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NUCLEAR PHOSPHOPROTEINS

III. INCREASE IN PHOSPHORYLATION DURING HISTONE–PHOSPHOPROTEIN INTERACTION

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SUMMARY

The effects of histones on the enzymatic phosphorylation of nuclear phosphoproteins have been extensively studied *in vitro*. Histones cause a 5- to 10-fold increase in the rate and extent of phosphorylation of nuclear phosphoprotein, with the lysine-rich histones being the most effective at stimulation. Evidence suggests that the histones are not being phosphorylated themselves, but rather act to make more sites available for phosphorylation in the phosphoprotein itself. The possible physiological significance of histone–phosphoprotein interactions is discussed.

INTRODUCTION

The enzymatic properties of a nuclear protein fraction rich in phosphorus have recently been described by KLEINSMITH AND ALLFREY[1,2]. These proteins, which are part of the non-histone chromatin acidic protein fraction, appear to account for roughly 4% of the dry weight of the calf thymus nuclei from which they are isolated, thus indicating that they represent a major structural component of the nucleus. *In vitro* studies have shown that these proteins can be phosphorylated by the terminal phosphate group of various nucleoside- and deoxynucleoside triphosphates. The phosphate incorporated into the protein is found mainly as phosphoserine, with small amounts of phosphothreonine also being detectable.

Experiments designed to elucidate the possible cellular functions of nuclear phosphoproteins have implicated them in the process of gene activation. When human lymphocytes are induced to grow and divide by the addition of phytohemagglutinin, one of the earliest events which can be detected is an increased rate of phosphorylation of nuclear proteins[3]. Likewise, during maturation of the avian erythrocyte, as the chromatin becomes structurally more compact and less active in nucleic acid synthesis, nuclear levels of protein-bound phosphorus and phosphoprotein kinase fall several-fold[4]. Thus changes in the metabolism and composition of

nuclear phosphoproteins seem to correlate with changes in the structure and metabolic activity of chromatin.

LANGAN[5], working with phosphoproteins purified from rat liver nuclei, has found that they are capable of forming complexes with histones. The formation of such complexes diminishes the ability of histones to inhibit DNA-dependent RNA synthesis *in vitro*, suggesting the possibility that interactions between histones and phosphoproteins might play an important role in the regulation of RNA synthesis *in vivo*. Previous studies on the enzymology of the phosphorylation of nuclear phosphoproteins have been performed in purified systems in the absence of histones[1,5]. Since histone–phosphoprotein interactions may be of physiological importance, and since phosphoproteins are normally found in close association with histones in cell nuclei, we thought it important to study the effects of histones on the enzymatic phosphorylation of nuclear phosphoproteins.

Since histones are widely recognized as general inhibitors of enzymatic reactions, it might be expected that the addition of histones would simply inhibit the phosphorylation reaction under study. Therefore, it was somewhat surprising to find that the addition of histones stimulated the *in vitro* phosphorylation of nuclear phosphoproteins from 5- to 10-fold. All histone subfractions tested were capable of stimulating the rate and extent of phosphorylation, although the f_1 histone worked best. Additional observations suggested that the histones are not being phosphorylated themselves, but are acting in some way to make more phosphorylation sites available in the phosphoprotein.

METHODS

Preparation of protein fractions

Nuclei were isolated from fresh calf thymus according to the procedure of ALLFREY *et al.*[6], and the nuclear phosphoprotein fraction prepared as described by GERSHEY AND KLEINSMITH[7]. For preparation of total histones, nuclei were first washed with 0.01 M Tris–HCl (pH 7.1) containing 3 mM $MgCl_2$ in order to remove soluble nuclear proteins and ribosomes[8,9]. This was followed by a wash in 80% ethanol–0.01 M HCl to remove additional non-histone proteins, after which the histones were extracted in 0.1 M H_2SO_4 and precipitated in 10 vol. of acetone. Purity was monitored by electrophoresis at pH 9.0 on cellulose polyacetate strips[10]. Histone subfractions were isolated and purified by the technique of JOHNS[11]. Poly-L-amino acids were obtained from Sigma Chemical Co.

