Purification and Properties of a Nuclear Protein Kinase from Rat Mammary Gland

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A nuclear protein kinase that shows a high degree of substrate specificity for the phosphorylation of the acidic proteins casein, phosvitin and non-histone chromatin proteins, rather than the basic proteins histones and protamine, was partially purified from lactating-rat mammary gland. The enzyme is associated with the acidic protein fraction of chromatin. Nuclear kinase requires Co^{2+} for activity, and other bivalent cations such as Mg^{2+} and Mn^{2+} can substitute partially for Co^{2+} . The kinase is further activated (2–3-fold) by various salts, their concentration for maximum stimulation being: NaCl, 150 mM; KCl, 200 mM; sodium acetate, 300 mM. The sedimentation coefficient of the nuclear kinase is 8.9S and its mol.wt. is approx. 300 000 by gel-exclusion chromatography. The enzyme is not activated by cyclic AMP or cyclic GMP and is inhibited neither by the regulatory subunit of mammary cyclic AMP-dependent protein kinase nor by the heat-stable protein kinase inhibitor from ox heart. Analysis of ^{32}P -labelled protein products reveals that the kinase transfers the terminal phosphate of ATP to serine and threonine residues of proteins. The enzyme, however, has specificity for the phosphorylation of threonine in casein and serine in phosvitin. Molecular size and enzymic characteristics of the nuclear protein kinase are clearly different from those of the cytosol enzyme previously characterized.

It has been demonstrated that the phosphorylation of histones and nuclear acidic proteins in cultured mammary cells is under hormonal regulation and that these nuclear phosphoproteins may be involved in the regulation of gene expression in mammary gland (Turkington & Riddle, 1970). Subsequent studies from this laboratory have characterized a mammary cytosol cyclic AMP-dependent protein kinase which showed much greater substrate specificity for nuclear histones than for chromatin acidic proteins (Majumder & Turkington, 1971a). Cyclic AMP-dependent protein kinase (RC) of mammary gland and many other tissues is an inactive complex of a catalytic (C) and a regulatory (R) subunit, and cyclic AMP causes the activation of the holoenzyme by specifically binding with the regulatory subunit, thereby dissociating it from the catalytic subunit, which is the active form of the enzyme (Tao *et al.*, 1970; Reimann *et al.*, 1971; Gill & Garren, 1971; Miyamoto *et al.*, 1971; Erlichman *et al.*, 1971; Majumder & Turkington, 1972; Langan, 1973).

The hormone-dependent differentiation of mammary gland *in vivo* as well as *in vitro* is associated with a marked increase in the specific activity of a cytosol cyclic AMP-dependent protein kinase. It was shown that prolactin acts synergistically with insulin to induce rapidly the activities of both the subunits of the holoenzyme in mammary cells cultured *in vitro* in a chemically defined medium (Majumder & Turkington, 1971b).

Hormonally induced cytosol protein kinase may subsequently mediate the action of prolactin by causing phosphorylation of nuclear histones and multiple specific phosphoproteins of plasma membranes and ribosomes (Majumder & Turkington, 1972; Turkington *et al.*, 1973). In the present studies I purified and characterized a protein kinase from the acidic protein fraction of mammary chromatin. The enzyme is not activated by cyclic AMP or cyclic GMP and it preferentially phosphorylates nuclear acidic proteins rather than histones. The properties of the nuclear enzyme are clearly different from those of the cytosol protein kinase, indicating that the nuclear protein kinase is different from the cytoplasmic counterpart.

Experimental

Materials

ATP, ox heart protein kinase inhibitor, protamine (salmon spermatozoa), phosvitin, bovine serum albumin and γ -globulin were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [γ - ^{32}P]ATP (21–27 Ci/mmol) was a product of International Chemical and Nuclear Corp. (Irvine, CA, U.S.A.). Whole and fractionated histones from calf thymus and trypsin (crystallized twice) were from Worthington (Freehold, NJ, U.S.A.). Bovine casein, ovalbumin and density-gradient grade (ribonuclease-free) sucrose were obtained from Mann Research (New York, NY, U.S.A.). Cyclic AMP, *O*-phosphoserine

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and *O*-phosphothreonine were products of Calbiochem (La Jolla, CA, U.S.A.). DEAE-cellulose (Cellex-D) and phosphocellulose were from Bio-Rad Laboratories (Richmond, CA, U.S.A.). Sephadex G-200 and DEAE-Sephadex were products of Pharmacia Fine Chemicals (Uppsala, Sweden). Cyclic GMP, cyclic UMP and cyclic CMP were obtained from Boehringer Mannheim Corp. (New York, NY, U.S.A.).

