Phosphorylation of Non-histone Proteins in the Regulation of Chromosome Structure and Function [1]

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ABSTRACT Non-histone chromosomal proteins are phosphorylated and dephosphorylated within the intact nucleus by two independent sets of reactions, a protein kinase reaction which transfers the terminal phosphate group of a variety of nucleoside and deoxynucleoside triphosphates to serine and threonine residues in the proteins, and a phosphatase reaction which cleaves these phosphoserine and phosphothreonine bonds and releases inorganic phosphate. Several lines of evidence are consistent with the hypothesis that the phosphorylation and dephosphorylation of these proteins is involved in gene control mechanisms, including the findings that phosphorylated non-histone proteins are highly heterogeneous and their phosphorylation patterns are tissue specific, changes in their phosphorylation correlate with changes in chromatin structure and gene activity, addition of phosphorylated non-histone proteins increases RNA synthesis in vitro, and phosphorylated non-histone proteins bind specifically to DNA.

Cyclic AMP has both stimulatory and inhibitory properties on non-histone protein phosphorylation, depending on the enzyme fraction and substrate employed. A specific protein component whose phosphorylation is inhibited by cyclic AMP has been found to be associated with RNA polymerase. The cyclic AMP-induced decrease in the phosphorylation of this protein correlates with an enhancement of RNA synthesis in vitro. These results suggest that both phosphorylation and dephosphorylation of chromatin-associated proteins may be involved in the control of gene readout.

The specific regulation of the process of gene transcription is thought to play a key role in both the development and normal functioning of the cells of higher organisms. Since the DNA of each cell in a multicellular animal appears to be the same, and yet each cell type performs different functions, this means that various cells must use the information encoded in their DNA differently. One way in which such control may be exerted is through the activation and inactivation of particular regions of the genome for RNA synthesis, depending on the particular needs of the cell at the time.

Although the mechanism by which such control is achieved is yet to be determined in eukaryotic cells, considerable progress has been made on this problem in recent years in microbial systems. A number of DNA-associated proteins have been isolated, such as the *lac*, lambda, *gal*, *trp*, and *hut* repressors, as well as the cyclic AMP-receptor protein, all of which bind to DNA and serve to regulate transcription (Gilbert and Mueller-Hill, '67; Ptashne, '67; Nakanishi et al., '73; Shimizu et al., '73; Hagen and Magasanik, '73; Riggs et al., '71). The DNA-associated proteins of the eukaryotic chromosome have also come under intensive study, but their role in the regulation of transcription seems less certain. The proteins involved in this case fall into two general classes, the highly basic histones and the mildly acidic non-histones. The histones are known to act as general inhibitors of RNA synthesis, but their limited heterogeneity and relative constancy between diverse cell types and under different physiological conditions has led to the general conclusion that they are not involved in regulating the activity of specific genes (for review see Hnilica, '72). On the other hand the non-histone proteins exhibit considerable heterogeneity and variability, and a number of lines of evidence, including chromatin reconstitution experiments, suggest an important role for these proteins in the specific regulation of gene transcription (for review see Stein et al., '74).

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In this presentation I would like to discuss one special aspect of these non-histone proteins which I feel may have important implications for this genetic regulatory role, and that is the extensive occurrence of phosphorylation and dephosphorylation reactions within this protein class. I would first like to review some of the older evidence which was obtained concerning the nature of the phosphorylation and dephosphorylation reactions, and then describe some of the functional properties of non-histone phosphoproteins which are compatable with a role in gene regulation.

Phosphorylation of non-histone proteins in intact nuclei

The first studies demonstrating the existence of protein phosphorylation in the intact nucleus were performed employing isolated calf thymus nuclei incubated in the presence of ^{32}P-orthophosphate (Kleinsmith et al., '66a). These experiments demonstrated that incorporation of ^{32}P into nuclear proteins occurs in a reaction which is energy dependent, since it is blocked by inhibitors of ATP formation such as iodoacetate and 2,4-dinitrophenol. The major site of protein phosphorylation was found to be the hydroxyl group of serine residues, which accounts for about 90% of the incorporated ^{32}P. The remaining 10% of the radioactivity incorporated into protein is found as phosphothreonine. In order to determine whether these phosphorylated amino acids are incorporated into the nuclear proteins as part of the process of protein synthesis or after the polypeptide chains have been completed, experiments were performed to determine the effects of the protein synthesis inhibitor puromycin on protein phosphorylation. Under conditions where protein synthesis was inhibited up to 90%, no change in the incorporation of ^{32}P into nuclear proteins was observed, indicating that the phosphate groups are put on the protein molecules after the polypeptide chains have been completed.

