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HETEROGENEITY AND SPECIFICITY OF NON-HISTONE NUCLEAR PHOSPHOPROTEINS

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Abstract—1. Extensive heterogeneity has been observed amongst the non-histone chromatin phosphoproteins from a variety of species, tissues and cell types.

2. Each tissue and each cell type exhibits a unique protein pattern and radioactivity profile when labeled with [^{32}P].

3. The differences in nuclear phosphoproteins of liver from different species were found to increase as the species being compared diverged evolutionarily.

INTRODUCTION

It is now generally believed that chromatin-associated proteins play a key role in regulating the pattern of gene expression in higher organisms. Although histones have been extensively studied as potential gene regulators in this regard, they do not seem to exhibit the properties required for modulating the activity of a large number of specific genes. Histones are present in only five major types, and in spite of some limited microheterogeneity which has been observed within a few of these fractions, this small number of histone classes and their relative constancy in different tissues and organisms has led to the general conclusion that they are not responsible for regulating the activity of individual genes (see Hnilica, 1972 for review).

The non-histone (or "acidic") chromosomal proteins, on the other hand, exhibit a number of properties expected of genetic regulatory molecules (for review see Stein *et al.*, 1974). In contrast to the histones, these non-histone proteins exhibit a considerable degree of heterogeneity, and the distribution of these various protein species appears to differ significantly in different tissues (Loeb & Creuzet, 1970; Platz *et al.*, 1970; Wang, 1971; Wu *et al.*, 1973). Among the various components of this heterogeneous fraction of proteins are a large number of proteins which are extensively phosphorylated (Kleinsmith *et al.*, 1966a; Kleinsmith & Allfrey, 1969a; Platz *et al.*, 1970; Teng *et al.*, 1971; Rickwood *et al.*, 1973; Kleinsmith, 1975). These phosphorylated proteins exhibit a number of properties expected of genetic regulatory molecules, including the following: they can bind specifically to DNA (Teng *et al.*, 1971; Kleinsmith *et al.*, 1970; Kleinsmith, 1973), they can induce alterations in RNA synthesis when added to cell-free systems (Langan, 1967; Teng *et al.*, 1971; Kamiyama *et al.*, 1972; Shea & Kleinsmith, 1973) and changes in their phosphorylation can be shown to correlate with changes in differentiation, growth, and gene transcription *in vivo* (Kleinsmith *et al.*, 1966b; Gershey & Kleinsmith, 1969b; Turkington & Riddle, 1969; LeStourgeon & Rusch, 1971; Ahmed & Ishida, 1971; Platz *et al.*, 1973; Platz & Hnilica, 1973; Allfrey *et al.*, 1973; Kleinsmith, 1974).

If these phosphorylated non-histone proteins are indeed gene regulators, then in view of the large number of different genes which need to be controlled, one would expect them to be highly heterogeneous. In addition, tissue-specific differences in gene readout should be reflected in tissue-specific differences in the non-histone phosphoproteins. Preliminary data from our laboratory on a small number of different tissues have suggested that such heterogeneity and tissue specificity do in fact exist (Platz *et al.*, 1970), a conclusion which has received some independent support from other investigators (Teng *et al.*, 1971; Rickwood *et al.*, 1973). The present paper examines the heterogeneity of phosphorylated proteins in several non-histone protein subfractions, and compares preparations obtained from a broad spectrum of different species, tissues and cell types in order to provide a firm basis for conclusions regarding both the heterogeneity and specific distribution of these nuclear proteins.

METHODS

Materials

Fresh liver was obtained from laboratory raised rat (Sprague–Dawley), chicken, and deer mouse (*Peromyscus maniculatus*), and from sea lamprey (*Petromyzon marinus*) trapped in mid-May at the Hammond Bay Station, Roger City, Michigan. Mouse plasma cell tumor tissue was maintained in BALB/c mice. Frozen liver was used from freshly caught snapping turtle (*Chelydra serpentina*) and frog (*Rana pipiens*) and from opossum (*Didelphis marsupialis*) and monkey (*Rhesus macacamulatta*) after laboratory acclimation for at least 1 month. With the exception of the frog tissue which had been stored at −20°C for 1 yr, frozen tissues were used within 1 week after freezing at −20°C or −70°C. Beef liver was purchased from a local slaughter house.

Isolation of nuclei

Rat liver nuclei were isolated by modification of the dense sucrose procedure of Pogo *et al.* (1966). Ten grams

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of liver were minced with scissors and homogenized in 3 vol of 0.32 M sucrose. 3 mM $MgCl_2$ in a Dounce homogenizer using four strokes with a loose pestle (0.127 mm clearance) and three strokes with a tight pestle (0.076 mm clearance). The homogenate was centrifuged for 7 min at 1000 *g*, the pellet resuspended in homogenization medium, and sedimented at 1000 *g*. The nuclear pellet was then suspended in 110 ml of 2.4 M sucrose, 1 mM $MgCl_2$ and centrifuged 1 hr at 100,000 *g* in an SW 27 rotor. The purified nuclear pellets were taken up in 10 ml of 0.25 M sucrose, 4 mM $MgCl_2$, 0.01 M Tris–HCl, pH 7.5 (TMS) and centrifuged for 10 min at 1000 *g* in graduated conical tubes. The nuclei were washed twice more by resuspending in TMS followed by centrifugation for 7 min at 1000 *g*. A small sample of the last nuclear suspension was stained with crystal violet (0.5% in 0.25 M sucrose) and examined under a microscope for morphological integrity of the nuclei and evidence of cytoplasmic contamination.

The livers of monkey, frog and turtle had too much connective tissue to permit passage of the tight pestle in the Dounce homogenizer. Satisfactory yields of clean nuclei were obtained using seven to nine strokes with a loose pestle, followed by filtration of the homogenate through two layers of gauze.