Assay of enzymatic phosphorylation of nuclear phosphoprotein

The standard incubation mixture contained 37.5 μg phosphoprotein, 1.25 μmoles $MgCl_2$, 15 mmoles Tris–HCl (pH 7.5), 2.0 nmoles [γ-^{32}P]ATP (500–1500 mC/mmole), and varying concentrations of histone in a final volume of 0.5 ml. Samples were incubated for 10 min at 37°, and the reaction terminated by the addition of cold 25% trichloroacetic acid. 1 mg of phosvitin was added to each tube as carrier, and the precipitates were washed two times with cold 25% trichloroacetic acid and once with 20% ethanol in ether. The final precipitate was dissolved in 1.0 ml of 1.0 M NaOH, and the incorporation of ^{32}P into alkali-labile phosphate was determined as previously described[8].

Measurement of turbidity of histone–phosphoprotein mixtures

Mixtures of phosphoprotein with varying concentrations of histone were prepared in the same proportions as in the standard incubation media, except the final volume was reduced to 0.25 ml, and the ATP was not included. After the histone was added, the solution was diluted with 1.0 ml of distilled water and shaken vigorously. After standing for 10 min at room temperature, the turbidity was determined by measuring the absorption at 400 nm in a spectrophotometer.

Acrylamide gel electrophoresis

Phosphoproteins were labeled by incubation with [γ-^{32}P]ATP as described above. At the end of 10 min the reaction was stopped by adding solid urea to a final concentration of 4.0 M, followed by dialysis at 4° against 0.01 M sodium phosphate buffer (pH 7.0) containing 4.0 M urea and 0.1% β-mercaptoethanol. Sodium dodecyl sulfate was introduced by a subsequent dialysis at 20° against 0.01 M sodium phosphate buffer (pH 7.0) containing 0.1% sodium dodecyl sulfate and 0.1% β-mercaptoethanol. Electrophoresis was performed in a 10% sodium dodecyl sulfate–acrylamide gel as described by WEBER AND OSBORN[12]. Gels were sliced at 0.5 or 1 mm intervals and counted in the scintillation fluid of BRAY[13].

RESULTS

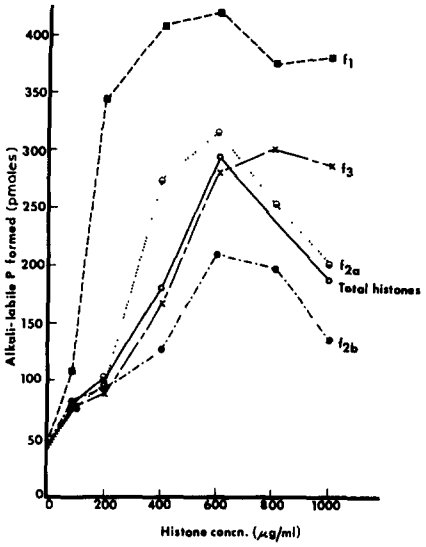
Effects of histones on rate of phosphorylation reaction

When the purified nuclear phosphoprotein fraction is incubated in the presence of [γ-^{32}P]ATP and Mg^{2+}, a rapid incorporation of ^{32}P into the phosphoprotein occurs[1]. When varying concentrations of histone are added to this reaction mixture, a striking increase in the rate of the reaction occurs (Fig. 1). Total histone as well as all histone subfractions tested have been found to be capable of stimulating this reaction. The extent of stimulation varies from 5- to 10-fold, with the f_1 histone fraction being effective at the lowest concentration and causing the greatest stimulation.

Optimal stimulation for most of the histone fractions tested occurs at a histone concentration of 600 μg/ml, with higher concentrations generally causing some inhibition. The occurrence of this optimum is apparently not an artifact resulting from the toxicity of higher concentrations of histone, since a sharp optimum still occurs when the histone concentration is held constant at 600 μg/ml and the phosphoprotein concentration is varied (Fig. 2). Thus the degree of stimulation obtained depends critically on the ratio of histone to phosphoprotein.