Female Sprague-Dawley rats which had been lactating for 15–25 days were the source of all tissue used. Cyclic AMP-dependent cytosol mammary protein kinase and its catalytic (C) and regulatory (R) subunits were isolated by the procedure described previously (Majumder & Turkington, 1972).

Fractionation of nuclei

Lactating-rat mammary glands were homogenized in 0.25 M-sucrose containing 0.1 M-KCl, 0.01 M-Tris/HCl (pH 7.4) and 3 mM-MgCl₂ in an all-glass homogenizer, and the homogenate was filtered through four layers of cheesecloth and then centrifuged at 1000 *g* for 10 min. The crude nuclear pellet was washed twice in the same buffer. The pellet was then dispersed in 2.0 M-sucrose and centrifuged at 35000 *g* for 60 min to sediment purified nuclei (Chauveau *et al.*, 1956). The pellet was washed by resuspending in the above buffer and centrifuging at 1000 *g* for 10 min. These preparations of nuclei appeared highly purified by phase-contrast microscopy. For the extraction of histones, nuclei were stirred in 0.25 M-HCl at 4°C for 4 h and then centrifuged at 27000 *g* for 30 min (Majumder & Turkington, 1971a). The soluble proteins of the nuclear sap were removed by washing the nuclei with NaCl/EDTA by the method of Ueda *et al.* (1968). The nuclei obtained from 10 g of mammary gland were extracted successively with 4 ml each of Tris/HCl buffer (0.01 M, pH 7.4, and 0.05 M, pH 7.8), NaCl (75 mM)/EDTA (24 mM, pH 8.0) and Tris/HCl buffer (0.05 M, pH 8.0), followed each time by centrifugation for 10 min at 20000 *g*, and all the extracts were pooled (nuclear sap). Chromatin was extracted from the final pellet with 0.05 M-Tris/HCl, pH 7.5, containing 1 M-NaCl, by homogenization in a Teflon/glass homogenizer and shearing for 1 min (20 s at a time) in a Polytron homogenizer (Brinkmann, Westbury, NY, U.S.A. at setting 3, followed by centrifugation at 80000 *g* for 60 min (Takeda *et al.*, 1971). The salt concentration of the supernatant (chromatin preparation) was lowered by the addition of 1.5 vol. of 0.05 M-Tris/HCl, pH 7.5, and the resulting precipitate (nucleohistone) was removed by centrifugation at 100000 *g* for 60 min. The supernatant was dialysed overnight against 0.05 M-Tris/HCl, pH 8.0, containing 0.01 M-KCl and the turbid dialysed solution was centrifuged at 10000 *g* for 10 min to obtain a clear supernatant containing the nuclear acidic-protein fraction.

Isolation of nuclear protein kinase

Purified nuclei isolated by the procedure described above were suspended in 0.14 M-NaCl and centrifuged at 10000 *g* for 10 min. The pellet was dispersed in 0.05 M-Tris/HCl, pH 7.5, by homogenization in a glass/Teflon homogenizer and the suspension was adjusted to 1 M-(NH₄)₂SO₄ by the addition of a 4 M-(NH₄)₂SO₄ solution with stirring. The suspension was homogenized in a glass/Teflon homogenizer, left in ice for 10 min and centrifuged at 27000 *g* for 30 min. The pellet was discarded and the supernatant was dialysed overnight against 0.05 M-Tris/HCl, pH 8.0, containing 0.01 M-KCl and then centrifuged at 27000 *g* for 10 min. The resulting supernatant (step 1) was applied to a column (0.9 cm × 38 cm) of DEAE-Sephadex previously equilibrated with 50 mM-Tris/HCl, pH 8.0, containing 0.01 M-KCl. The column was washed with 25 ml of the equilibrating buffer and then further eluted with a linear gradient of KCl (0.01–1.5 M) in 50 mM-Tris/HCl, pH 8.0, in a total volume of 260 ml. The flow rate was 12 ml/h and 3.5 ml fractions were collected (step 2). All the steps were carried out at 0–4°C.