Isolation of nuclei from the more fibrous opossum and beef livers required the use of different homogenization procedures. Liver was minced well with scissors and homogenized in 10 vol of 0.32 M sucrose, 3 mM $MgCl_2$ in the Omni-mixer at 6000 rev/min for 2 min using the small blades. The homogenate was filtered through a single layer of flannelette, and centrifuged for 10 min at 1000 *g*. The crude nuclear pellet was washed twice in homogenization medium and the washed pellet suspended in dense sucrose.

Nuclei were isolated from frozen beef kidney and brain by adapting the procedure of Løvtrup-Rein & McEwen (1966). Minced tissues were homogenized in 10 vol of 0.32 M sucrose, 1 mM $MgCl_2$, 1 mM potassium phosphate, pH 6.5, 0.25% Triton X-100 using a modified Waring blender at 2000 rev/min for 2 min. The homogenate was filtered through two layers of gauze and centrifuged at 850 *g* for 10 min. The resulting pellet was washed twice by resuspending the nuclei in 10 vol of homogenization medium without Triton followed by centrifugation at 850 *g* for 8 min. The crude nuclear pellet was then suspended in 2.0 M sucrose, 1 mM $MgCl_2$, 1 mM potassium phosphate, pH 6.5. Following centrifugation at 53,500 *g* for 45 min, the purified nuclear pellets were taken up and washed 3 times in 0.25 M sucrose, 0.78 mM $MgCl_2$, 0.78 mM potassium phosphate, pH 6.5.

Procedures for the isolation of nuclei and extraction of the nuclear proteins from Burkitt lymphoma and mouse L1210 cells have been described in detail by Weisenthal & Ruddon (1972). Nuclei from [^{32}P]-labeled HeLa cells were isolated as described by Stein & Borun (1972). Nuclei prepared in this fashion are free of visible cytoplasmic contamination when examined by phase-contrast microscopy. After washing with 0.14 M NaCl, 0.01 M Tris–HCl, pH 8.0, nuclei from HeLa cells were stored as a frozen pellet at −70°C for less than 5 days.

Fractionation of nuclear proteins and purification of non-histone phosphoproteins

The procedure used to isolate the non-histone phosphoprotein fraction has been described in detail elsewhere (Langan, 1967; Gershey & Kleinsmith, 1969a; Kish & Kleinsmith, 1974), and is outlined briefly here. Purified nuclei were washed once in 0.14 M NaCl and the chromatin was solubilized in 1.0 M NaCl. Dilution of this extract with 1.5 vol of 0.02 M Tris–HCl, pH 7.5 precipitated the DNA and histones out of solution (nucleohistone pellet) leaving the bulk of the non-histone chromatin proteins (NHCP) in the supernatant. After centrifugation for 60 min at 105,000 *g*, any histones remaining in the supernatant were removed by batchwise extraction with Bio-Rex 70 (Na^+) (Bio-Rex pellet). The phosphoproteins were then removed by adsorbtion on calcium phosphate gel. The gel was recovered by centrifugation leaving the rest of the non-histone proteins in the calcium phosphate supernatant. Additional proteins were removed by washing the gel with 1.0 M ammonium sulfate (ammonium sulfate supernatant). The phosphoproteins were then recovered in solution by gently homogenizing the calcium phosphate gel in 0.3 M EDTA, 0.33 M $(NH_4)_2SO_4$. Centrifugation at 27,000 *g* for 20 min removed the insoluble residue (calcium phosphate residue) and the supernatant representing the phosphoprotein fraction was dialyzed overnight against 0.05 M Tris–HCl, pH 7.5. In some cases the supernatant was dialyzed directly against 0.01 M sodium phosphate, pH 7.0, 0.1% SDS, 0.1% β-mercaptoethanol, in preparation for electrophoresis.

Labeling of phosphoproteins with radioactive phosphate

For *in vivo* labeling experiments, male Sprague Dawley rats were injected with [^{32}P] or [^{33}P] orthophosphate in 0.9% NaCl at a dose of 1–8 mCi/100 g body weight. At intervals ranging from 30 min to 8 hr, the animals were killed and tissues removed into cold homogenization medium.

In vitro labeling of the phosphoproteins in the presence of [γ-^{32}P] ATP and Mg^{++} utilized the endogenous kinase activity of the purified phosphoprotein (Kleinsmith & Allfrey, 1969a). The standard incubation mixture of 0.9 ml was comprised as follows: 0.7 ml of purified phosphoprotein (100 to 200 μg) in 0.05 M Tris, pH 7.5; 0.1 ml of 0.25 M $MgCl_2$; and 0.1 ml of [γ-^{32}P]ATP (100 μCi/ml, 1200–3650 mCi/mmole). After incubation for 15 min at 37°C in a shaking water-bath, the reaction was stopped by placing the tubes in ice and adding 0.26 gm solid urea to make the solution 4 M with respect to urea. The incubation mixture was dialysed at 4°C against 0.01 M sodium phosphate buffer, pH 7.0 containing 4 M urea and 0.1% β-mercaptoethanol. Sodium dodecyl sulfate (SDS) was introduced by a subsequent dialysis at 20°C against 0.01 M sodium phosphate, pH 7.0 containing 0.1% SDS and 0.1% β-mercaptoethanol.