Since phosphate groups are thus at-

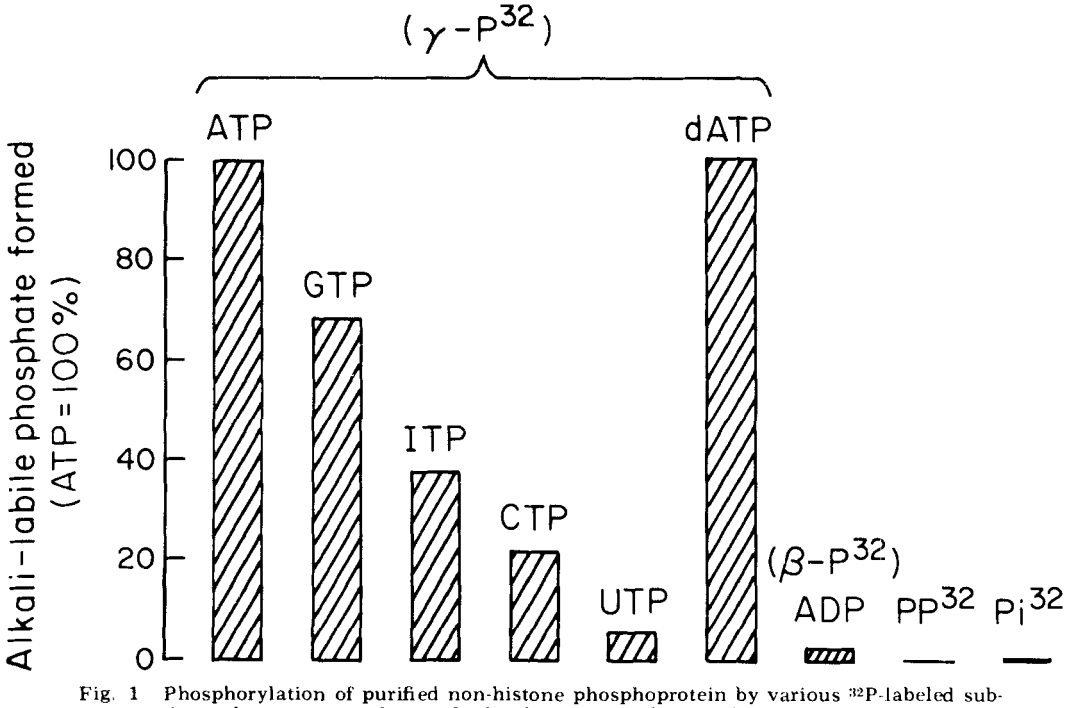

Fig. 1 Phosphorylation of purified non-histone phosphoprotein by various ^{32}P-labeled substrates in vitro. The reaction volume of 0.5 ml contained 2.5 μmoles of $MgCl_2$, 20 μmoles of Tris-HCl (pH 7.4), 0.5 μmoles of [γ-^{32}P] ATP, and 60 μg of purified non-histone phosphoprotein. After incubation for 10 min at 37°, radioactivity bound to protein and releasable in 1.0 N NaOH was assayed as a measure of protein phosphorylation (Kleinsmith and Allfrey, '69a).

tached to nuclear proteins independent of protein synthesis, the question arose as to whether phosphate groups are also removed from these proteins independent of protein degradation. This problem was approached in pulse-chase experiments, where it was found that 70–80% of previously incorporated ^{32}P is lost from the nuclear proteins independent of any protein breakdown.