Specificity of histone-mediated stimulation

The observed stimulation appears to be relatively specific for histones, since we have not been able to observe similar effects with addition of unrelated proteins such as cytochrome *c*, serum albumin, or hemoglobin (Table I). Poly-L-lysine, which is often used as a model compound for studying the physical and metabolic effects of histones[14–16], is almost as effective as the f_1 histones in stimulating the reaction, while poly-L-arginine is effective to a lesser degree. Other amino acid polymers inhibited the reaction.



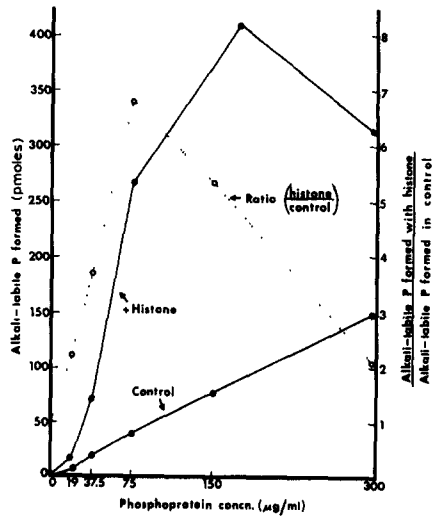


Fig. 1. Effects of varying concentrations of total histone and various histone subfractions on the rate of phosphorylation of nuclear phosphoprotein. After 10 min of incubation with [γ-^{32}P]ATP, the incorporation of ^{32}P into alkali-labile phosphate was determined. While all histone fractions are seen to stimulate the rate of phosphorylation, the f_1 fraction is the most effective, causing a 10-fold increase in rate.

Fig. 2. Effect of a constant amount of total histone (600 μg/ml) on the rate of nuclear protein phosphorylation with varying amounts of phosphoprotein. The dotted line shows the ratio of incorporation with histone present to that without histone at each concentration of phosphoprotein. Note the existence of a sharp optimum for maximal stimulation.

Absence of histone phosphorylation

Since histones themselves are phosphorylated proteins[8,17,18], it is important to determine whether the histone-induced stimulation of the phosphorylation rate might be accounted for simply by incorporation of ^{32}P into the added histone. Several lines

TABLE I

SPECIFICITY OF THE HISTONE-MEDIATED STIMULATION OF NUCLEAR PHOSPHOPROTEIN PHOSPHORYLATION

Nuclear phosphoprotein was incubated with [γ-^{32}P]ATP for 10 min and the incorporation into alkali-labile phosphate determined. Final concentrations were: phosphoprotein (75 μg/ml), $MgCl_2$ (2.5 μmoles/ml), Tris–HCl, pH 7.5 (30 mmoles/ml), [γ-^{32}P]ATP (4.0 nmoles/ml), and a protein to be tested at a concentration of 400 μg/ml.

	Alkali-labile $^{32}P_i$ formed (pmoles)	*Degree of stimulation* $\frac{\text{experimental rate}}{\text{control rate}}$
Control	51.2	—
+ f_1 histone	404.0	7.9
+ poly-L-lysine	312.0	6.1
+ poly-L-arginine	169	3.3
+ poly-L-aspartate	20.5	0.4
+ cytochrome *c*	56.3	1.1
+ serum albumin	61.4	1.2
+ hemoglobin	63.4	1.2

of investigation have indicated that this is not the case. First, histone phosphorylation is known to be extremely sensitive to sulfhydryl reagents, with $1 \cdot 10^{-4}$ M *N*-ethylmaleimide causing greater than 90% inhibition of the reaction[19]. However, concentrations of *N*-ethylmaleimide as high as $1 \cdot 10^{-3}$ M were found to have no inhibitory effect on the rate of protein phosphorylation either in the presence or absence of histone (Table II). Second, cyclic 3',5'-AMP is known to cause a 4- to 6-fold increase in the rate of histone phosphorylation[18]. However, in our system the addition of cyclic 3',5'-AMP produced no significant alteration in the rate of phosphorylation either in the presence or absence of histone (Table II). Thus, the histone-stimulated phosphorylation of nuclear phosphoprotein is not affected by conditions which are known to inhibit or activate the phosphorylation of histone.

