DIFFERENTIAL PHOSPHORYLATION OF RAT LIVER NUCLEAR NON-HISTONE PROTEINS IN VITRO

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Summary.
An in vitro incubation system for rat liver slices is employed to study the kinetic incorporation of [^{32}P] into non-histone proteins. The phosphate incorporation reaches a stable maximum after two hours of incubation and is shown to represent the phosphorylation of serine and threonine residues. The kinetic phosphorylation is found to be molecular weight dependent with the smaller molecular weight non-histone proteins (about 10,000-22,000) preferentially phosphorylated before the larger molecular weight non-histone proteins (about 22,000-160,000). In addition, the data suggest that more than one type of phosphate may be present in the non-histone proteins.

Introduction. The ability of non-histone nuclear proteins to bind to homologous DNA (1,2,3) and to stimulate transcription in vitro (4,5,6,7,8) increases concomitantly with increasing phosphorylation and decreases upon enzymatic hydrolysis of their phosphate esters (9). Kleinsmith et al. (10,11,12) have found that the phosphate, once incorporated into serine or threonine, was subject to very rapid turnover. This high turnover has been correlated with metabolic activity (13,14), hormonal (15,16,17) and mitogenic (18) induction, RNA synthesis associated with euchromatin (19); and thus, in a general sense, with gene activation (20).

In this report, an in vitro system for the phosphorylation of NHP* is described and characterized. Additionally, we present data which show that the phosphorylation of smaller molecular weight NHP precedes the phosphorylation of larger molecular weight NHP.

Materials and Methods.

A) Tissue Preparation and In Vitro Incubation - Livers were obtained from male Holtzman rats (415-455 g) following decapitation and brief exsanguination. Livers were excised, dipping each briefly into cold, isotonic saline

* NHP - non-histone proteins

and placing on ice before slicing. Uniform liver slices were prepared (0.5 mm thickness) using a Stadie-Riggs tissue slicer, taking care to discard the first slice. These slices were placed into sterile nalgene flasks containing [^{32}P] enriched Eagle's Minimal Essential Medium (MEM). Each flask was prepared aseptically to contain the following incubation mixture* at a final pH of 7.4: 50.0 ml phosphate-free Eagle's MEM, 0.1 M HEPES buffer (Sigma), 2% Newborn calf serum (Flow Labs), 6.6 mCi [^{32}P] orthophosphate (H_3PO_4 in water, carrier free, ICN), 2500 I.U. Penicillin, 2500 μg Streptomycin (Flow Labs). This medium was pre-warmed to 37C, gassed with 85% O_2/15% CO_2 and 7.0 g of liver slices were added. This atmosphere was maintained in a Dubnoff shaker for the duration of the incubation. The incubation period of each flask (0, 1, 2, 4, 6 and 8 hrs.) was terminated by collecting slices on a Buchner funnel and rinsing (2x) with 5.0 ml cold 0.9% saline to remove excess medium. Tissues were maintained at 0-4C for all subsequent manipulations, except as noted.

B) Isolation of Nuclei and Extraction of Nuclear NHP - Nuclei were isolated using the Blobel and Potter (22) modification of the Changeaux sucrose gradient sedimentation velocity method. Final nuclear pellets were obtained by centrifugation for 40 min. at 75,000 x g_{av} (SW 25.2 rotor). The method of Grownow and Griffiths (23) was employed for the extraction of nuclear NHP. The urea extracted NHP were dialyzed against cold, distilled water, frozen at -70C, and lyophilyzed.

C) Electrophoresis of Nuclear NHP - Polyacrylamide gels containing urea and SDS** were used to electrophoretically separate the NHP into molecular weight fractions for further analysis. Polymerization and electrophoretic conditions were according to the Bhorjee and Pederson (24) modification of the Fairbanks' (25) procedure. SDS solubilized [^{32}P] NHP (100 μg in 0.05 ml) were electrophoresed for 6 hrs. at 8 ma per gel. Gels were fixed, stained with Coomassie Blue R, and destained essentially according to the method to Fairbanks (25).