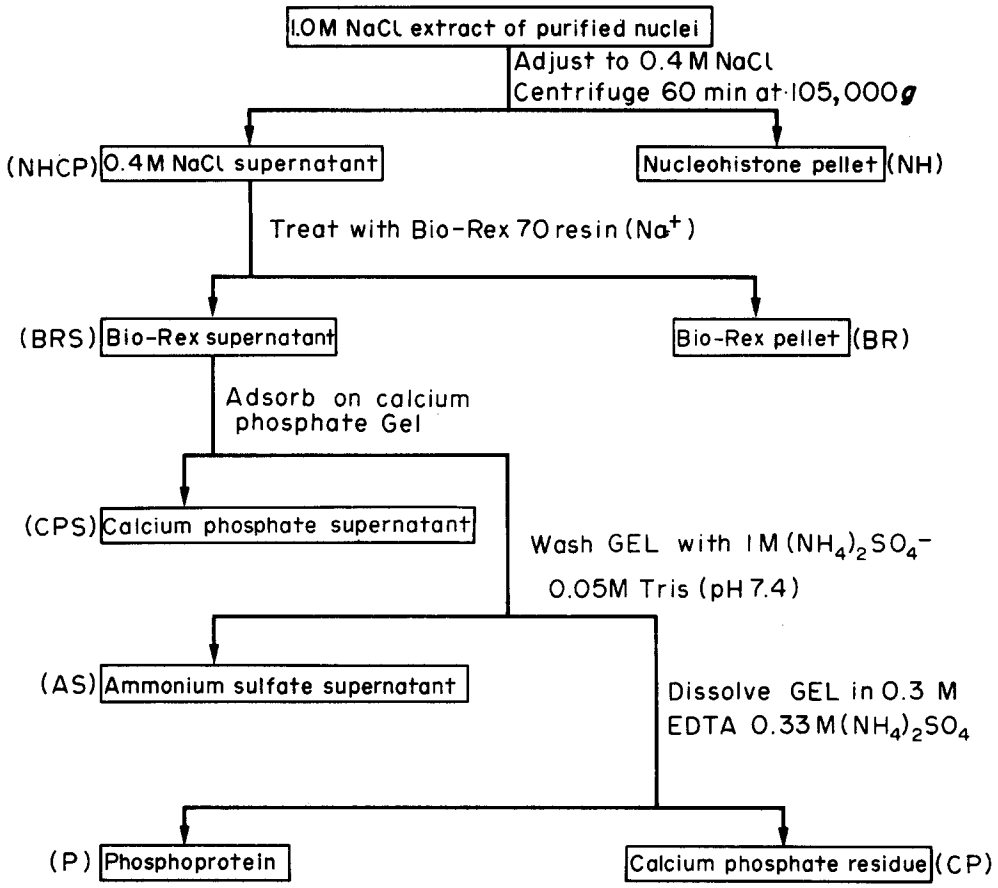
Acrylamide gel electrophoresis

Electrophoresis was performed in 10% acrylamide gels in the presence of SDS as described by Weber & Osborn (1969). Gels containing [^{32}P] were sliced at 0.5 mm intervals using a Mickel gel slicer (Brinkman Instruments). Slices were dried in scintillation vials at 60°C for 2 hr and counted in 5 ml of toluene-based scintillation fluid. Gels containing [^{33}P] were sliced and incubated overnight at 37°C in 5 ml of toluene cocktail containing 3% Protosol (New England Nuclear). After equilibrating for 3 hr at room temperature the vials were counted in a liquid scintillation spectrometer.

RESULTS

Heterogeneity of phosphorylated non-histone proteins

In order to analyze the distribution of phosphorylated species among the non-histone chromosomal proteins, rats were injected with 8 mCi of [^{33}P] orthophosphate, sacrificed 2 hr later, and the nuclear proteins fractionated as described under Methods. At various steps in the isolation procedure, fractions were retained for further analysis. The flow-diagram in Fig. 1 indicates the origin and identity of each fraction. Analysis of these materials by SDS-acrylamide gel electrophoresis indicates that each individual fraction is highly heterogeneous and distinctly different from the others. Although some proteins must



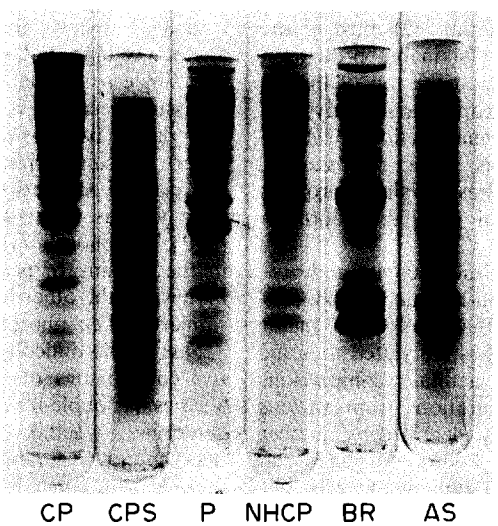


Fig. 1. Flow-diagram for the isolation of phosphoproteins from purified nuclei. The nucleohistone pellet (NH), Bio-Rex pellet (BR) and calcium phosphate residue (CP) were solubilized in 0.01 M sodium phosphate buffer, pH 7.0, 1% SDS, 1% β-merceptoethanol. All fractions were then dialyzed against SDS dialysis buffer and run on SDS-acrylamide gels. The heterogeneity of these protein fractions is obvious from the stained gel patterns. Note that the major protein bands in the purified phosphoprotein fraction (P) are only poorly resolved by electrophoresis of the starting material (NHCP).

certainly appear in more than one fraction, the overall heterogeneity is clearly greater than can be observed simply by examining the electrophoretic pattern of the starting material. For example, the purified phosphoprotein (P) fraction exhibits electrophoretic bands which are not even detectable in the starting non-histone chromosomal protein (NHCP) material.