Results from the experiments employing poly-L-lysine (Table I) also support the conclusion that histone phosphorylation is not the explanation for the stimulation of protein phosphorylation observed in the presence of histone. Poly-L-lysine was found to be almost as effective as the f_1 histone fraction in stimulating the phosphorylation reaction, yet the site of phosphorylation in histones is a serine residue[8,17,20], and thus poly-L-lysine, containing no serines, could not be acting as a substrate for phosphorylation in this reaction.

Finally, the most direct way to demonstrate the absence of histone phosphorylation would be to isolate the histones after the reaction has taken place and determine directly whether they had been phosphorylated. Unfortunately, due to the tendency of histones and phosphoprotein to form complexes, this is not a simple task, and standard histone fractionation procedures failed to yield unambiguous results. However, electrophoresis in sodium dodecyl sulfate–acrylamide gel did demonstrate that histone phosphorylation, if it does occur, is not sufficient quantitatively to account

TABLE II

EFFECTS OF VARIOUS CONDITIONS ON THE ENZYMATIC PHOSPHORYLATION OF NUCLEAR PHOSPHOPROTEIN

Nuclear phosphoprotein was incubated with [γ-^{32}P]ATP for 10 min and the incorporation of ^{32}P into alkali-labile phosphate determined. Final concentrations used were: phosphoprotein (75 μg/ml), total histone (600 μg/ml), *N*-ethylmaleimide ($1 \cdot 10^{-3}$ M), and cyclic 3',5'-AMP ($1 \cdot 10^{-4}$ M). The "complete system" contained phosphoprotein, Tris buffer, $MgCl_2$ and [γ-^{32}P]ATP as described in METHODS.

	Alkali-labile $^{32}P_i$ formed (pmoles)
Expt. A	
Complete system	40.8
+ *N*-ethylmaleimide	50.4
+ cyclic 3',5'-AMP	35.5
+ histone	260.0
+ histone and *N*-ethylmaleimide	297.0
+ histone and cyclic 3',5'-AMP	273.0
Expt. B	
Complete system	38.2
+ histone	250.0
+ histone heated 5 min at 60° before addition to complete system	270.0

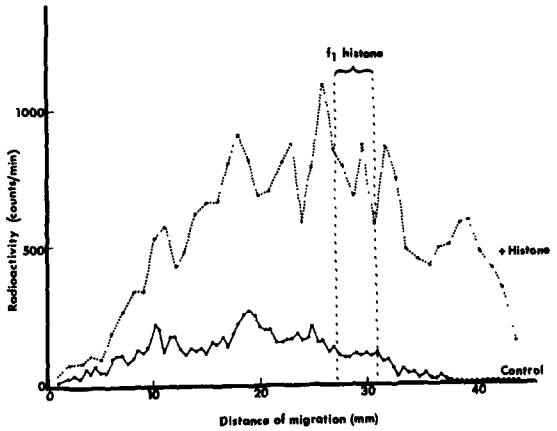


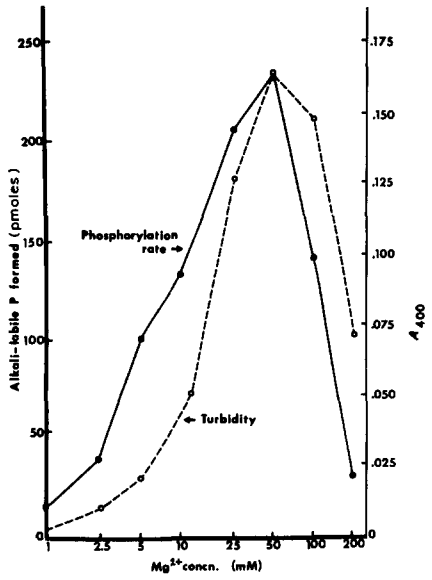
Fig. 3. Acrylamide gel electrophoresis patterns of phosphoprotein labeled with [γ-^{32}P]ATP in the presence or absence of f_1 histone. Note that the increase in radioactivity which occurs when phosphoprotein is labeled in the presence of f_1 histone is spread throughout the entire electrophoresis pattern, and is not localized in the region of the f_1 histone band.

