in suspension culture in Joklik-modified Eagle's minimal essential medium supplemented with 7% calf serum. To maintain exponential growth the cell concentration was kept between 2×10^5—5×10^5 cells/ml. Most experiments required approx. 6×10^9 cells.

Isolation of nuclei. Isolation and preparation of nuclear protein kinase fractions were carried out at 4°C. Cells were harvested by centrifugation at 600 g for 4 min and washed three times in Earle's balanced salt solution. The cell pellet was homogenized by 30--35 strokes with a motor-driven teflon pestle in 10 vols. of 2.2 M sucrose/1 mM $MgCl_2$. The mixture was then diluted to 1.8 M sucrose with 1 mM $MgCl_2$ and after thorough homogenization was centrifuged at 80 000 g for 1 h. The resulting nuclear pellet was homogenized in 0.14 M NaCl and centrifuged at 8000 g repeatedly until the nuclei appeared free of cytoplasmic material when examined by phase contrast microscopy.

Isolation of nuclear phosphoproteins. Purified nuclei at a DNA concentration of 2 mg/ml were vigorously homogenized in a Dounce homogenizer (using a B pestle) in 1.0 M NaCl/0.05 M Tris (pH 7.5). 1.5 volumes of 0.02 M Tris (pH 7.5) were added dropwise, and the mixture was briefly homogenized and then centrifuged at 80 000 g for 1 h. The proteins in the supernatant were treated with BioRex 70 (previously equilibrated with 0.4 M NaCl/0.02 M Tris, pH 7.5) at a ratio of 20 mg BioRex/mg protein. The suspension was stirred for 5—10 min and centrifuged at 6000 g for 5 min. Calcium phosphate gel (prepared according to the method of Keilen and Hartree [25]) was added to the resulting supernatant at a ratio of 0.45 mg gel/mg protein; the mixture was stirred for 5—10 min and centrifuged at 6000 g for 5 min. The pellet of calcium phosphate gel was washed in 40 ml of 1.0 M $(NH_4)SO_4$/0.05 M Tris (pH 7.5) and solubilized in 0.3 M EDTA (pH 7.5)/0.33 M $(NH_4)SO_4$ at a ratio of 0.2 ml of solution/mg gel. The insoluble residue was removed by centrifugation for 15 min at 33 000 g; the supernatant consisted of the protein fraction referred to as 'total phosphoproteins'. These proteins were dialyzed against several changes of 0.05 M Tris (pH 7.5)/0.1 M NaCl.

Separation of nuclear protein kinase fractions. Three milligrams of total phosphoprotein from HeLa cell nuclei were applied to a phosphocellulose

column (0.9 cm X 14 cm) that had been previously equilibrated overnight with 0.05 M Tris (pH 7.5)/0.1 M NaCl. The column was then eluted stepwise with 0.05 M Tris (pH 7.5) containing 0.1, 0.3, 0.5, 0.7 and 0.9 M NaCl. Twenty 1-ml fractions were collected with each buffer elution of the column. The first fractions eluted at 0.1 M NaCl represent protein which does not bind to the column (flowthrough). The optical density of the eluted fractions was measured and 5 distinct peaks of protein were resolved. The fractions constituting each peak were pooled and dialyzed overnight against 100 vols. of 0.05 M Tris (pH 7.5). It should be noted that each protein kinase fraction is a heterogeneous group of proteins and may contain more than one species of protein kinase. Further characterization of the protein kinase fractions was performed as described in Results.

Preparation of protein kinase modulator. Crude protein kinase modulator was prepared from dog heart by the method of Appleman [26]. Fifty grams of dog heart were minced and then homogenized in a Tekmar homogenizer at full speed. The homogenate was centrifuged at 15 000 g for 15 min and the supernatant was collected. The tissue extract was boiled with stirring for 20 min, cooled in ice and subsequently filtered through three layers of cheesecloth. One-ninth volume of 50% TCA (trichloroacetic acid) was added to the filtrate and the resultant precipitate was collected by centrifugation at 15 000 g for 15 min. The precipitate was solubilized in double-distilled H_2O, the pH was adjusted to 7.5 with 5 N NaOH and the solution was dialyzed overnight against 100 vols. of distilled H_2O at room temperature. The solution was then centrifuged at 15 000 g for 15 min. The supernatant was collected and is referred to as 'crude protein kinase modulator'.

Protein kinase activity assay. Each assay contained the following (unless otherwise indicated) in a total volume of 0.3 ml: 0.05 M Tris (pH 8.0); 20 mM $MgCl_2$; 0.20 ml protein kinase fraction; 6 nmol [γ-^{32}P]ATP (spec. act. 0.5 Ci/nmol ATP made according to the procedure described by Glynn and Chappell [27]) and when indicated, 5 μM cAMP, 5 μM cGMP, 40–100 μg heated total phosphoproteins, 50—130 μg histone or 60 μg protein kinase modulator. The assay mixtures were incubated for 10 min at 37°C and the reaction was terminated by the addition of 2 ml of 10% TCA/2% sodium pyrophosphate. Four milligrams of bovine serum albumin were added as carrier and the mixtures were extensively vortexed and then centrifuged at 900 g for 3 min. The pellets were washed three times (by vortexing and centrifugation) in 12 ml of 5% TCA/1% sodium pyrophosphate. The final pellet was solubilized in 0.5 ml of NCS reagent (Amersham Corp.), 3 ml of dioxane-toluene scintillation fluid (150 ml liquiflour; 1 l toluene, 1 l ethanol; 1 l dioxane; 240 g napthalene) were added and incorporation of ^{32}P was measured by liquid scintillation spectrometry.