The distribution of radioactivity and the specific activity of each fraction are summarized in Table 1. As can be seen, the [^{32}P] specific activity is the

Table 1. Incorporation of [^{32}P]orthophosphate into rat liver chromatin proteins. Three 90 g male rats were each injected with 8 mCi [^{32}P]orthophosphate and killed by decapitation 2 hr later. The total wet weight of livers was 14 g

Fraction	Total protein (mg)	Total radioactivity (counts/min × 10^3)	Distribution of radioactivity (%)	Specific activity (counts/min/mg)
Acid soluble pool	1076.0	2,327,000		2,163,400
Nucleohistone pellet (NH)	3.2	1,693	19.9	529,062
Non-histone chromatin protein (NHCP)	12.4	6,820	80.1	550,000
Bio-Rex pellet (BR)	1.7	383	5.1	225,352
Calcium phosphate supernatant (CPS)	5.12	2,442	32.5	476,953
Ammonium sulfate supernatant (AS)	2.1	564	7.5	268,402
Calcium phosphate residue (CP)	2.65	2,121	28.2	800,422
Phosphoprotein (P)	1.59	2,010	26.7	1,266,667

highest in the purified non-histone phosphoprotein (P) fraction. The relatively high specific activity of some of the other fractions (such as NH, BR and CP) is most likely explained by the presence of RNA. This conclusion is supported by two observations: (1) such radioactivity is not released as inorganic phosphate by treatment with alkali, thus indicating that it is not phosphoprotein in nature; (2) most of the radioactivity and none of the stainable protein in these fractions remains trapped at the top of the gel after SDS-acrylamide gel electrophoresis. Analysis of the various [^{32}P] labeled protein fractions by SDS-acrylamide gel electrophoresis (data not shown), demonstrates that no major phosphorylated proteins are lost during purification of the phosphoprotein fraction.

A closer examination of the radioactivity and protein profiles for the purified non-histone phosphoprotein fraction (Fig. 2) indicates that most of the major proteins in this fraction are phosphorylated to some extent *in vivo*. In order to facilitate comparisons between phosphoprotein preparations from various sources, 21 bands are routinely identified by number as is indicated in this figure. Additional bands can be identified by visual examination of the gels, but these 21 have been the most useful in terms of comparative studies. Inclusion of molecular weight standards during electrophoresis has indicated that the major protein species in this fraction are in the mol. wt range of 13,000 to >100,000.

Tissue specificity of phosphorylated non-histone proteins

The purified non-histone phosphoprotein fractions prepared from fresh rat kidney, brain and liver tissues were compared by SDS-acrylamide gel electrophoresis. As is evident from the densitometer tracings of the gels (Fig. 3A) each tissue exhibits a unique, reproducible pattern which differs quantitatively and qualitatively from the others. Phosphoproteins from brain are found predominantly in the high mol. wt region of the gel. The protein patterns of liver and kidney appear to be qualitatively similar, with liver having relatively more high molecular weight protein than kidney. Similar trends were also observed in the corresponding tissues from beef. Phosphoproteins from beef thymus are characterized by the relative absence of high mol. wt proteins and the dominance of a single peak (Platz *et al.*, 1970).

Tissue specificity is also apparent when the phosphorylation patterns of these proteins are compared. Figure 3B summarizes the results of electrophoretic analyses of non-histone phosphoproteins isolated from various rat tissues after *in vivo* labeling with [^{32}P]. The phosphorylation pattern in brain is unique in the labeling of peaks at 6 and 9 mm. Liver and kidney, on the other hand, appear to be qualitatively similar in their phosphorylation patterns.

Due to the difficulty in making accurate quantitative comparisons of materials run in separate gels, a series of double-label experiments were designed to determine whether the quantitative differences between rat liver and kidney were real. Rats were injected with either [^{32}P] or [^{33}P] and kidneys and livers were removed and mixed in various combinations. This double-label technique reveals quantitative differences in the phosphorylation of liver and kidney proteins which are not consistently discernible in comparing gels run separately (Fig. 4). Significant differences in the level of phosphorylation of peaks running at 12, 18, and 30 mm are evident, no matter whether one examines [^{32}P] liver versus [^{33}P] kidney run together, or vice versa.

Cellular specificity of phosphorylated non-histone proteins

Since organs such as brain, liver and kidney are heterogeneous populations of many cell types, it