for the massive stimulation of nuclear phosphoprotein phosphorylation which occurs in the presence of histones. In such an electrophoresis system, f_1 histone forms one major band, while the nuclear phosphoproteins exhibit great heterogeneity. As shown in Fig. 3, the ^{32}P-labeling pattern of phosphoprotein labeled and electrophoresed in the presence of f_1 histone shows increased radioactivity in all regions of the gel, and not just in the region where f_1 histone bands. Thus, phosphorylation of histone cannot account for the 10-fold increase in the rate of phosphorylation which occurs in the presence of histone.

Mechanism of histone-mediated stimulation

Since histone phosphorylation does not seem to account for the increased rate of phosphorylation, other possible mechanisms for explaining the histone effects must be considered. One possibility is that our histone fractions were simply contaminated with some phosphoprotein kinase activity, resulting in the addition of this exogenous kinase along with the histone. In order to rule against this possibility, the histone preparation was first heated to destroy any endogenous kinase activity which might be present, and was then added to the phosphorylating system. Under these conditions, the histones were still capable of stimulating the reaction to the same degree (Table II), indicating that addition of kinase along with the histone is not the explanation for the observed stimulation.

Another possibility which must be considered is that the histone preparations contain a phosphatase activity which removes phosphate groups from the phosphoprotein, thereby making more sites available for enzymatic phosphorylation. To test this, a phosphoprotein sample was labeled with [γ-^{32}P]ATP in the absence of histone, and was subsequently incubated with histone in the presence of a 1000-fold excess of non-radioactive ATP. No significant decrease in protein-bound ^{32}P could be detected under these conditions, indicating the absence of significant phosphatase activity. Along these same lines, we also considered the possibility that the histones



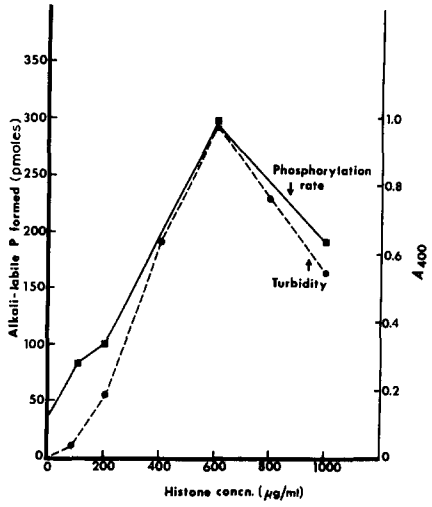
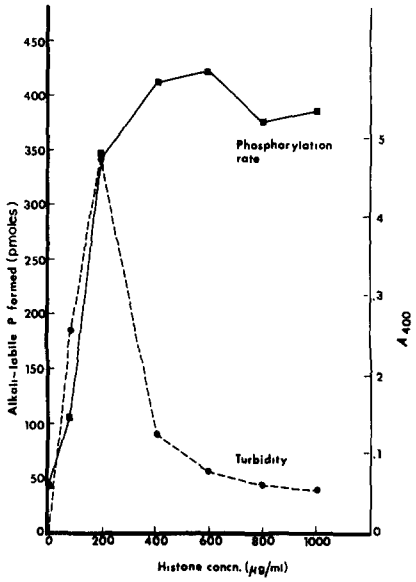


Fig. 4. Effect of varying Mg^{2+} concentration on the rate of phosphorylation and turbidity of nuclear phosphoprotein solutions. The phosphorylation rate was measured by determining the incorporation of [γ-^{32}P]ATP into alkali-labile phosphate during a 10 min incubation, and turbidity was measured by determining the absorbance at 400 nm. Note that the Mg^{2+}-induced change in phosphorylation rate is roughly parallel to the change in solubility of the phosphoprotein as measured by turbidity.