In addition to demonstrating the existence of protein phosphorylation and dephosphorylation in intact nuclei, these early studies also established that the major site of nuclear protein phosphorylation is the non-histone chromosomal proteins. A crude fractionation of nuclei into nuclear sap, histone, and non-histone proteins showed that over 90% of the phosphoprotein is in the non-histone protein fraction. Although the existence of histone phosphorylation has been confirmed and extensively studied by other investigators (for review see Hnilica, '72), it accounts for only a very small portion of nuclear protein phosphorylation in quantitative terms. The functional significance of histone phosphorylation is unknown, although it has been implicated in hormone action as well as in changes in chromosome condensation during the cell cycle (Langan, '69; Louie and Dixon, '73). The phosphorylation of non-histone proteins, on the other hand, is of special interest because of the previously mentioned evidence suggesting a role for non-histone proteins in specific gene regulation, and will therefore be the subject of the remainder of this article.

Purification and enzymatic studies of non-histone phosphoproteins

Most of the studies on the phosphorylation of non-histone proteins carried out in our laboratory have involved the purifica-

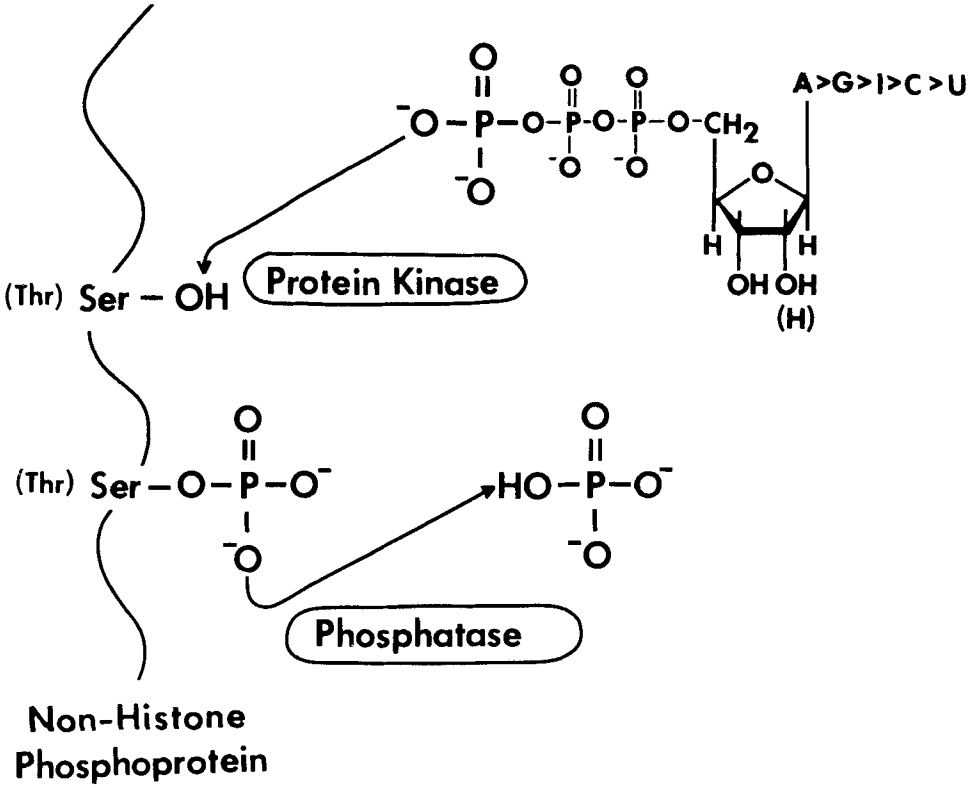

Fig. 2 Model summarizing the relationships between the phosphorylation and dephosphorylation reactions of non-histone phosphoproteins. Serine (and threonine) residues in the proteins are phosphorylated via the terminal phosphate of various nucleoside and deoxynucleoside triphosphates in a typical protein kinase reaction. In a separate phosphatase reaction the phosphoserine (and phosphothreonine) bonds are broken, releasing inorganic phosphate.