Examination of ^{32}P incorporation into phosphoproteins utilizing poly-acrylamide gel electrophoresis. To determine the ability of protein kinase

fractions to incorporate ^{32}P into nuclear phosphoproteins, each fraction was incubated with [γ-^{32}P]ATP and exogenous substrate consisting of heat-inactivated phosphoproteins from exponentially-growing HeLa cells. The phosphorylated substrate was then examined by polyacrylamide gel electrophoresis. Since it was not possible to remove the small quantity of endogenous substrate contained in each fraction, a large amount (150 μg; 20 100-fold excess) of endogenous substrate was used to dilute the endogenous protein.

Each protein kinase fraction was assayed in the following mixture with a total volume of 0.3 ml: 0.05 M Tris (pH 8.0)/20 mM MgCl$_2$/0.20 ml protein kinase fraction/6 nmol [γ-^{32}P]ATP (spec. act. of 0.5 Ci/nmol ATP) and 100–150 μg of heat-inactivated total nuclear phosphoproteins from HeLa cells. The assay mixtures were incubated for 10 min at 37°C and the reaction was terminated by the addition of solid urea to a final concentration of 5 M and then extensively dialyzed against either 0.1% sodium dodecyl sulfate (SDS)/10 mM sodium phosphate (pH 7.0)/0.1% β-mercaptoethanol or 0.0675 M Tris/0.1% SDS/0.1% β-mercaptoethanol (pH 6.8). Details of the electrophoresis procedures used in these studies are given in the legend of Figs. 2 and 4. The data are presented as percent of total radioactivity, reflecting the relative extent of phosphorylation of various molecular weight classes of non-histone chromosomal polypeptides. Listed in each legend are the counts per minute represented by 1% total counts.

RESULTS

The starting material we have been using for the isolation and separation of protein kinase activity in HeLa S$_3$ cells is a nuclear phosphoprotein fraction prepared by a procedure developed by Langan [28]. This fraction of nuclear proteins has been shown to be rich in phosphorus-containing proteins and endogenous protein kinase activity [18,28] and does not contain significant levels of phosphatase or protease activity (unpublished observations). It is this protein fraction which several laboratories have used to study the correlation between phosphorylation of non-histone chromosomal proteins and variations in gene expression in many biological systems [5,8,12,13,18,23,29]. These nuclear phosphoproteins can be resolved into several distinct protein kinase fractions. Kish and Kleinsmith [18], using a gradient elution procedure, obtained 12 protein kinase fractions from calf thymus and Thomson et al. [23], using a batch elution procedure, obtained 6 reproducible fractions from several neoplastic tissues. Both elution techniques yield fractions containing multiple species of protein and perhaps more than one protein kinase.

By applying 'total phosphoproteins' isolated from nuclei of exponentially-growing HeLa cells to a phosphocellulose column and eluting with buffers containing 5 stepwise increases in NaCl concentration (0.1 M, 0.3 M, 0.5 M, 0.7 M, 0.9 M NaCl), it was possible to subdivide the HeLa phosphoproteins
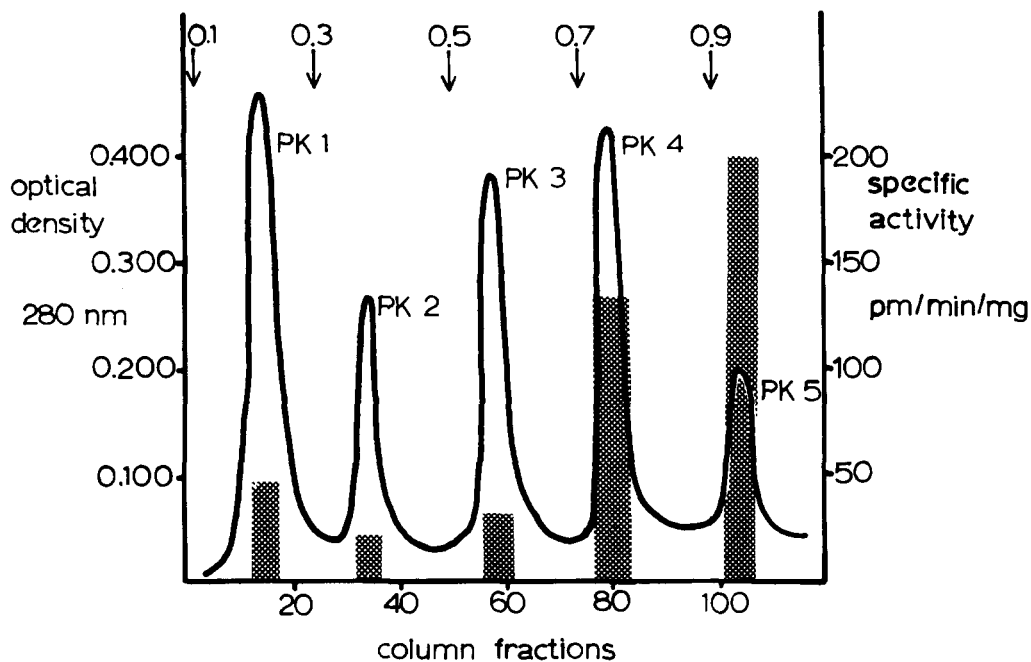
Fig. 1. Absorption profile of nuclear phosphoproteins and specific activity of protein kinase activity eluted from phosphocellulose. Total phosphoproteins from HeLa cell nuclei were applied to a phosphocellulose column and eluted as described in Materials and Methods. The resulting absorption profile of the eluted fractions and the specific activity of each peak is shown (pmol/min/mg protein). Protein concentrations were determined by the method of Layne [51].