Densiometric tracings were recorded at 550 nm on a Gilford Model 2400 linear transport spectrophotometer. After gels were photographed they were frozen (-70C), sliced transversely into sequential 1 mm discs, and allowed to swell and diffuse into 10 ml Bray's solution overnight. Samples were counted for 20 min. or 5% error on a Beckman LS-355 scintillation counter using external standards.

D) Characterization of Nuclear NHP - It is well established that serine and threonine are phosphorylated *in vivo* (10). In order to determine the validity of this *in vitro* phosphorylation procedure, the NHP were acid hydrolyzed and by high voltage electrophoresis, phosphoserine and phosphothreonine were separated and assayed for [^{32}P] activity. In addition, these proteins were subjected to enzymatic hydrolysis with alkaline phosphatase preceding acid hydrolysis and high voltage electrophoresis in order to show specific enzymatic cleavage of phosphate-ester bonds.

High voltage electrophoresis was used to separate phosphoserine, phosphothreonine, and free phosphate which were anticipated products of the acid hydrolysate. Nuclear phosphoprotein (0.5 mg) was mixed with 1.0 ml 6.0 M HCl and hydrolyzed in sealed ampules at 105C for 7 hrs. The hydrolysate was evaporated to dryness, and dissolved in 10.0 μl electrophoresis buffer (2.3% formic acid, 7.8% acetic acid). Free phosphate and the phosphorylated amino acids were electrophoretically separated on a water-cooled Camag High Voltage unit at 3000 V for 70 min. The paper was dried, sprayed with ninhydrin, and heated (26). The paper was cut into strips and the [^{32}P] activity meas-

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* Essentially that of Liberti *et al.* (21)

** SDS - sodium dodecyl sulfate

ured on a Packard Radiochromatogram scanner (Model 270, 2π Geometry). These radiochromatographic scans were found to be colinear with the ninhydrin identified standards, confirming the presence of [^{32}P] labelled phosphoserine and phosphothreonine.

The NHP were treated with alkaline phosphatase in a final reaction mixture of 0.6 ml containing 0.01 M Tris, pH = 8.2; 10 μg E. coli alkaline phosphatase (Sigma) and 2.0 mg NHP. This reaction mixture was incubated for 48 hrs. at 37C in a Dubnoff shaker. The reaction was terminated by dialyzing against cold distilled water.

The phosphatase hydrolyzed protein was lyophilyzed and subjected to acid hydrolysis, high voltage electrophoresis, and radiochromatographic scanning at the same time as the non-enzymatically treated NHP. In order to determine if there was proteolysis during the incubation of NHP with the phosphatase, a ninhydrin assay (27) was performed on aliquots taken before and after incubation.

Results. The isolated nuclei are a homogenous population free of microscopically visible cytoplasmic contamination. The inner nuclear membrane appears intact and retains nucleosol. The endoplasmic reticulum which is continuous with the outer layer of the nuclear membrane appears to be adequately sheared by the partitional sucrose gradient; and thus, these nuclei are probably free of endoplasmic or associated ribosomal contamination. Extraction of polysomal proteins and characterization by molecular weight electrophoresis has confirmed the absence of ribosomal proteins in the final nuclear protein preparation.

These labelled nuclei have been shown by SDS polyacrylamide gel electrophoresis to contain at least 29 urea-soluble nuclear NHP (Fig. 1). The molecular weights of the NHP fractions shown in Fig. 1 and 3 were estimated by comparison with co-electrophoresed standards.

The kinetic incorporation of [^{32}P] into total NHP is shown in Fig. 2. It is noteworthy that the total activity of the NHP in this system stabilizes after 2 hrs. However, the incorporation of [^{32}P] into specific molecular weight protein fractions shows temporal phosphorylation permutations during a 6 hr. in vitro incubation as shown in Fig. 3. During this period of time, there was no evidence for a change in the protein pattern shown in Fig. 1. For the first hr. of incubation there is essentially no differential phosphate labelling of these proteins. This is illustrated by the consistantly low and uniform [^{32}P] activity of the electrophoretically separated proteins