Assay of protein kinase

The activity of protein kinase was measured by a modification of the method described by Majumder & Turkington (1972). The standard assay medium contained 10 μmol of sodium glycerophosphate/HCl (pH 7.0), 2.0 mg of bovine casein, 1 nmol of [γ -³²P]-ATP (containing 1.5×10^5 – 7.0×10^5 c.p.m.), 2 μmol of NaF, 40 μmol of NaCl, 0.4 μmol of CoCl₂ and protein kinase in a total volume of 0.2 ml. The incubation was carried out at 30°C for 20 min and the reaction was terminated with the addition of 5 ml of cold 10% (w/v) trichloroacetic acid. The resulting precipitate was processed for the assay of radioactivity as described by Majumder & Turkington (1971a). One unit of protein kinase activity was defined as the amount of the enzyme that causes the transfer of 1 pmol of ³²P from [γ -³²P]ATP to the recovered protein during 20 min under the standard assay conditions.

Preparation of ³²P-labelled casein and phosvitin

Casein and phosvitin were phosphorylated enzymically by the isolated nuclear protein kinase. The reaction mixture contained 25 μmol of sodium glycerophosphate/HCl, pH 7.0, 5 μmol of NaF, 100 μmol of NaCl, 2 μmol of CoCl₂, 6 mg of casein or 2 mg of phosvitin, 6 nmol of [γ -³²P]ATP (5×10^6 c.p.m.) and 55 units of nuclear protein kinase in a total volume of 550 μl. The mixture was incubated at 30°C for 30 min and the reaction was stopped by the addition of 5 ml of 10% (w/v) trichloroacetic acid. The precipitate

was collected by centrifugation at 1000 *g* for 10 min and washed successively with 10% (w/v) trichloroacetic acid (three times), ethanol, ethanol/diethyl ether (1:3, v/v) and diethyl ether.

Sucrose-density-gradient sedimentation

Isokinetic sucrose gradients (5–23.7%, w/w) were prepared as described by McCarty *et al.* (1968). The sucrose solution contained 5 mM-sodium glycerophosphate/HCl buffer, pH 7.0, and 0.2 mM-dithiothreitol. Protein kinase preparation (600 units in 0.2 ml) was layered over 4.5 ml of sucrose gradient, and after centrifugation at 46000 rev./min for 13 h at 4°C in a Spinco SW50.1 rotor the tube bottom was punctured and 10-drop fractions (about 0.15 ml) were collected and assayed for enzyme activity.

Determination of molecular weight of protein kinase

Molecular weight of the nuclear protein kinase was determined by the method of Whitaker (1963) on a column (0.9 cm × 53 cm) of Sephadex G-200. The gel was equilibrated with 50 mM-sodium glycerophosphate/HCl buffer, pH 6.5, containing 0.2 mM-dithiothreitol and the enzyme activity was eluted with the same buffer. Standard proteins and Blue Dextran were detected by absorption at 280 nm and the activity of the kinase was measured under the standard assay conditions. The mol.wts. of the proteins used as standards were bovine γ -globulin (156000), bovine serum albumin (70000), ovalbumin (45000) and trypsin (24000).