into 5 reproducible fractions of protein kinase activity. The 5 fractions were defined both by absorbance at 280 nm and by protein kinase activity assayed using endogenous phosphoprotein as substrate. Fifty-eight percent (58%) of the applied total protein kinase activity was recovered in the 5 eluted peaks. Peaks 1—5 accounted for 5%, 4.4%, 18.8%, 48.3% and 23.2% respectively, of the recovered activity. Figure 1 shows a typical absorbance and specific activity profile of nuclear phosphoproteins eluted from a phosphocellulose column. As is evident from Fig. 1, peak 4 represents the highest percentage of recovered activity (48.3%) and peak 5 contains the highest specific activity (194 pmol phosphate incorporated/min/mg protein). Several characteristics of these 5 electrophoretically heterogenous fractions, perhaps each containing more than one kinase, were then investigated, both to determine if each fraction contained unique species of kinase activity and to define the properties of these fractions. Each fraction was assayed in the presence of varying concentrations of Mg^{2+} and Mn^{2+} and the optimal concentrations for each peak were determined by the incorporation of ^{32}P from $[\gamma\text{-}^{32}P]ATP$ into TCA precipitable material, using endogenous

TABLE I

CHARACTERISTICS OF PROTEIN KINASE FRACTIONS FROM HeLa CELL NUCLEI

All assays were performed as described in Materials and Methods. Optimal conditions were determined by the concentration that resulted in the highest incorporation of ^{32}P into TCA-insoluble material. Ranges of concentrations tested are as follows: pH, 5.0—9.0; Mg^{2+}, 10—50 mM; Mn^{2+}, 0.4—2.0 mM. No kinase activity was demonstrated in the absence of Mg^{2+} and Mn^{2+}. Added substrate (line 4) was 40 μg histone plus 40 μg phosphoprotein. All assays were done in duplicate and all values are the average of at least three experiments. Percentage of control values (lines 8, 9 and 10) were determined by assaying the fraction in the presence of $[\gamma\text{-}^{32}P]ATP$ with or without added protein (40 μg histone or 40 μg phosphoprotein) and dividing the counts incorporated in the presence of the added protein by the counts incorporated in its absence. Blank assays (containing all components except the kinase fraction) were 200—600 cpm and assays containing kinase fractions were at least 6—10 times higher in incorporated radioactivity than blank assays.

		Fraction					
		Total phospho-protein	Protein kinase fraction				
			1	2	3	4	5
1	NaCl concentration of elution buffer (M)	—	0.1	0.3	0.5	0.7	0.9
2	Spec. act. (pmol/min/mg) A [a]	535	45.0	16.1	30.8	132	194
	B [b]	—	66.1	23.7	47.1	232	725
3	% of total recovered activity assayed with endogenous substrate	100	5 (range 2—10)	4.4 (range 2—7)	18.8 (range 10—30)	48.3 (range 32—67)	23.2 (range 18—40)
4	% of total recovered activity assayed with added substrate	100	5.4	3.3	7.4	30.9	50.6
5	Optimal pH	8.0	—	—	—	—	—
6	Optimal Mg^{2+}	20	15	25	15	40	25—30
7	Optimal Mn^{2+}	1.6	1.4	1.4	1.6	1.6	1.4
8	% activity retained when Mg^{2+} replaced with Mn^{2+}	90	115	86	37	13	6
9	% of control with the addition of histone	—	755	183	106	104	54
10	% of control with the addition of heat-inactivated phospho-protein	—	335	188	194	300	186

[a] A = endogenous protein
[b] B = added protein

substrate. Table I lists the results of these studies and indicates that the 5 protein kinase peaks have optimal Mg^{2+} concentrations of 15, 25, 15, 40 and 25 30 mM respectively and optimal Mn^{2+} concentrations of 1.4, 1.4, 1.6, 1.6 and 1.4 mM.

The substrate specificity of each of the kinase fractions was then examined. Both histones and non-histone chromosomal proteins were assayed for their abilities to serve as substrates. A complicating factor in investigating the non-histone protein substrate specificity is that each protein kinase fraction contains endogenous phosphoprotein that can be utilized as substrate and this endogenous protein is not identical in each fraction. Therefore, to determine any difference in the specificity of non-histone protein phosphorylation by the protein kinase fractions, it was necessary to provide an excess of the same exogenous substrate to each of the fractions to dilute the contribution of the endogenous protein substrate as much as possible. The exogenous substrate used to assay the kinase fractions was 'total phosphoprotein' which had been heated at 60°C for 10 min to eliminate greater than 95% of the endogenous protein kinase activity. After incubation with the kinase fraction the heat-inactivated phosphoproteins were examined by SDS-polyacrylamide gel electrophoresis, and the distribution of ^{32}P radioactivity among the various molecular weight classes of phosphoproteins was determined. As is shown in Fig. 2, each protein kinase fraction exhibits a different pattern of protein phosphorylation. All kinase fractions phosphorylated nuclear proteins which electrophoresed in the 100 000–150 000 molecular weight region of the gel, however, substantial increases can be seen in the phosphorylation of lower molecular weight (35 000—55 000) proteins by fraction 3, and in the intermediate molecular weight (55 000—100 000) proteins by fraction 4. In addition, fraction 5 exhibits decreased phosphorylation of a high molecular weight protein band as compared with the other four fractions.

The protein kinase fractions were also examined for their preference for either heat-inactivated phosphoproteins or histones as substrates. In this determination two methods were employed. The first was a comparison of the incorporation of ^{32}P into TCA-insoluble material of each fraction utilizing endogenous protein, heat-inactivated phosphoprotein, or histone as substrate (Table I). Protein kinase peak 1 showed a higher percentage increase of kinase activity when histone, as compared with added heat-inactivated phosphoprotein, was added as exogenous substrate. Protein kinase peak 2 showed approximately equal increases of activity when either histone or phosphoprotein was added as compared with endogenous substrate. Protein kinase peaks 3, 4 and 5 showed a greater increase when heat-inactivated phosphoproteins, as compared with histones, were added, suggesting that these three protein kinase fractions preferentially use non-histone proteins as substrates.