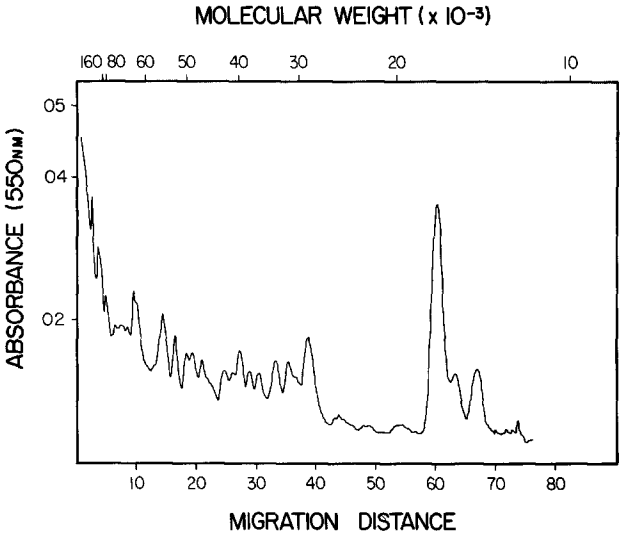

Figure 1. Densiometric tracing of NHP separated into molecular weight fractions by SDS polyacrylamide electrophoresis. Molecular weight scale derived from standards which were electrophoresed at the same time. Migration distance is in mm.

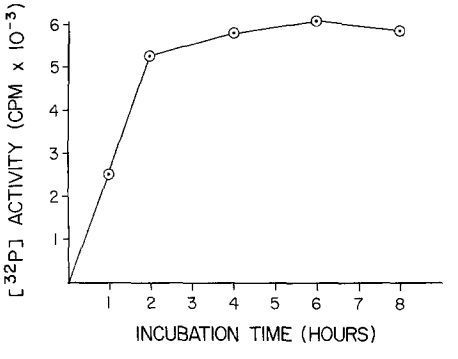

Figure 2. In vitro kinetic incorporation of [^{32}P] into total NHP. Total [^{32}P] activity is expressed as CPM/100 μg NHP vs incubation time. All samples were from electrophoretic gels shown in Figure 3 and were counted and normalized for decay as in text.

(Fig. 3A and 3B). At 2 hrs. (Fig. 3C) the proteins demonstrate a selective phosphorylation which is virtually restricted to a low molecular weight class of about 10,000-22,000. At 4 hrs. (Fig. 3D) there is a delocalization of

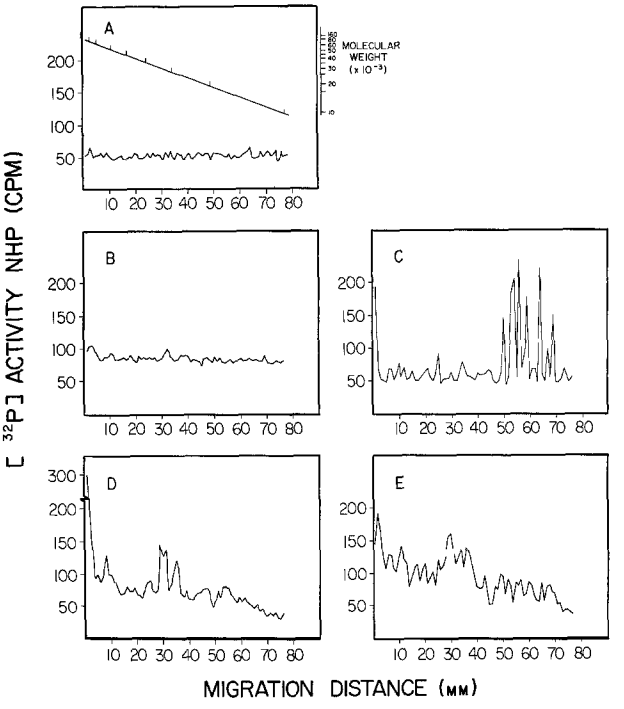

Figure 3. [^{32}P] activity of NHP separated by SDS plyacrylamide gel electrophoresis. Each gel represents a different incubation period from the same experiment. All gels were co-electrophoresed, sliced, counted, and normalized for decay as described in text. A) 0 hr., B) 1 hrs., C) 2 hrs., D) 4 hrs., E) 6 hrs. Molecular weight scale was derived from standards which were electrophoresed at the same time.

phosphate from these low molecular weight proteins to larger proteins. This shift is still prominent at 6 hrs. (Fig. 3E) when there is a phosphorylation of proteins in the 22,000-160,000 molecular weight range.