tion of a subfraction of the non-histone proteins which is enriched in phosphoproteins. This purification procedure has been described in detail elsewhere (Langan, '67; Gershey and Kleinsmith, '69a; Kish and Kleinsmith, '74), and will only be briefly outlined here. In this scheme nuclei are first washed with dilute salt solutions to remove the soluble proteins, after which the chromatin is solubilized with 1.0 M NaCl. This extract is then diluted back to a final salt concentration of 0.4 M, at which point the DNA and histones precipitate out of solution, leaving the bulk of the non-histone phosphoprotein in the supernatant. The phosphoproteins are then further purified by adsorption with calcium phosphate gel. The final product usually averages about 1.2% phosphorus by weight, which is enough to phosphorylate 4 or 5 out of every 100 amino residues present. As can be seen in table 1, which summarizes the yield of purified non-histone phosphoprotein from several sources, this material is a major component of the cell nucleus, accounting for 5–10% of the total nuclear mass.

Once this purification procedure had been developed, we then had a tool with which we could answer some of the basic questions about the nature of the phosphorylation and dephosphorylation reactions. The way I originally viewed the situation these non-histone phosphoproteins could have fit into one of two general functional categories. On one hand they might be intermediates in phosphate transfer reactions, in which case the origin and fate of the phosphate groups would be the focus of

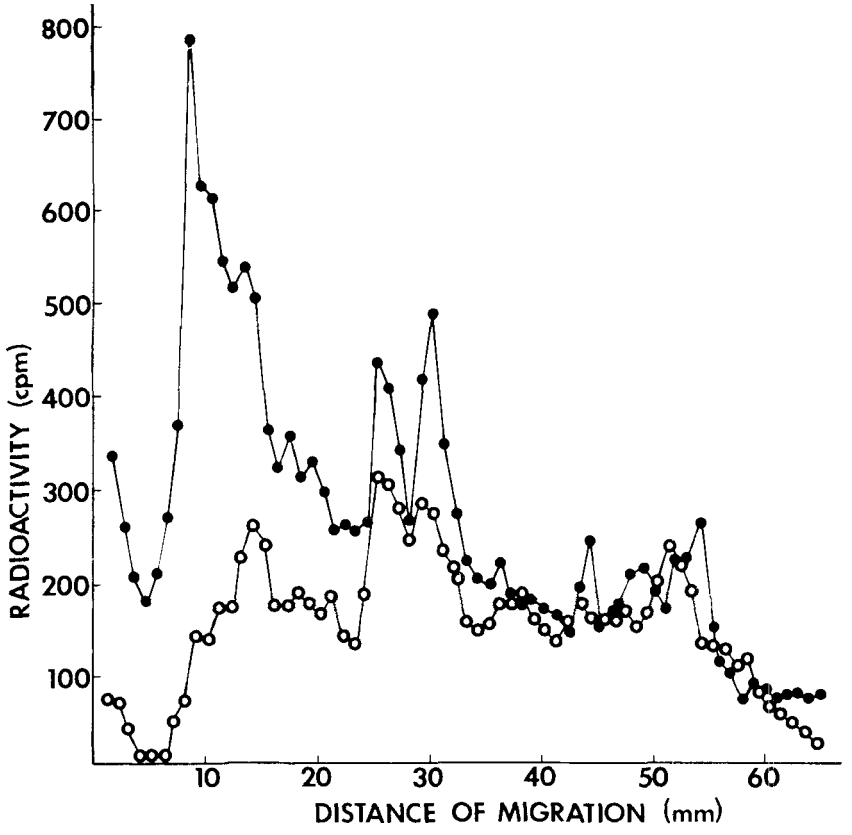

Fig. 3 The incorporation of ^{32}P into non-histone phosphoproteins of WI-38 (o——o) and SV-40 transformed WI-38 (●——●) human fibroblasts. Cells were labeled for one hour with radioisotope, the non-histone phosphoprotein fraction isolated, and electrophoresis of the proteins performed in 10% polyacrylamide gels containing 0.1% SDS. The radioactivity in each gel slice is plotted as a function of the distance of migration. Note the increased radioactivity in the high molecular weight components of the transformed fibroblasts.

interest, with the proteins simply serving as intermediate agents which transfer the phosphate groups. On the other hand the addition and removal of phosphate groups might be serving the function of altering the structure and function of the proteins themselves. In this case changes in the non-histone proteins would be the focus of interest, with the phosphate groups serving merely as agents which induce such changes. It is obvious that in order to distinguish between these two possibilities one must know both the origin and fate of the phosphate groups involved in non-histone protein phosphorylation and dephosphorylation.