An important question which arose was whether the histones themselves were acting as substrate or whether they were stimulating the phosphoryla-
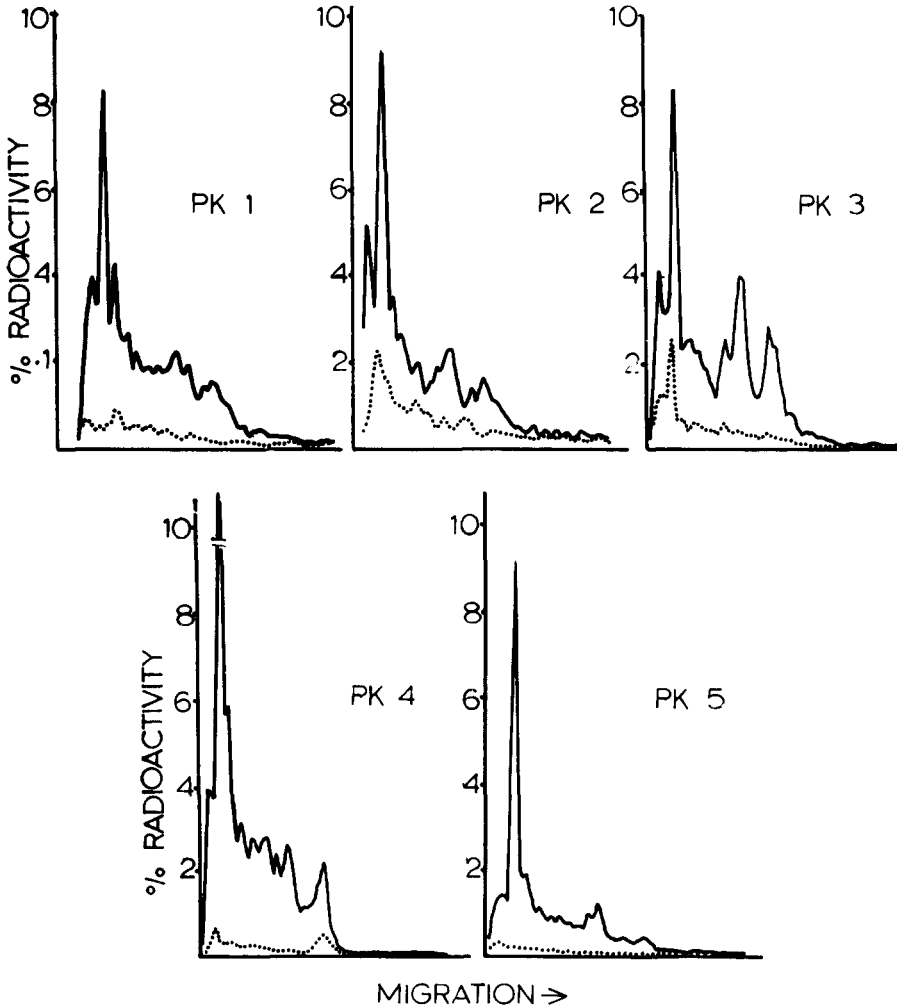
Fig. 2. Distribution of ^{32}P among nuclear phosphoproteins induced by each isolated protein kinase fraction. Each protein kinase fraction was assayed in the presence of [γ-^{32}P]ATP and exogenous substrate (heat-inactivated phosphoproteins) as described in Materials and Methods. After termination of the reaction by the addition of solid urea to a final concentration of 5 M, the reaction mixtures were dialyzed extensively against 0.1%SDS/0.01 M NaPO$_4$ (pH 7.0)/0.1% β-mercaptoethanol, and subsequently fractionated electrophoretically according to molecular weight on 7.5% acrylamide, 0.28% bisacrylamide gels according to the procedure previously reported [9,52]. Electrophoresis was for 6.5 h at 8 mA per gel. The gels were fixed overnight in 12.5% trichloroacetic acid/7% acetic acid/40% ethanol, and stained in 0.25% Coomassie brilliant blue in the above solution for 5 h. After the gels were destained in a solution of 7% acetic acid/10% ethanol and scanned at 590 nm, the gels were sectioned into 1-mm slices and each slice examined for the incorporation of ^{32}P. The dotted lines represent the ^{32}P incorporated into the endogenous substrate when no additional protein was added as substrate to the assay mixtures. PK 1-PK 5 represent protein kinase fractions eluted from phosphocellulose with 0.1, 0.3, 0.5, 0.7 and 0.9 M NaCl, respectively.
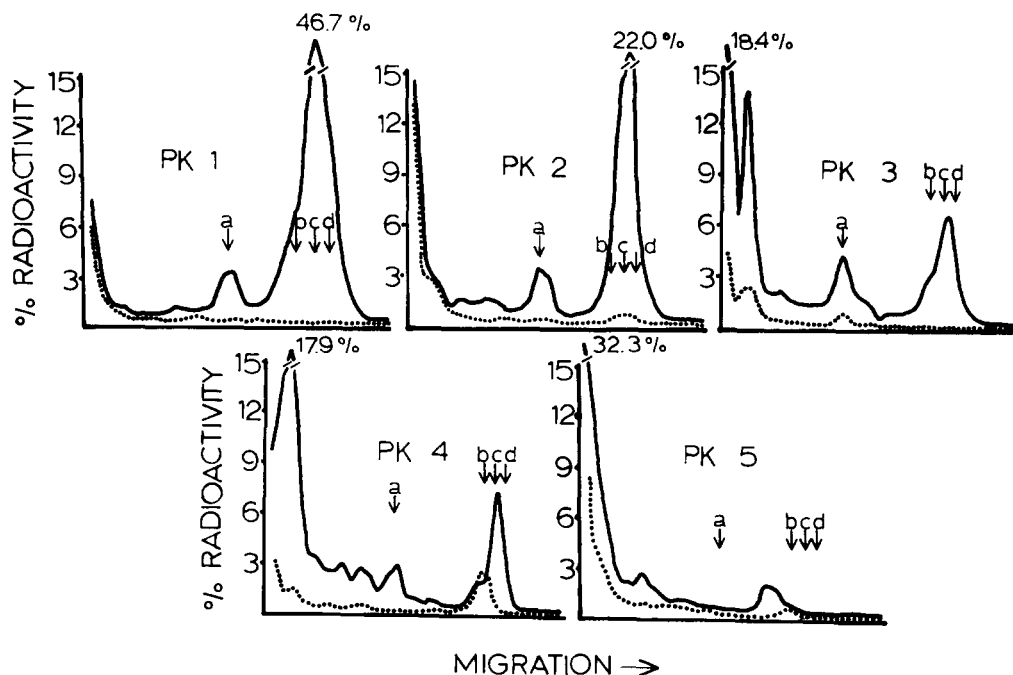
Fig. 3. Ability of kinase fractions to phosphorylate histones. Each protein kinase fraction was assayed in the presence of [γ-^{32}P]ATP and 40 μg of HeLa histone as described under Materials and Methods. After termination of the reaction, the assay mixtures were examined by polyacrylamide gel electrophoresis as described in the legend of Fig. 2. The dotted lines represent the ^{32}P incorporation into endogenous phosphoprotein when no histone was added. Migration of histone standard H1 is marked by the (a) arrow, H3 and H3B by the (b) arrow, H2A by the (c) arrow, and H4 by the (d) arrow. One percent radioactivity is 103, 109, 288, 1276 and 67 cpm for PK 1, PK 2, PK 3, PK 4 and PK 5, respectively.