Other analytical methods

³²P-labelled proteins were hydrolysed with 2 M-HCl and the hydrolysates were subjected to high-voltage electrophoresis for the measurement of radioactivity in the phosphoserine and phosphothreonine spots as described by Majumder & Turkington (1971a). The protein contents of all samples were determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Purification of nuclear protein kinase

One peak of protein kinase activity was demonstrated in the mammary nuclear extract with bovine casein as the protein substrate by DEAE-Sephadex chromatography (Fig. 1). Assay of the individual chromatography fractions with and without the addition of calf thymus histones (0.5 mg) as an exogenous protein substrate failed to detect any additional kinase peaks. Active fractions (fractions 30–38) were pooled, concentrated by ultrafiltration

through an Amicon PM-30 membrane and finally preserved in 40% (v/v) glycerol containing 2 mM-sodium glycerophosphate/HCl buffer, pH 6.5, and 0.2 mM-dithiothreitol at –25°C. This preparation of the enzyme was used in the studies reported here unless otherwise stated.

Results of a typical purification procedure are shown in Table 1. Cyclic AMP (2 μ M) had no appreciable effect on the protein kinase activities of all the fractions tested. Protein kinase activity was purified from nuclei approx. 34-fold. Kinase activity was not detectable in the intact nuclei, and there was a marked inhibition (approx. 90%) of the activity of the isolated enzyme when purified nuclei (100 μ g of protein) were added to it, indicating the presence of component(s) in nuclei that interfere with the protein kinase-catalysed reaction.

Kish & Kleinsmith (1974) demonstrated the presence of at least 12 species of protein kinase in the non-histone chromatin-phosphoprotein fraction from ox liver nuclei by using phosphocellulose chromatography. Mammary non-histone chromatin proteins were extracted, subjected to phosphocellulose chromatography as described by Kish & Kleinsmith (1974) and the chromatography fractions were assayed for protein kinase activity without and with exogenous protein substrate (casein) in the presence and absence of 2 μ M-cyclic AMP. The fractions contained no significant protein kinase activity for phosphorylation of endogenous protein substrates, whereas a single peak of protein kinase activity, eluted with 50 mM-Tris/HCl (pH 8.1)/0.6 M-NaCl buffer, was detected with casein as protein substrate. Partially purified nuclear kinase preparation as obtained by DEAE-Sephadex chromatography was also subjected to phosphocellulose chromatography (Kish & Kleinsmith, 1974), and there was no evidence for the presence of more than one species of kinase activity in the nuclear preparation. It was further observed that the recovery of protein kinase activity after phosphocellulose chromatography was low (approx. 25%) because of the instability of the enzyme.

Properties of nuclear protein kinase

The amount of casein phosphorylated by mammary nuclear protein kinase increased linearly with time for at least 30 min. Proportional increase in the activity of the enzyme was observed with as much as 50 units of protein kinase. The maximum activity of the enzyme was observed at pH 7.0–7.5. The activity of the kinase was inactivated almost completely (approx. 95%) when heated at 70°C for 10 min. The amount of phosphorylation of endogenous proteins by the nuclear kinase preparation was insignificant under the standard assay conditions.

Protein substrate concentration. The effects of varying the concentrations of casein and phosvitin on

Fig. 1. DEAE-Sephadex chromatography pattern of mammary nuclear protein kinase

Nuclear extract (15mg of protein) was applied to a column (0.9cm×38cm) of DEAE-Sephadex and 50μl of each fraction was assayed for protein kinase activity in the presence of 2μM-cyclic AMP under the standard assay conditions with bovine casein as the protein substrate. O, Protein kinase activity; ----, A_{280} ; —, KCl concentration.

Table 1. Summary of purification of nuclear protein kinase from lactating rat-mammary gland

The activities of all the fractions were measured under standard assay conditions with casein (2mg) as the protein substrate. The results have been duplicated. N.D., Not detectable. The activity of protein kinase was not detectable in purified nuclei and therefore the specific activity of the enzyme in the nuclei was calculated by assuming that the nuclei contain 20000 units of the enzyme as recovered in the nuclear extract (step 1).

Fraction	Protein (mg)	$10^{-3} \times$ Total activity (units)	Specific activity (units/mg of protein)	Purification (fold)	Yield (%)
Nuclei	50	N.D.	400	1	—
Step 1: Nuclear extract	15	20	1330	3.3	100
Step 2: DEAE-Sephadex eluate	1.2	16	13300	34.0	80

Fig. 2. Effect of concentrations of casein and phosvitin on the activity of mammary nuclear protein kinase

Standard assay conditions were used except for the variation in the nature and amount of protein substrate; O, casein; Δ, phosvitin.

the activity of mammary nuclear protein kinase are shown in Fig. 2. The apparent K_m values of the enzyme for casein and phosvitin as estimated by the Lineweaver-Burk plot of these data were 4.2mg/ml and 1.9 mg/ml respectively.