The fate of the protein-bound phosphate groups was studied by adding purified phosphoprotein labeled with ^{3}H-serine and ^{32}P-orthophosphate to unlabeled nuclei, and determining the subsequent fate of these isotopes. Although it was found that the serine label was completely stable to incubation, indicating the absence of proteolysis, the ^{32}P was rapidly released as inorganic phosphate (Kleinsmith and Allfrey,

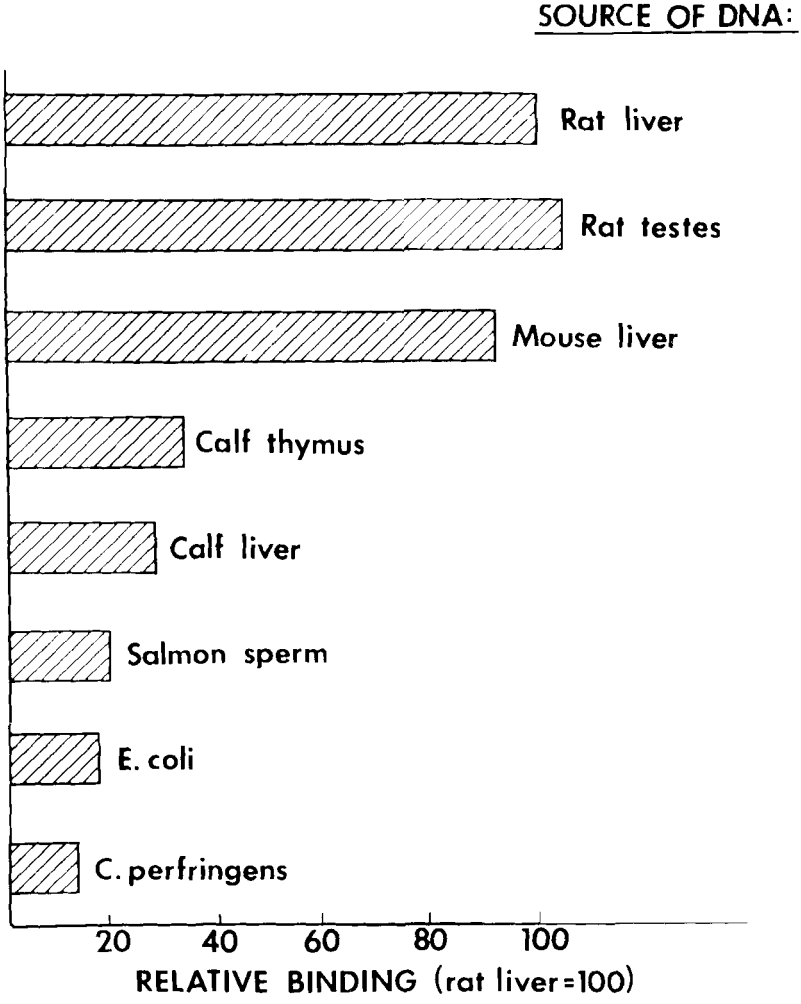

Fig. 4 Binding of phosphorylated non-histone proteins to DNA prepared from various sources. The ^{32}P-labeled phosphoprotein fraction prepared from rat liver was reacted with DNA-cellulose at an ionic strength of 0.14 M NaCl, and bound material eluted at 0.6 M NaCl. The bars show the relative binding of these proteins to various DNAs, with rat liver DNA set equal to 100 as a basis for comparison (Kleinsmith, '73).

TABLE 1

Yield of non-histone phosphoproteins extracted from various tissues

	Calf thymus [1]	Rat liver [1]	Beef kidney
Phosphorus content			
Total nuclear protein	0.07%	0.14%	0.12%
Purified phosphoprotein	1.2 %	1.3 %	1.1 %
Purification	17 ×	9 ×	9 ×
Yield	18 %	25 %	18 %
Phosphoprotein content expressed as percent of:			
Dry wt nucleus	5 %	9 %	8 %
Total acidic proteins	15 %	50 %	36 %

[1] Kleinsmith and Allfrey, '69a.