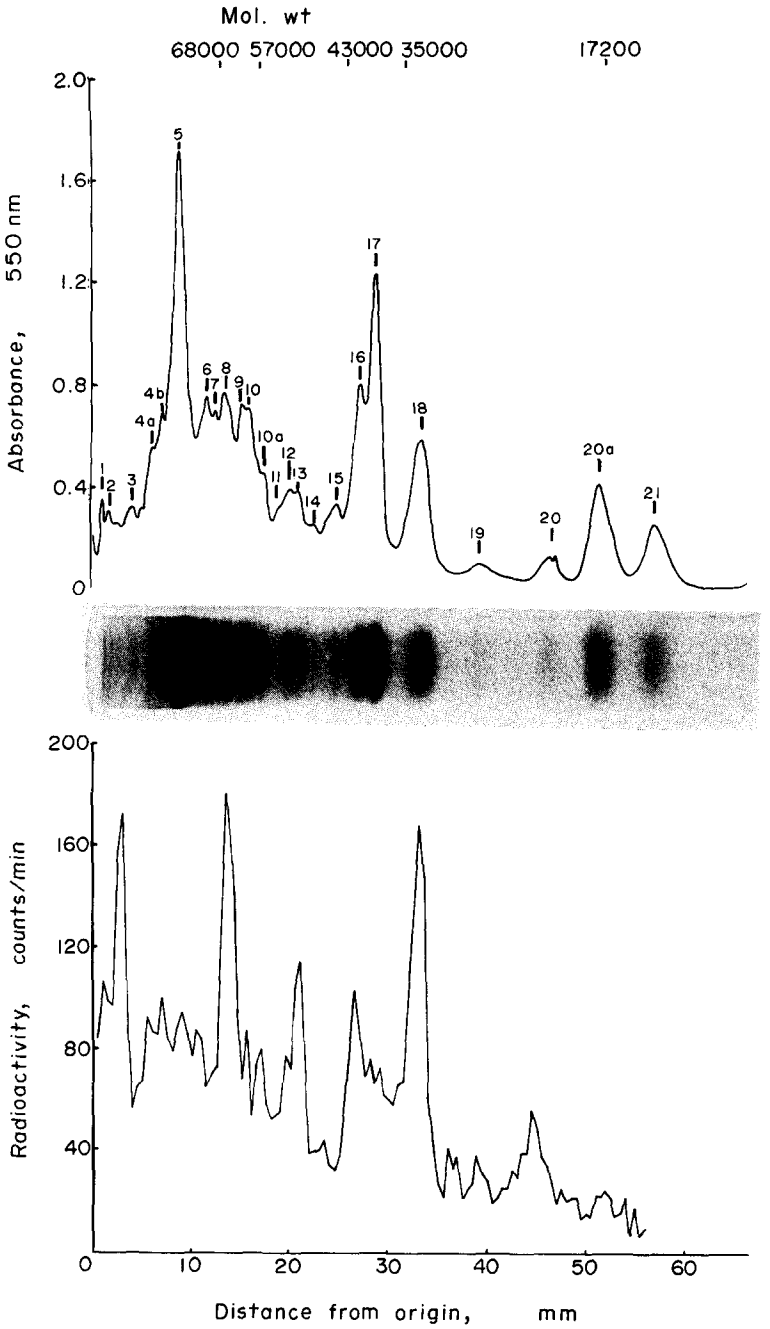


Fig. 2. Distribution of protein and [^{32}P] in acrylamide gels after electrophoretic separation in SDS. The purified non-histone phosphoprotein fraction was isolated from 10 g of liver after a 4-hr pulse of [^{32}P] (1 mCi/100 *g*). Note that most of the proteins in this fraction are phosphorylated. Molecular weight standards indicated across the top are: albumin 68,000; pyruvate kinase 57,000; ovalbumin 43,000; pepsin 35,000; myoglobin 17,200.

could be argued that the heterogeneity observed in the nuclear phosphoproteins from these tissues is a reflection of the multiple cell types which make them up. In order to demonstrate that heterogeneity and specificity of non-histone phosphoproteins exist at the cellular level, this protein fraction was isolated from several established cell lines (HeLa, Burkitt lymphoma, L1210 mouse lymphoma, and a mouse plasma cell tumor). As shown in Fig. 5A, the phosphoproteins from all these cell lines are heterogeneous and exhibit striking quantitative differences from each other. Labeling of these protein fractions by *in vitro* incubation with [γ-^{32}P]ATP followed by gel electrophoresis demonstrates that the non-histone proteins from each cell type are characterized by a unique pattern of phosphorylation (Fig. 5B).