Metal ions. Table 2 shows that the activity of the mammary nuclear kinase is dependent on a bivalent metal ion such as Co^{2+} , Mg^{2+} or Mn^{2+} , the degree of activation being maximum in the presence of Co^{2+} , whereas Ca^{2+} inhibits the activity of the enzyme. NaCl cannot replace a bivalent metal ion for the activation of the enzyme, but in the presence of Co^{2+} , 100mM-NaCl has a marked stimulatory effect (2.5-fold). The data also indicate that the thiol compound dithiothreitol had no effect, and that the addition of EDTA strongly inhibits the kinase activity. Cyclic nucleotides, namely cyclic AMP, cyclic GMP, cyclic

Table 2. General properties of mammary nuclear protein kinase

The complete system contains all the reactants as defined in the standard assay except that NaCl was omitted. The results have been duplicated. N.D., Not detectable. A 100% enzyme activity was 20 units.

System	Protein kinase activity (%)
Complete	100
- CoCl ₂	N.D.
- CoCl ₂ + MgCl ₂ (5 mM)	50
- CoCl ₂ + MnCl ₂ (5 mM)	40
- CoCl ₂ + NaCl (5 mM)	N.D.
- CoCl ₂ + NaCl (100 mM)	N.D.
+ NaCl (100 mM)	250
+ CaCl ₂ (8 mM)	75
+ CaCl ₂ (20 mM)	40
+ EDTA (5 mM)	10
+ Dithiothreitol (1 mM)	105
+ Cyclic AMP (2 μM)	100
+ Cyclic GMP (2 μM)	105
+ Cyclic UMP (2 μM)	98
+ Cyclic CMP (2 μM)	102

Fig. 3. Effect of Co²⁺ and Mg²⁺ concentrations on the activity of mammary nuclear protein kinase
Standard assay conditions were used except for the variation in the nature and amount of the bivalent metal ion. ○, Co²⁺; △, Mg²⁺.

CMP and cyclic UMP, had no appreciable effect on the enzyme activity in the concentration range 0.01–2 μM under conditions of the standard assay.
The effect of varying the concentrations of Mg²⁺ or Co²⁺ on the protein kinase activity is shown in Fig. 3. The maximum stimulation by the bivalent metal ion

Fig. 4. Effects of NaCl, KCl and sodium acetate concentrations on the activity of mammary nuclear protein kinase
Effects of various concentrations of NaCl (○), KCl (△) and sodium acetate (□) on the activity of nuclear protein kinase measured under the standard assay conditions.

Table 3. Substrate specificity of mammary nuclear protein kinase

Mammary nuclear acidic protein fraction was obtained by the procedure described in the text and it was subjected to heat treatment at 80°C for 5 min to inactivate the endogenous protein kinase activity before use as protein substrate of the purified nuclear enzyme. Standard assay conditions were used except for the replacement of 2 mg of casein by the various substrates indicated. The results have been duplicated.

Substrate	Amount (mg)	Incorporation of ³² P into protein (pmol/20 min)
Casein	0.6	24
	0.2	13
Phosvitin	0.6	26
	0.2	16
Mammary nuclear acidic proteins	0.6	5.0
	0.2	3.0
Protamine	0.6	0.5
	0.2	0.4
Calf thymus histones	0.6	0.6
	0.2	0.4
Mammary histones	0.6	0.5
	0.2	0.4

was observed at a concentration range of 1.5–2.8 mM for Co²⁺ and 9–15 mM for Mg²⁺, and at higher concentrations (above 3 mM-Co²⁺ and above 25 mM-Mg²⁺) the stimulatory effects were markedly decreased. The apparent K_m values of the enzyme for Co²⁺ and Mg²⁺ were 0.7 and 2.0 mM respectively.
As shown in Fig. 4 high concentrations of NaCl, KCl or sodium acetate markedly increased the

Table 4. Distribution of [32 P]phosphoserine and [32 P]-phosphothreonine in 32 P-labelled casein and 32 P-labelled phosvitin

32 P-labelled casein and 32 P-labelled phosvitin were hydrolysed with 2M-HCl and the hydrolysate was subjected to high-voltage electrophoresis by the procedure described in the Experimental section. Approx. 70% of the radioactivity of the [32 P]phosphoproteins was recovered in the O-phosphoserine and O-phosphothreonine spots. The results have been duplicated.