'69b). Since the release of no other labeled product could be detected, it seems unlikely that the non-histone proteins transfer their phosphate groups to other molecules.

The question of the phosphate donors involved in non-histone protein phosphorylation was studied in a cell-free system where the purified phosphoprotein fraction was incubated in the presence of magnesium and [γ-^{32}P] ATP. Under such conditions the phosphorylation of serine and threonine residues occurs without the addition of any other enzyme, demonstrating that protein kinase activity is present in the non-histone phosphoprotein fraction (Kleinsmith and Allfrey, '69a). In addition to ATP a variety of other nucleoside and deoxynucleoside triphosphates were found to be capable of phosphorylating the non-

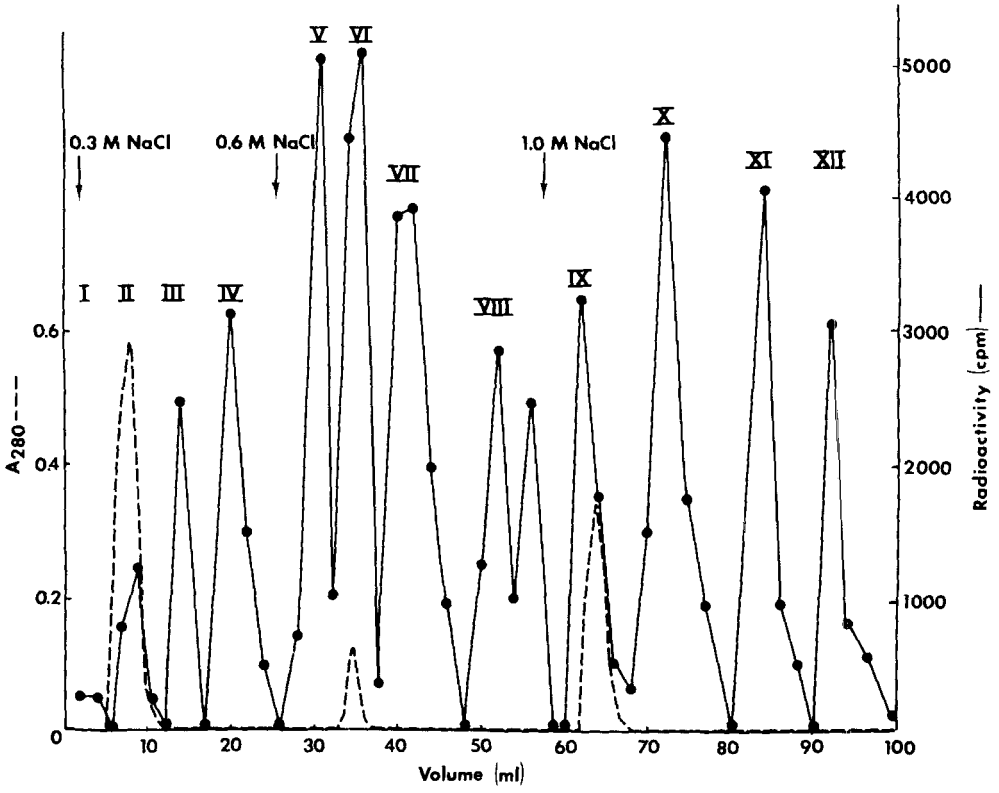

Fig. 5 Fractionation of protein kinase activities from purified non-histone phosphoprotein of beef liver. Purified non-histone phosphoproteins were applied to a phosphocellulose column equilibrated with 0.05 M Tris-HCl (pH 7.5) − 0.3 M NaCl, and eluted with steps of 0.05 M Tris-HCl (pH 8.1) − 0.6 M NaCl and 0.05 M Tris-HCl (pH 8.1) − 1.0 M NaCl. Protein was measured by absorption at 280 nm (– – –). Protein kinase activity was measured as incorporation of radioactivity from [γ-^{32}P] ATP into endogenous protein substrate (●——●). Twelve distinct protein kinase fractions can be resolved under these conditions (Kish and Kleinsmith, '74).