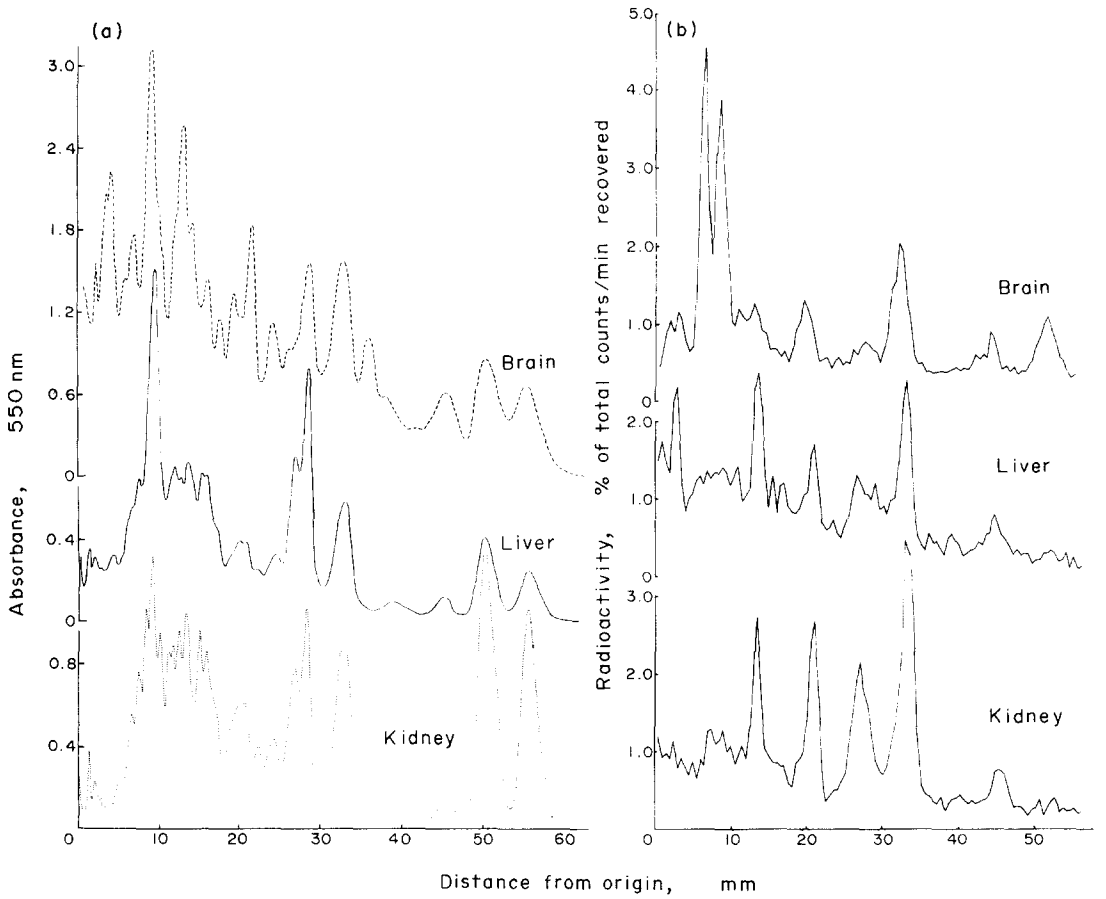


Fig. 3. (A) Densitometer tracings of SDS gels of phosphoproteins isolated from fresh rat tissues: brain, liver, and kidney. The traces have been aligned to point out the striking differences in pattern between tissues. (B) Distribution of radioactivity after electrophoresis of phosphoproteins in SDS gels. The radioactivity in 0.5 mm slices is plotted as a function of migration distance. Liver and kidney were labeled *in vivo* with a 4-hr pulse of [^{32}P] (1 mCi/100 g body weight). Minced brain tissue was incubated for 60 min at 37°C in the presence of [^{32}P]orthophosphate (1 mCi/ml).

Species specificity of phosphorylated non-histone proteins

If the non-histone phosphoproteins are regulators of gene expression, then one might expect to see basic similarities in this protein fraction isolated from the same tissue from different animals. Consequently, we have compared the protein and phosphorylation profiles of non-histone phosphoproteins of liver obtained from animals representing five different classes of vertebrates. Densitometer tracings of gels of non-histone phosphoprotein from fresh livers of lamprey, chicken, and rat (Fig. 6A) show that both major similarities and significant differences exist in phosphoproteins from different vertebrate classes. All three patterns show major peaks at 9 and 34 mm, while the rat and chick patterns share major peaks at 4.5, 21 and 28 mm. The differences seen in these liver proteins from widely divergent species contrast with the close similarity observed when these proteins are compared in closely related species, such as rat and deer mouse (data not shown).

Densitometer profiles of phosphoproteins obtained from frozen livers of turtle, opossum, monkey, rat and frog are compared in Fig. 6B. These protein patterns share some features in common, but each has a distinctive profile. Major bands are consistently present at 30, 47, and 52 mm. The overall reduction in the amount of heterogeneity of high mol. wt material is typical of phosphoprotein preparations from frozen tissue.

DISCUSSION

The present results demonstrate both heterogeneity and specificity in the components of the non-histone phosphoprotein fraction when analyzed via SDS-acrylamide gel electrophoresis. This technique of separation has at least three inherent limitations which could result in an underestimation of the actual heterogeneity present. First of all, this system separates polypeptide chains solely on the basis of size. If two proteins existed with the same amino acid backbone but with differing side chain modifications, they would still band together in this system. This is especially important when studying phosphorylated proteins which may vary greatly in number of sites with attached phosphate groups. Another limitation of SDS-electrophoresis is that the detergent treatment

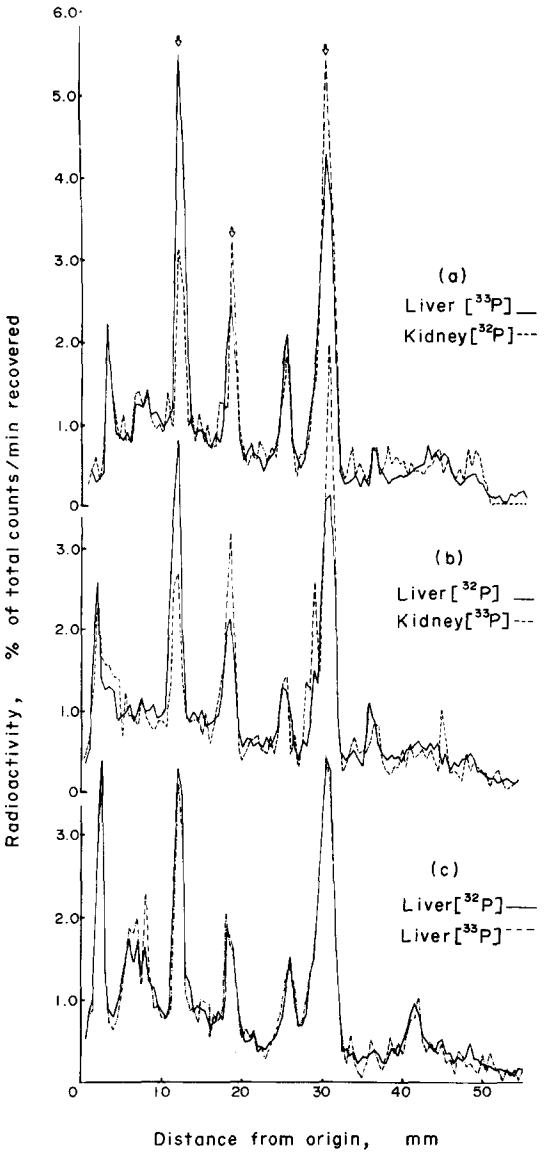


Fig. 4. Differences in phosphorylation of rat liver and kidney phosphoproteins as demonstrated by double-labeling with [^{33}P] and [^{32}P]. Isotopes were injected for a 4-hr pulse ([^{32}P]-1 mCi/100 g body weight, [^{33}P]- 3 mCi/100 g weight). Kidney and liver tissues labeled with different isotopes were removed, pooled together, and non-histone phosphoproteins isolated. Electrophoresis was performed in SDS-acrylamide gels. (A) Comparison of [^{33}P]-liver and [^{32}P]-kidney phosphoproteins. (B) Comparison of [^{32}P]-liver and [^{33}P]-kidney phosphoproteins. (C) Control comparison of [^{32}P]- and [^{33}P]-labeled liver. Note that in spite of the close similarity in labeling patterns of liver and kidney, three peaks (arrows) show consistent quantitative differences in the double-labeled mixtures of the two tissues.

dissociates any subunit structure which might be present in the native proteins. If subunit structure does exist, then the association of subunits in varying combinations would allow for additional heterogeneity. Finally, a third drawback in demonstrating heterogeneity via SDS-electrophoresis is that components present in relatively small amounts would most likely go undetected.