tion of the phosphoprotiens, as has been shown in some systems (W. Criss, pers. comm.). To address this question, the protein kinase fractions were assayed in the presence of endogenous phosphoprotein as substrate or in the presence of endogenous phosphoprotein plus histones, and following in vitro incubation in the presence of [γ-^{32}P]labeled ATP, the samples were fractionated electrophoretically according to molecular weight in SDS polyacrylamide gels. The distribution of radioactivity among the various molecular weight classes of chromosomal proteins was determined and is shown in Fig. 3. The radioactivity profile induced by protein kinase fractions 1 and 2 exhibited a significant level of ^{32}P incorporation in the molecular weight region of the gel where histone standards migrate, only when histones were present in the assay. The radioactivity profile induced by protein kinase fraction 3 indicated that when histones were present in the assay mixture ^{32}P was incorporated into the histone region of the gel and in addition,

^{32}P incorporation was increased in the higher molecular weight region of the gel. When histone was present in the assay mixtures containing protein kinase fractions 4 and 5, very little ^{32}P incorporation was found in the histone region of the gel, however, there was a substantial increase in incorporation of ^{32}P in the higher molecular weight region.

The responsiveness of the protein kinase fractions to the cyclic nucleotides cAMP and cGMP was examined. Each fraction was assayed in the presence or absence of 5 μM cAMP or cGMP, and the ^{32}P incorporated into TCA insoluble material was measured. The results are presented in Table II. There was no significant change in the total activity of any protein kinase fraction with the addition of either cAMP or cGMP. However, it was considered possible that exposure to cyclic nucleotides might alter the substrate specificity of the kinase activity. Such changes might not be

TABLE II

EFFECT OF THE PRESENCE OF CYCLIC NUCLEOTIDES ON THE ABILITY OF PROTEIN KINASE FRACTIONS TO INCORPORATE ^{32}P FROM [γ-^{32}P]ATP INTO TCA INSOLUBLE MATERIAL

All assays were performed as described in Materials and Methods. Percentage of control values were calculated by assaying the fraction in the presence of [γ-^{32}P]ATP and substrate (endogenous or added as indicated) with or without the cyclic nucleotide and dividing the cpm incorporated in the presence of the cyclic nucleotide by the cpm incorporated in its absence. Added substrate consists of 40 μg of histone plus 40 μg of phosphoprotein (heat-inactivated). All assays were done in duplicate and all values are the average of at least two experiments. Blank assays (containing all components except the kinase fraction) were 200—600 cpm and assays containing kinase fractions were at least 6—10 times higher in incorporated radioactivity than blank assays. 60 μg of protein kinase modulator were added where indicated.