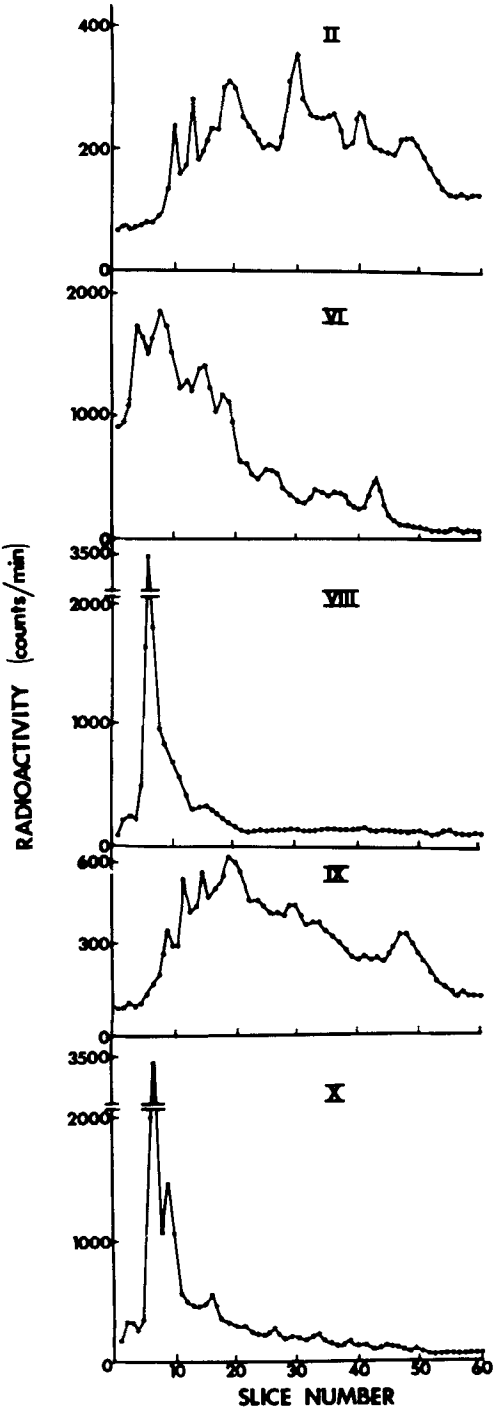

Figure 6

histone phosphoproteins in this system (fig. 1).

Another problem which could be studied in this cell-free system concerns the nature of the enzymatic activity involved in the dephosphorylation reaction. We have already seen that the phosphorylating (protein kinase) activity is carried along with the non-histone phosphoprotein during purification, and the question arises as to whether the dephosphorylating activity is also part of this same complex. Studies on phosphate turnover in the cell-free system, however, showed that no detectable loss of previously incorporated ^{32}P could be observed (Kleinsmith and Allfrey, '69a). This result suggests that the enzymes involved in protein phosphorylation and dephosphorylation are different from each other, and led to the general model of protein phosphate metabolism summarized in figure 2.

Having established that phosphorylation and dephosphorylation of non-histone proteins occurs via two independent sets of reactions, the next obvious question concerns the functional significance of these processes. The fact that the phosphate donor is a high energy compound such as ATP while the phosphate is released in the low energy form of inorganic phosphate indicates that considerable energy is being expended. The above data rule against the possibility of a phosphate transfer function, leaving the alternative possibility that the addition and removal of phosphate groups is serving to alter the structural and functional properties of the proteins. There is now ample evidence that such protein modification reactions may be important regulatory mechanisms for other proteins (Krebs, '72; Segal, '73), and so the hypoth-

Fig. 6 Demonstration of differences in the phosphorylation pattern of non-histone proteins phosphorylated by various protein kinase fractions. The non-histone phosphoprotein fraction employed as substrate was preheated at 60° for 3 min to inactivate its endogenous protein kinase activity. These phosphoproteins were labeled by incubation with [γ-^{32}P] ATP in the presence of the appropriate protein kinase fraction (designated by Roman numerals referring to fractions obtained as shown in fig. 5), and then electrophoresed in 10% polyacrylamide gels containing 0.1% SDS. Note the different patterns of phosphorylation.[2]

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esis has arisen that the phosphorylation and dephosphorylation of non-histone proteins is involved in modifying their structural and functional interactions in chromatin in a way as to provide a mechanism for the regulation of gene activity. This hypothesis can be used to generate a number of specific predictions about the behavior and properties of these proteins which can be easily tested.