Although the existence of heterogeneity in the non-histone proteins is not generally disputed, the extent and interpretation of that heterogeneity appears to vary amongst investigators. The concept of "limited heterogeneity" of non-histone proteins has been suggested by Elgin & Bonner (1970, 1972), since under their conditions of isolation a relatively small number of different components account for the bulk of the non-histone protein fraction. There are several limitations in this concept of limited heterogeneity, however, which need to be stressed. First of all, it clearly refers only to the quantitative species of non-histone proteins and does not concern itself with the extensive heterogeneity which may exist in protein species present in small amounts. The material isolated by Elgin & Bonner, for example, exhibits a very low level of phosphorylation, suggesting the loss of many of the species of non-histone proteins present in our preparations. Since regulatory proteins might be expected to be present in relatively small amounts, they would require purification and concentration before they would become detectable above the bulk of the non-histone proteins. Our present data indicate that steps involving selective extraction and concentration do reveal greater heterogeneity. A second limitation in the "limited heterogeneity" concept is its heavy dependence on SDS-acrylamide gel electrophoresis, which as discussed above may severely underestimate the actual degree of heterogeneity. And finally, Elgin & Bonner, as well as many other investigators, have employed frozen tissue as starting material. Our experience indicates that non-histone proteins isolated from frozen tissues show a marked decrease in heterogeneity and a loss of high molecular weight components (compare Fig. 6A with 6B). Thus, the concept of limited heterogeneity may be useful in pointing out the existence of some major species of non-histone proteins which are routinely encountered, but it should not be interpreted to exclude the existence of extensive heterogeneity of other less abundant components.

The cell, tissue and species specificity shown here for the phosphorylated non-histone proteins is consistent with their proposed role as gene regulators. However, it should be emphasized that in many cases the similarities between material obtained from different sources were as striking as the differences. This is not surprising, since specific genetic regulatory molecules would be expected to be present in relatively small quantities. Thus, a large portion of the non-histone phosphoprotein fraction may consist of enzymatic or structural proteins which serve similar functions in different cell types.

The present experiments demonstrate, then, that the phosphorylated non-histone proteins exhibit two basic properties expected of genetic regulatory molecules, that is, heterogeneity and cellular specificity. While these properties are consistent with the proposed regulatory role, they do not in themselves prove an involvement of these proteins in specific gene control. It should be pointed out in this context, however, that phosphorylated non-histone proteins have also been shown to exhibit a number of other properties expected of genetic regulatory molecules. As mentioned in the introduction, changes in their phosphorylation rate have been shown to correlate