Protein substrate	[32 P]Serine radioactivity		[32 P]Threonine radioactivity	
	(c.p.m.)	(%)	(c.p.m.)	(%)
Casein	470	20	1790	80
Phosvitin	1620	76	500	24

activity of the nuclear protein kinase. The maximum stimulatory effects of these salts on the enzyme were almost identical (approx. 150% increase). The concentration ranges of the salt for the maximum activation were: 150–200mM-NaCl, 200–250mM-KCl, 300–400mM-sodium acetate, and at higher concentrations the stimulatory effects of the salts were greatly decreased.

Substrate specificity. The ability of mammary nuclear protein kinase to phosphorylate various protein substrates was evaluated (Table 3). The acidic proteins bovine casein and phosvitin were phosphorylated by nuclear protein kinase at considerably faster rates than were the basic proteins protamine and mammary and calf thymus histones. The rate of phosphorylation of mammary nuclear acidic proteins is much lower than for those of casein and phosvitin. However, the substrate specificity of the nuclear kinase for mammary nuclear acidic proteins is much greater than for the mammary histones.

Amino acid analysis of the 32 P-labelled protein products of nuclear protein kinase demonstrates that the enzyme catalyses the phosphorylation of both serine and threonine residues of casein and phosvitin (Table 4). The kinase, however, preferentially phosphorylates threonine residues (80%) in casein and serine residues (76%) in phosvitin.

Sedimentation coefficient and molecular weight. Mammary nuclear kinase was subjected to sucrose-density-gradient centrifugation along with bovine serum albumin (4.4S) as an internal standard (Fig. 5). Nuclear kinase sedimented as a single peak of activity at 8.9S. By molecular sieving on Sephadex G-200 one peak of enzyme activity was detected, which has an approximate mol.wt. of 300000.

Effect of protein kinase inhibitor and regulatory subunit. A heat-stable protein that causes inhibition of cyclic AMP-dependent protein kinase occurs in many tissues (Ashby & Walsh, 1972). The effect of

Fig. 5. Isokinetic sucrose-density-gradient profile of mammary nuclear protein kinase

Sucrose-density-gradient fractionation of nuclear protein kinase was carried out by the procedure described in the Experimental section. A 50 μ l portion of each fraction was assayed for protein kinase activity under the standard assay conditions. The arrow indicates the position of bovine serum albumin marker (4.4S).

various concentrations of heart protein kinase inhibitor on the activities of mammary nuclear protein kinase and cytosol cyclic AMP-dependent protein kinase is shown in Fig. 6(a). Kinase inhibitor decreased the activity of the cytosol protein kinase, but it had no appreciable effect on the nuclear kinase activity.

As shown in Fig. 6(b), regulatory subunit (R) of mammary cyclic AMP-dependent protein kinase (RC), which markedly inhibits the activity of the catalytic subunit (C), has no significant effect on the nuclear protein kinase activity. Cyclic AMP (2 μ M) has no effect on the activity of the nuclear protein kinase in the presence of either regulatory subunit or protein kinase inhibitor. These studies thus demonstrate that, unlike the cytosol cyclic AMP-dependent protein kinase, nuclear kinase activity is inhibited neither by protein kinase inhibitor nor by the regulatory subunit of the holoenzyme.