|	% of control with the addition of cAMP		% of control with the addition of cGMP		
	Endogenous substrate	Added substrate	Endogenous substrate	Added substrate	Added substrate plus protein kinase modulator
80 000 g supernatant	96.9	—	—	—	—
total phosphoproteins	103	—	—	—	—
protein kinase					
fraction 1	106	95	101	91	107
fraction 2	104	114	76	97	102
fraction 3	100	97	97	91	97
fraction 4	117	109	107	103	101
fraction 5	100	113	101	88	91
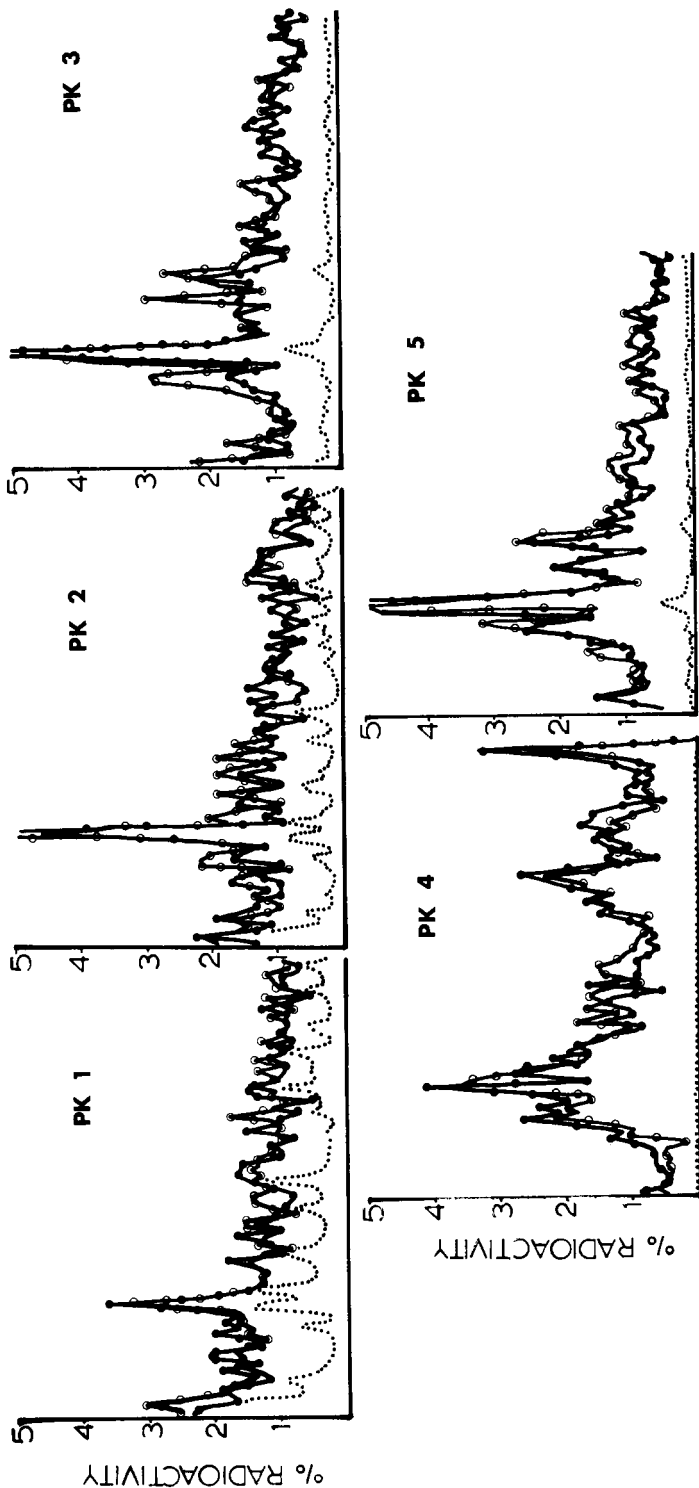
Fig. 4. Distribution of ^{32}P among nuclear proteins induced by each isolated protein kinase fraction in the presence or absence of cyclic AMP. Each protein kinase fraction was assayed in the presence of [γ-^{32}P]ATP, exogenous substrate (heat-inactivated phosphoproteins and histones), and where indicated 5 μM cAMP. After termination of the reaction with the addition of solid urea to a concentration of 5 M, the reaction mixtures were dialyzed extensively against 0.0625 M Tris (pH 6.8)/0.1% SDS/0.1% β-mercaptoethanol, and subsequently fractionated electrophoretically on a slab gel apparatus according to the procedure of Laemmli [30]. The slab gel was 3 mm thick and contained 2 cm of 3% acrylamide/0.08% biscarylamide stacking gel and 8 cm of 8.75% acrylamide/0.23% bisacrylamide resolving gel. Electrophoresis was for 6 h at 30 mA per gel slab. The gel was fixed overnight in 12.5% trichloroacetic acid/7% acetic acid/40% ethanol, and stained in 0.25% Coomassie brilliant blue in the above solution for 5 h. After the gel was destained on a solution of 7% acetic acid/10% ethanol and scanned at 590 nm, the gel was sectioned into 1 mm slices and each slice examined for the incorporation of ^{32}P. The solid circles represent the distribution of ^{32}P among the various proteins when the assay did not contain cAMP, and the open circles represent the distribution when the assays contained 5 μM cAMP. The dotted lines represent the ^{32}P incorporated into the endogenous substrate when no additional protein was added to the assay mixtures. One percent radioactivity is 40, 59, 77, 1429 and 101 cpm PK 1, 2, 3, 4 and 5, respectively.
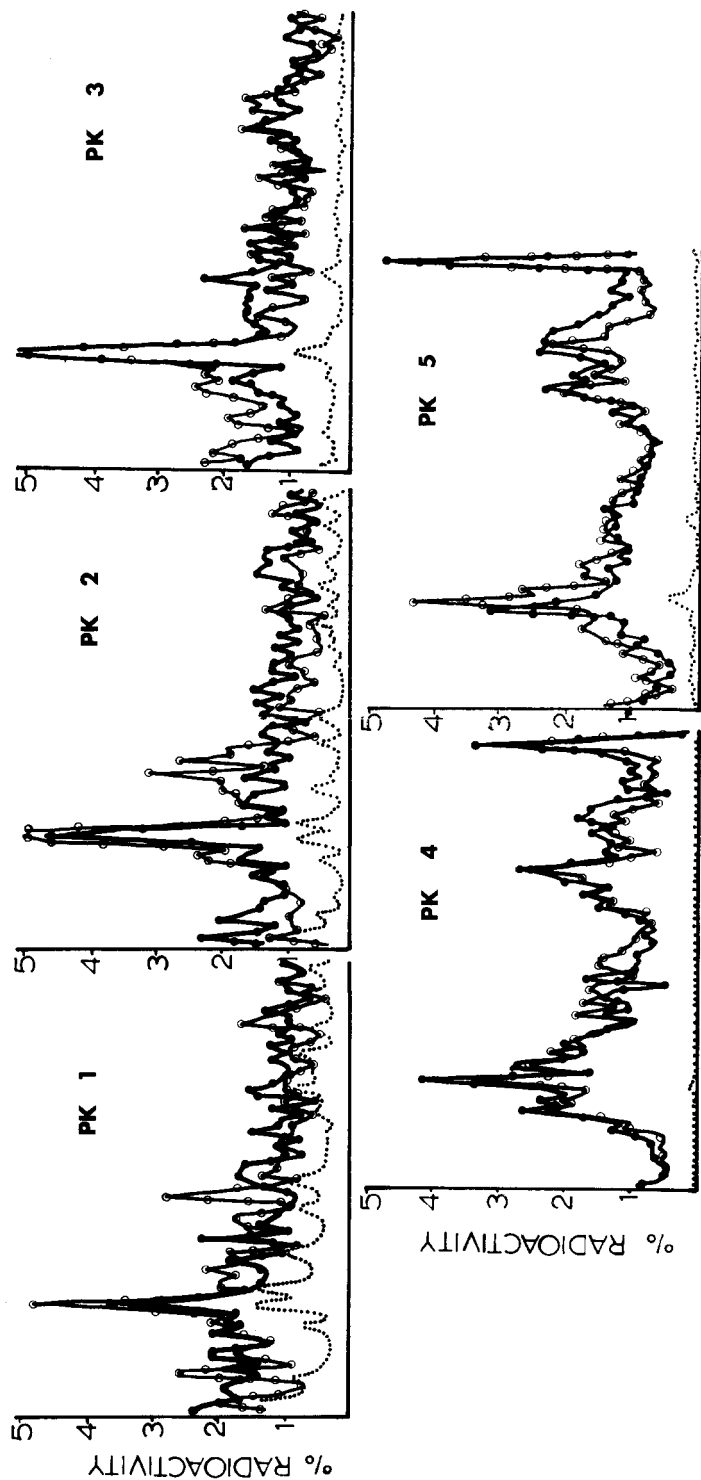
Fig. 5. Distribution of ^{32}P among nuclear proteins induced by each isolated protein kinase fraction in the presence or absence of cyclic GMP. Each protein kinase fraction was assayed in the presence of $[\gamma^{-32}P]ATP$, exogenous substrate (heat-inactivated phosphoproteins and histones), and where indicated 5 μM cGMP. After termination of the reaction with the addition of solid urea to a concentration of 5 M, the reaction mixtures were dialyzed extensively against 0.0625 M Tris (pH 6.8)/0.1% SDS/0.1% β-mercaptoethanol, and subsequently fractionated electrophoretically on a slab gel apparatus according to the procedure of Laemmli [30]. The slab gel was 3 mm thick and contained 2 cm of 3% acrylamide/0.08% bisacrylamide stacking gel and 8 cm of 8.75% acrylamide/0.23% bisacrylamide resolving gel. Electrophoresis was for 6 h at 30 mA per gel slab. The gel was fixed overnight in 12.5% trichloroacetic acid/7% acetic acid/40% ethanol, and stained in 0.25% Coomassie brilliant blue in the above solution for 5 h. After the gel was destained in a solution of 7% acetic acid/10% ethanol and scanned at 590 nm, the gel was sectioned into 1 mm slices and each slice examined for the incorporation of ^{32}P. The solid circles represent the distribution of ^{32}P among the various proteins when the assays did not contain 5 μM cGMP. The dotted lines represent the ^{32}P incorporated into the endogenous substrate when no additional protein was added to the assay mixtures. One percent radioactivity is 40, 65, 77, 4000 and 160 cpm for PK 1, 2, 3, 4 and 5, respectively.