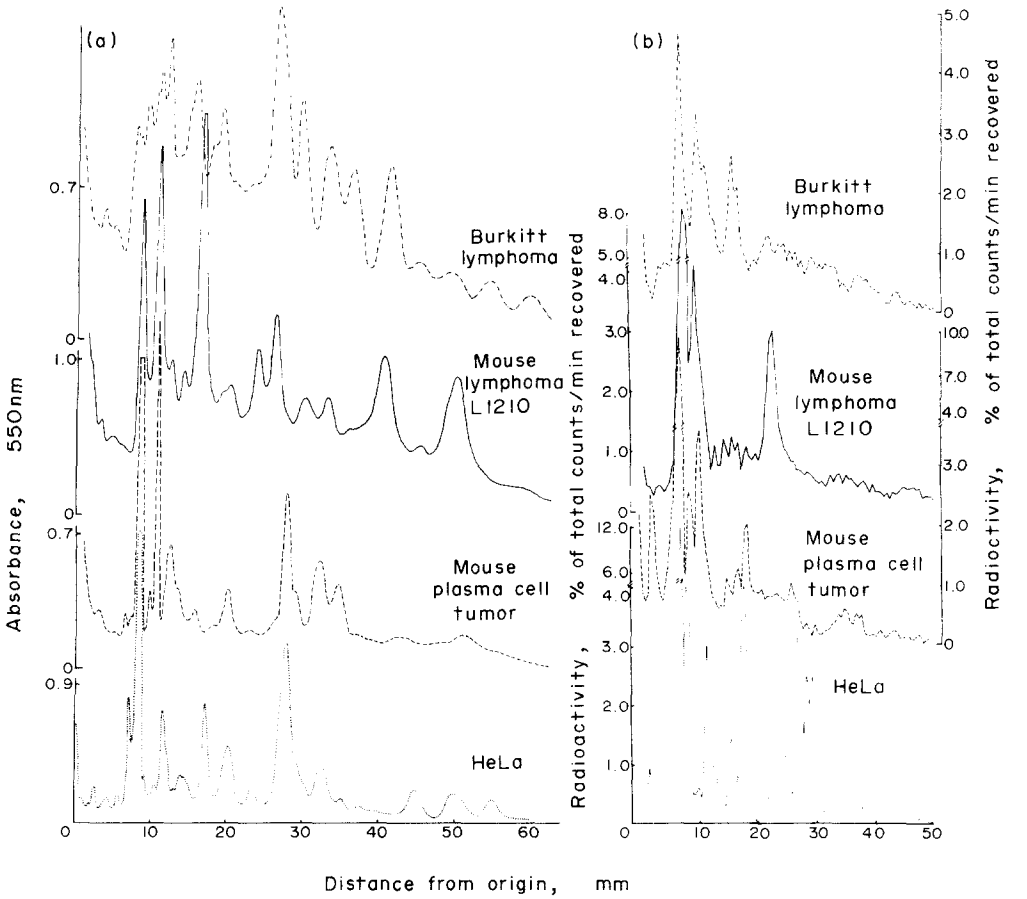


Fig. 5. (A) Densitometer tracings of SDS gels of phosphoproteins isolated from four homogeneous cell populations. The traces have been aligned to point out the differences in patterns between cell types. (B) Distribution of radioactivity in gels of phosphoproteins isolated from four homogeneous cell populations. Phosphoproteins from Burkitt lymphoma and L1210 mouse plasma cell tumor were labeled by *in vitro* incubation with [γ-^{32}P]ATP. HeLa cells were labeled in phosphate-free medium by incubation with [^{32}P]orthophosphate (10 mCi/100 ml) for 1 hr at 37°C.

with changes in differentiation and gene activity in a variety of systems; they have been shown to alter the rate of RNA synthesis *in vitro*, and a small fraction of them have been shown to be capable of binding specifically to host DNA sequences. In addition, they are subject to rapid phosphorylation reactions which may be modulated by cyclic AMP (Johnson & Allfrey, 1972; Kish & Kleinsmith, 1974) or steroid hormones (Ahmed & Ishida, 1971; Allfrey *et al.*, 1973; Kleinsmith, 1974), which in turn provides a mechanism for modulation and control of their structural and functional properties (Kleinsmith *et al.*, 1966a; Kleinsmith & Allfrey, 1969b; Kaplowitz *et al.*, 1971; Kleinsmith, 1975). Finally, it has been shown that removal of the phosphate groups from phosphoproteins prior to reconstitution of HeLa cell chromatin, specifically decreases the number of initiation sites available for transcription (Kleinsmith *et al.*, 1975). Thus, evidence obtained from several different approaches points to a role of these phosphorylated proteins in gene regulation, a conclusion which is reinforced by the current studies on their heterogeneity and tissue specificity.

SUMMARY

The heterogeneity and specificity of non-histone chromatin phosphoproteins has been studied in a variety of species, tissues and cell types. A large amount of heterogeneity has been observed amongst these phosphorylated nuclear proteins with each tissue and each cell type exhibiting a unique protein pattern and radioactivity profile when labeled with [^{32}P]. The differences in the nuclear phosphoproteins of one extensively studied tissue, namely liver, were found to increase as the species being compared diverged evolutionarily. The present results on phosphoprotein heterogeneity and specificity are consistent with the proposed role of the non-histone chromatin phosphoproteins in gene regulation.

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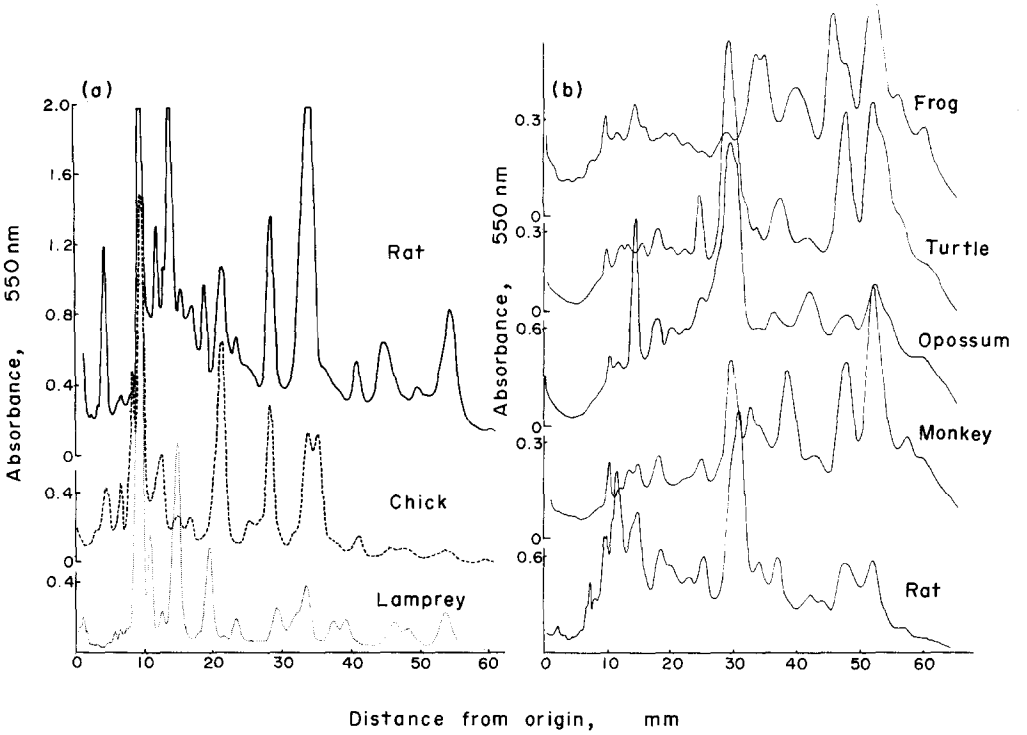

Fig. 6. (A) Densitometer tracings of SDS gels of phosphoproteins isolated from fresh livers of rat, chick, and lamprey. The traces have been aligned to compare the profiles of liver phosphoproteins from different species. (B) Densitometer tracings of SDS gels of phosphoproteins isolated from frozen livers of five species. Frog, turtle, opossum and monkey phosphoproteins were isolated in parallel. The effect of freezing is apparent from comparing the rat preparations in 6A from fresh liver with that shown in 6B from frozen liver.

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