Nuclear fractionation. As shown in Table 5 approx. 95% of the activity of the nuclear protein kinase is tightly bound to the acidic protein fraction of chromatin and approx. 5% of the enzyme activity was present in the nuclear sap. A small amount of protein kinase activity which is more specific for histones and

Fig. 6. Effect of protein kinase inhibitor and regulatory subunit of cyclic AMP-dependent protein kinase on the activities of mammary nuclear and cytosol protein kinase

(a) Effect of various concentrations of ox heart protein kinase inhibitor on the activities of mammary nuclear protein kinase (●) determined under the standard assay conditions with casein as protein substrate. The action of the kinase inhibitor on mammary cytosol cyclic AMP-dependent protein kinase (▲) was measured with calf thymus histones as protein substrate as described previously (Majumder & Turkington, 1972). (b) Effect of regulatory subunit of cyclic AMP-dependent mammary protein kinase on the activities of nuclear protein kinase (○) measured under the standard assay conditions. Role of protein kinase regulatory subunit on the activity of catalytic subunit (Δ) of mammary cyclic AMP-dependent protein kinase was determined by using calf thymus histones as protein substrate (Majumder & Turkington, 1972).

activated by cyclic AMP was detected in the nuclear sap. Cyclic AMP, however, had no appreciable effect on the activity of the enzyme in other nuclear fractions. The enzyme associated with the purified chromatin or nuclear acidic protein fraction can cause a significant amount of phosphorylation of the endogenous phosphoproteins. There was no detectable activity of protein kinase in intact nuclei and nucleohistone fraction for the phosphorylation of either endogenous proteins or exogenous proteins casein and histones. Removal of nuclear sap and nucleohistone fraction greatly increased protein kinase activity associated with the nuclear acidic protein fraction.

Discussion

The present studies characterize a rat mammary nuclear protein kinase which is associated with the acidic protein fraction of chromatin. The enzyme has high degree of substrate specificity for the phosphorylation of the acidic proteins casein and phosvitin rather than the basic proteins histones and protamine. The activity of the nuclear kinase (8.9S) is dependent on a bivalent metal ion (Co^{2+} , Mg^{2+} or Mn^{2+}) and it

is further activated (2–3-fold) by various salts. Cyclic AMP, the regulatory subunit of cyclic AMP-dependent protein kinase, and heat-stable protein kinase inhibitor had little effect on the activity of nuclear kinase, indicating that the nuclear enzyme is not the catalytic subunit of a cyclic AMP-dependent protein kinase. The enzyme catalyses the transfer of the terminal phosphate group of ATP to serine and threonine residues of proteins and it has specificity for the phosphorylation of threonine residues in casein and serine residues in phosvitin. Molecular size, substrate specificity and other enzymic characteristics of the nuclear kinase are markedly different from those of the cytosol cyclic AMP-dependent protein kinase (Majumder & Turkington, 1971a, 1972), indicating that the nuclear enzyme is clearly different from the cytosol kinase. Nuclear kinase showed higher substrate specificity for chromatin-bound acidic proteins, whereas the cytosol enzyme preferentially phosphorylates histones *in vitro*.

Multiple forms of protein kinase have been detected in purified liver chromatin of several species of animals (Takeda *et al.*, 1971; Desjardins *et al.*, 1972; Ruddon & Anderson, 1972; Higashino & Takeda,

Table 5. *Distribution of protein kinase activity in nuclear subfractions of lactating rat mammary gland*

Nuclear sap as obtained by the procedure described in the text was concentrated by dehydration with Sephadex G-200 at 4°C. All the fractions were dialysed against 4mM-sodium glycerophosphate/HCl buffer, pH 6.5, containing 30% (w/v) glycerol, before assay of protein kinase activity without and with casein (2mg) or calf thymus histones (0.5mg) as protein substrate in the presence of 2 μ M-cyclic AMP under the standard assay conditions. The results have been duplicated. N.D., Not detectable. Values in parentheses represent percentage recovery of the enzymic activity by assuming the activities recovered in nuclear sap and nuclear acidic protein fraction to be 100.