The incorporation of [^{32}P] into NHP via the formation of phosphoserine and phosphothreonine is shown in Fig. 4A. When these proteins are treated with E. coli alkaline phosphatase preceding acid hydrolysis and high voltage electrophoresis, the labelled phosphate esters of phosphoserine and phosphothreonine are cleaved (Fig. 4B). In addition, a ninhydrin assay could not detect any evidence of proteolysis during the NHP - E. coli alkaline

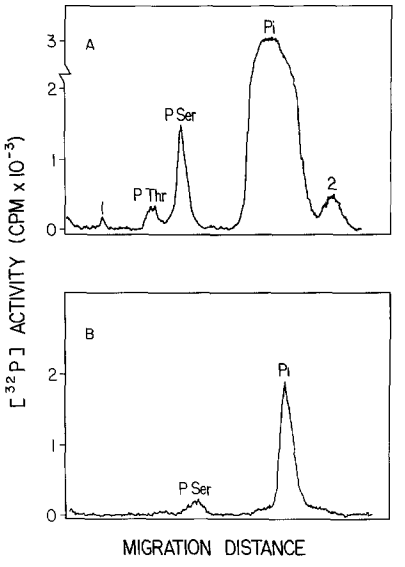

Figure 4. Radiochromatographic scan of labelled phosphate species following high voltage paper electrophoresis. [^{32}P] NHP were obtained from the same sample as shown in Fig. 3C. A) Acid hydrolyzed [^{32}P] NHP, B) Acid hydrolyzed [^{32}P] NHP following E. coli alkaline phosphatase treatment and dialysis. PThr = phosphothreonine, PSer = phosphoserine, P_i = orthophosphate. Peaks 1 and 2 are currently unidentified and are elaborated in discussion.

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phosphatase incubation period.*

Discussion. The application of this in vitro incubation system allows the incorporation of significant levels of isotope into this organelle subfraction without manipulating the massive amounts of [^{32}P] required for whole body labelling. Additionally, with this system one can control the tissue environment, permitting both reproducible kinetic studies and the possibilities for a wide range of experiments. These liver slices are metabolically viable in this virtually phosphate-free medium, as evidenced by the continuance of both RNA and protein synthesis for at least 6 hrs. as determined by [^{3}H] uridine and [^{14}C] leucine uptake into total cellular RNA and protein, respectively.*

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* Unpublished data

The specific phosphorylation of the lower molecular weight NHP precedes the phosphorylation of the higher molecular weight species in this in vitro system (Fig. 3). Also, the total phosphate incorporated approaches a maximum at 2 hrs. (Fig. 2), due to phosphorylation of the lower molecular weight species (Fig. 3C). The question arises as to the mechanism(s) responsible for the loss of phosphate from the lower molecular weight species and the phosphorylation of the higher molecular weight species (Fig. 3C, 3D, 3E), an event occurring subsequent to the time at which total phosphate incorporation has virtually ceased. Possible mechanisms include (1) a direct transfer of phosphate from lower to higher molecular weight species, (2) phosphorylation of the higher molecular weight proteins and concomitant dephosphorylation of the smaller proteins, (3) a precursor relationship whereby lower molecular weight phosphoproteins polymerize to form larger phosphoproteins.

The high voltage electrophoretic profile of the acid hydrolyzed NHP (Fig. 4A) consistently reveals two [^{32}P] labelled species (1 and 2) in addition to the phosphoserine, phosphothreonine, and free orthophosphate. The nature of these two phosphate species is unknown. Peak 2 represents a more negatively charged, ninhydrin negative, phosphate species. This raises the possibility of phosphate bonds other than esters existing in NHP. The existence of, e.g. polyphosphate (28) and specific polyphosphate phosphatases in rat liver nuclei (29), polyphosphates in mammalian liver (30) and neural tissue (31); and the catalytic activity of polyphosphates on polynucleotide polymerization (32), is not inconsistent with this speculation.

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