observed in measurements of ^{32}P incorporation into TCA-insoluble material. To examine this possibility, each fraction was assayed with exogenous substrate and $[\gamma\text{-}^{32}P]ATP$ in the presence or absence of cAMP and in the presence or absence of cGMP, and assay mixtures were examined by poly-acrylamide gel electrophoresis. The distribution of ^{32}P radioactivity among the various molecular weight classes of nuclear proteins was determined. Slab gel electrophoresis (using the method of Laemmli [30]) was utilized to afford better resolution and therefore increase the possibility of detecting small changes. No distinct differences in the phosphorylation of nuclear proteins could be seen with the addition of the cyclic nucleotides. as is shown in Figs. 4 and 5.

Protein factors have been shown to alter the sensitivity of kinases to cyclic nucleotides [31 -33], and there are reports of a protein kinase modu-lator which dramatically increases the responsiveness of cGMP-sensitive protein kinases to cGMP. This heat-stable protein has been shown to increase 2—5-fold the cGMP stimulation of cGMP-sensitive protein kinases in vitro. The mechanism of this stimulation is not yet understood. Our protein kinase fractions were assayed in the presence of this protein kinase modulator in order to investigate fully the cyclic nucleotide sensitivity of the protein kinase fractions The protein kinase fractions were assayed in the presence of protein kinase modulator isolated from dog heart as describ-ed by Appleman [26]. The ^{32}P incorporation into TCA insoluble material was measured. Even in the presence of protein kinase modulator, however, no significant response to cGMP could be detected in any of the protein kinase fractions. The results are presented in Table II.