Fraction	Exogenous protein substrate ...	Protein kinase activity (units/mg of protein)		
		None	Casein	Histones
Nuclei		N.D.	N.D.	N.D.
Nuclear sap		N.D.	20 (5)	40
Chromatin		20	80	20
Nucleohistone		N.D.	N.D.	N.D.
Nuclear acidic proteins		60	1200 (95)	25

1974; Kish & Kleinsmith, 1974). The studies of Kish & Kleinsmith (1974) demonstrated the presence of at least 12 species of protein kinase in the acidic protein fraction of chromatin from ox liver. These kinases can cause phosphorylation of the endogenous nuclear acidic phosphoproteins, and a complex interaction of kinase, protein substrate and cyclic AMP was demonstrated. It was further observed by Kish & Kleinsmith (1974) that nuclear protein kinase profiles of ox liver and kidney are markedly different. The present studies could not detect the occurrence of more than one species of protein kinase in mammary chromatin fraction. It is thus possible that the process of cell differentiation confers on cells a tissue-specific pattern of nuclear protein kinase distribution.

It was noteworthy that protein kinase activity of intact nuclei was undetectable for the phosphorylation of either endogenous phosphoproteins or the exogenous protein casein or histones (Tables 1 and 5), despite the presence of a large amount of chromatin-bound protein kinase activity. The addition of intact nuclei or a preparation of nucleohistone markedly inhibited the activity of purified nuclear kinase, indicating the presence of interfering component(s) in nuclei causing inhibition of the activity of the chromatin-bound protein kinase (G. C. Majumder, unpublished work). Preliminary studies demonstrate that the nucleohistone fraction of chromatin is rich

in adenosine triphosphatase activity, which may cause marked inhibition of the phosphorylation of endogenous nuclear proteins or exogenous protein substrates by the nuclear protein kinase (G. C. Majumder, unpublished work). It thus appears that this strikingly high activity of adenosine triphosphatase in chromatin may have a novel regulatory role for the phosphorylation of proteins and the transcriptional activity of nuclei.

A large body of evidence has now been gathered from several laboratories to support the general concept that acidic chromatin proteins may serve as gene regulators (Kleinsmith & Allfrey, 1969; Kleinsmith *et al.*, 1970; Spelsberg & Hnilica, 1970; Teng *et al.*, 1971; Kadohama & Turkington, 1973). Mammary acidic chromatin proteins are extremely heterogeneous and are tissue- and species-specific. Six of these protein species present in differentiated cells from lactating mice were not detectable in undifferentiated cells from virgin mice. These new protein species are formed in the undifferentiated mammary cells in response to insulin and subsequently may participate in chromatin reconstitution during DNA replication, thereby determining new states of gene expression in the daughter cells (Kadohama & Turkington, 1973; Turkington *et al.*, 1973). The occurrence of the nuclear kinase almost exclusively in the chromatin acidic protein fraction and its ability to phosphorylate preferentially these acidic proteins rather than nuclear histones suggests strongly that nuclear acidic proteins may serve as physiological protein substrates of the kinase. The earlier finding that hormonally induced mammary cytosol protein kinase has markedly low substrate specificity for chromatin acidic proteins as compared with histones (Majumder & Turkington, 1971a) is consistent with this view. The observation that the phosphorylation of nuclear acidic proteins of cultured mammary epithelial cells is under hormonal regulation (Turkington & Riddle, 1970) and that the phosphorylation of this class of proteins directly affects the rate of RNA synthesis *in vitro* (Teng *et al.*, 1971; Kamiyama *et al.*, 1972; Shea & Kleinsmith, 1973) are consistent with the possibility that nuclear protein kinase may have an important regulatory role in determining new patterns of gene expression in mammary epithelial cells.

Both mammary (Fig. 4) and liver (Desjardins *et al.*, 1972) nuclear protein kinases of the rat are markedly stimulated by NaCl, KCl and sodium acetate at 0.15–0.30M. The mechanism of this salt-mediated activation of the nuclear protein kinase is not clear at present. It is possible that the salts at higher concentrations affect the conformation of the protein kinase or its protein substrates. Shifts in the concentrations of Na⁺ ions in *Chironomus* salivary nuclei under different physiological conditions have been reported by Lezzi *et al.* (1976). It

thus appears possible that nuclear Na⁺ and K⁺ ions may have an important physiological role in the regulation of the activity of the chromatin-bound protein kinase.

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