Fig. 5. Effect of varying concentration of total histone on the rate of phosphorylation and turbidity of nuclear phosphoprotein. Experimental details are the same as described in Fig. 4. Note that the histone-induced changes in phosphorylation rate follow quite closely the changes in the solubility of the phosphoprotein.

inhibit a dephosphorylating enzyme present in the phosphoprotein preparation. However, previous experiments have shown that such an enzyme activity is not present in the purified phosphoprotein[1].

While working with the *in vitro* phosphorylating system, we have noted that the addition of Mg^{2+} (which is required for the reaction to take place) causes the appearance of turbidity in the reaction mixture, indicating a decreased solubility of the phosphoprotein. When varying concentrations of Mg^{2+} are added, the increase in phosphorylation rate roughly follows the increase in turbidity (Fig. 4). Thus it appears as if the phosphoprotein is more readily phosphorylated in the insoluble state. Since the addition of histone to phosphoprotein is also known to cause the formation of an insoluble complex[5], the possibility arises that the histone stimulation is simply the result of the decreased solubility of the phosphoprotein in the presence of histone. In fact, if one compares the degree of stimulation caused by various concentrations of total histone with the increase in turbidity, the curves are found to fit quite closely (Fig. 5). However, this correlation with change in solubility cannot be the sole explanation for the mechanism of histone stimulation, since the turbidity and stimulation curves for f_1 histone show that maximal stimulation of phosphoryl-



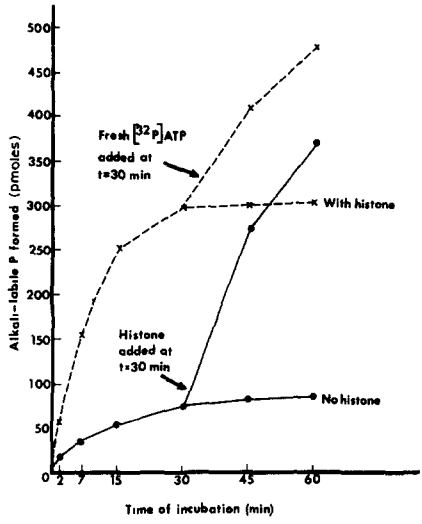


Fig. 6. Effect of varying concentration of f_1 histone on the rate of phosphorylation and turbidity of nuclear phosphoprotein. Experimental details are the same as described in Fig. 4. Note that at high concentrations of f_1 histone, maximal stimulation of phosphorylation rate is achieved while turbidity falls off, indicating a return of the phosphoprotein into solution. Thus, the f_1-mediated stimulation of nuclear protein phosphorylation does not depend on decreased solubility of the phosphoprotein.

Fig. 7. Time-course of phosphorylation of nuclear phosphoprotein by [γ-^{32}P]ATP with and without histone present. After the reactions had reached a plateau at 30 min of incubation, histone (600 μg/ml) was added to one set of tubes which had been incubating without histone, and fresh [γ-^{32}P]ATP was added to a set of tubes which had been incubating with histone. Note that the plateau reached in the absence of histone is not due to either depletion of precursor or inactivation of the enzyme, since the addition of histone reinitiates the phosphorylation reaction.

ation with high concentrations of f_1 histone is accompanied by a decrease in turbidity almost back to control values (Fig. 6).

The final possibility which was tested experimentally is that the histones act by causing an alteration in the conformation of the phosphoprotein which allows new sites to become available for phosphorylation. The time-course of labeling of phosphoprotein with and without histone present indicates that the phosphorylation reaction levels off at an appreciably higher plateau in the presence of histone (Fig. 7). The leveling off at relatively high values seen in the presence of histone only reflects a depletion of precursor [γ-^{32}P]ATP, since the addition of fresh radioisotope initiates incorporation again. On the other hand, the plateau at a relatively low level of phosphorylation which is reached in the absence of histone is not caused by either depletion of precursor or inactivation of the enzyme system, since the addition of histone to such a system causes the initiation of subsequent phosphorylation (Fig. 7). These results suggest that when histones are added, new sites for phosphorylation in the phosphoprotein become available.