DISCUSSION

The role that phosphorylation of nuclear protein plays in the complex mechanisms involved in the control of gene regulation in eukaryotic organisms has recently been receiving considerable attention. Nuclear phos-phoproteins have been shown to possess the characteristics that one would expect of molecules whose function is involved in the modulation of gene expression. These characteristics include tissue and species specificity [34—36], changes throughout the cell cycle [7,10,37,38], the ability to bind to homologous DNA, high rates of turnover, changes correlated with the metabolic state of the cell, and changes in both protein species and degree of phosphorylation induced by a variety of gene-activating stimuli such as hormones [3,6,11,39]. Differences in the phosphorylation of nuclear proteins have also been demonstrated during carcinogenesis [5,40] and viral transformation [41]. More direct evidence for the involvement of nuclear phosphoproteins in the control of gene transcription is suggested by studies concerned with the control of the histone genes in HeLa S_3 cells. In this system evidence has been presented which suggests that a component of the nuclear phosphoproteins isolated from S phase HeLa cells may be

involved with determining the availability of histone genes from transcription. Other results suggest that the ability of S phase chromatin to transcribe the histone genes in vitro can be reduced 70% by enzymatic partial dephosphorylation of the non-histone chromosomal proteins [12].

In intact cells the nuclear phosphoproteins are enzymatically phosphorylated and dephosphorylated by protein phosphokinases and protein phosphatases respectively, which are present in the nucleus [4,14,16—23]. It is possible that phosphoproteins can function as modulators of gene expression only when they are in a specific state of phosphorylation. The presence of specific phosphoproteins in the nucleus may not by itself determine availability or nonavailability of specific genes for transcription but rather the controlling factor might rest with the enzymes that are responsible for the phosphorylation or dephosphorylation of these proteins. Several investigators have studied nuclear protein kinases in various tissues [14,16,17,19—22], but only a few have demonstrated significant heterogeneity of the kinases [18,23] or have investigated any correlations of kinase activity with various biological conditions such as neoplasia [23,42,43]. Also only limited studies have been directed toward nuclear protein kinases in tissue culture cells [44—66]. We have therefore begun to isolate and partially characterize the nuclear protein kinase fractions from HeLa S_3 cells, which the aim of studying variations in nuclear protein kinases and their activities during the cell cycle.

A method of fractionation of nuclear protein kinase activity using gradient elution was employed by Kish and Kleinsmith [18] to obtain 12 fractions of kinase activity from calf thymus nuclear phosphoproteins. However in HeLa S_3 cells, due to limited availability of material, a stepwise elution gradient was employed. It is possible with this methodology to isolate 5 reproducible fractions of kinase activity from the nuclei of HeLa S_3 cells. However each fraction does not necessarily contain only one kinase. The majority of the activity recovered was found in fractions 3, 4 and 5 which were eluted with 0.05 M Tris (pH 7.5) containing 0.5, 0.7 and 0.9 M NaCl, respectively. Fraction 4 exhibited the highest percentage of recovered activity. This fraction has been shown in other systems to be substantially increased in activity in highly proliferating tissues [23]. All 5 kinase fractions differed with respect to specific activity, optimum Mg^{2+} concentration and substrate specificity, indicating that the fractions represented different species of kinase activity. However, the possibility should not be dismissed that protein factors or sub-units may be shared by various nuclear protein kinase fractions that we have identified. It should be noted that an additional small peak of activity with characteristics similar to those of the tumor-associated enzyme peak described in other systems [23] was occasionally eluted with the 0.3 M NaCl buffer. Since this peak was found only occasionally, it was not possible to characterize it in the manner that was used in the study of the other fractions. Based on the absence of cytoplasmic material in our nuclear preparations, it is unlikely that significant

amounts of cytoplasmic protein kinase were present in our nuclear protein kinase fractions. However, because nuclei were subjected to aqueous solutions during isolation, some loss of nuclear protein kinases may be a possibility.

Considering the importance of cyclic nucleotides to the activity of many cytoplasmic protein kinases the 5 fractions were analyzed for their responsiveness to cAMP and cGMP. It would have been of interest to have observed any response to cGMP since it has been shown that the concentrations of cGMP increases during the entry of the cell into mitosis [47]. However, no response was seen in any protein kinase fraction to either cAMP or cGMP, even in the presence of protein kinase modulator which is thought to be necessary to observe stimulation of some protein kinases by cGMP [31]. This is in agreement with many other reports concerning nuclear kinases [16 21,48,49]. However, a regulatory subunit may be lost in the preparation of the phosphoprotein; if so, this subunit must be lost in the initial isolation steps involving high salt extraction since the supernatant of the first centrifugation after this extraction shows no change in kinase activity when exposed to cyclic nucleotides. Additionally the possibility cannot be dismissed that due to the heterogeneity of the enzyme fractions and substrates cyclic nucleotide dependent alterations in protein kinase activity may be masked. The absence of a response of our nuclear kinases to cyclic nycleotides is consistent with the absence of cytoplasmic protein kinases in the nuclear phosphoprotein preparation, the cyclic nucleotide dependence of cytoplasmic protein kinases has clearly been documented.

Although our nuclear protein kinase fractions do not appear to be responsive to cyclic nucleotides, it is possible that the kinases themselves may be activated by other cyclic AMP-dependent protine kinases -- nuclear or cytoplasmic. Recent results suggest that in several biological systems, protein kinases which do not require cyclic AMP for their catalytic activity must initially be phosphorylated by a cyclic AMP-dependent protein kinase [50]. Having obtained some degree of fractionation of the HeLa nuclear protein kinases and accumulated information concerning their characteristics, studies are now underway to investigate these enzymes under various biological conditions It is anticipated that further study of these enzymes will offer insight into the interrelationship of phosphorylation of nuclear proteins and the control of gene expression in eukaryotic cells.

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