DISCUSSION

The results show that histones are potent stimulators of the phosphorylation of non-histone chromatin acidic proteins *in vitro*. This stimulatory effect is relatively specific for histones, since other naturally occurring proteins tested in this system had no effect. Especially significant is the lack of stimulation seen with cytochrome *c*, since this protein is similar to histones in both its small size and high isoelectric point. The stimulation seen with poly-L-lysine, and to a lesser extent poly-L-arginine, is not surprising in view of the fact that many of the metabolic effects of histones can be mimicked with these compounds[14–16]. This finding probably indicates that lysine and arginine residues in the histone molecules are playing a key role in the physical interactions of these proteins. There are two reasons why it seems reasonable to conclude that the histone-mediated stimulation of protein phosphorylation seen in our system is of potential physiological significance: (1) the histones are normally found in close association with the acidic phosphoproteins in chromatin *in vivo*, and thus this physical proximity indicates the potential for such interactions, and (2) other proteins tested in our system did not have a similar stimulatory effect on protein phosphorylation, indicating the relative specificity of the histone effect.

These findings indicate that histone–phosphoprotein interactions should be considered when discussing metabolic and structural properties of chromatin. It is important to note in this regard that the ratio of histone to phosphoprotein required for maximal stimulation shows a rather sharp optimum, indicating that local changes in the distribution of histones in chromatin could have a pronounced effect on the rate of phosphorylation of the non-histone proteins. A related finding of considerable interest is that in the presence of constant histone concentration, an increase in concentration of phosphoprotein inhibits the degree of histone-mediated stimulation. Thus the final rate of phosphorylation is extremely sensitive to local changes in both histone and phosphoprotein concentration.

The most likely explanation for the histone-mediated stimulation is that the histone induces a conformational change in the phosphoprotein which allows new sites to become available for phosphorylation. In the absence of histone, the maximum increase in phosphate content which can be achieved by incubating phosphoprotein with ATP is in the order of 2–3% of the phosphate already present in the phosphoprotein. Current experiments have shown that this limitation is not due to either depletion of substrate or inactivation of the kinase. However, in the presence of histone the phosphate content of the phosphoprotein can be increased up to 20–30%, indicating that new sites can become available for phosphorylation. These results indicate one of the dangers inherent in interpreting data obtained from highly purified systems. On the basis of earlier experiments on the phosphorylation of purified nuclear phosphoprotein *in vitro*, it had been concluded that the phosphate acceptor sites of the phosphoprotein are nearly saturated *in vivo*[20]. However, it now appears that this is not the case, since in the intact nucleus the presence of histone would be expected to change the properties of the phosphoprotein so as to allow more sites to be available for phosphorylation.

It has been previously postulated that phosphorylation of nuclear proteins is associated with changes in chromatin structure, with phosphorylation of the non-histone chromatin proteins being involved in the displacement of histone from the

DNA template during the process of gene activation[8]. This hypothesis has been supported by observations on changes in protein phosphorylation which correlate with changes in gene activity and chromatin structure in avian erythrocytes[4] and human lymphocytes[3]. The present findings indicate that histone–phosphoprotein interactions may result in significant changes in the properties of the phosphoproteins, and specifically may lead to an increase in the degree of phosphorylation. This increase in phosphorylation results in an increased negative charge on the phosphoprotein, and would therefore serve to strengthen the ionic binding between the phosphoprotein and the positively charged histones. Thus, when phosphoprotein and histone come together *in vivo*, one would expect this interaction to lead to phosphorylation of the phosphoprotein, resulting in a rapid increase in the strength of attraction between phosphoprotein and histone. Such an increased attraction might be sufficient to displace the histone from the DNA double helix, thereby allowing gene transcription to take place. Thus the current findings reinforce the earlier model of KLEINSMITH *et al.*[8] concerning the role played by phosphoproteins in chromatin structure and gene activation, and indicate that phosphoprotein–histone interactions may play a critical role *in vivo*.

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