Heterogeneity and tissue specificity of non-histone protein phosphorylation

If the phosphorylated non-histone proteins are involved in specific gene regulation, then one would expect this protein fraction to be heterogeneous and exhibit specific differences in tissues and cell types where differences in gene expression occur. Employing the technique of SDS-acrylamide gel electrophoresis we have been able to observe the occurrence of considerable heterogeneity in this protein fraction (Platz et al., '70). At least 25–30 bands are routinely resolvable, with this number almost certainly representing a lower limit estimate of the number of components present. The overall banding patterns from tissue to tissue have been found to share many features in common, but each has a unique, reproducible pattern which differs both quantitatively and qualitatively from the others. Labeling with ^{32}P indicates that most of these bands represent phosphorylated proteins, and their phosphorylation patterns have also been found to be tissue specific. These studies, which are consist-

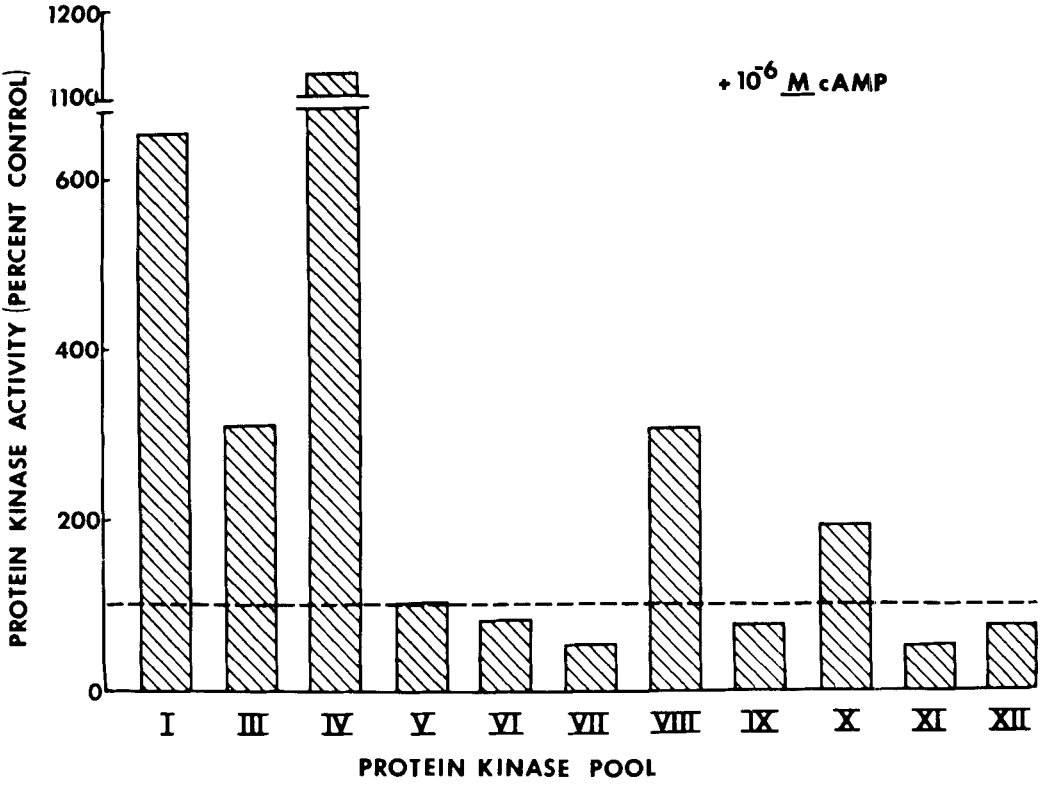

Fig. 7 Effects of 10^{-6} M cyclic AMP on non-histone phosphoprotein phosphorylation catalyzed by various protein kinase fractions. The non-histone phosphoprotein fraction employed as substrate was pre-heated at 60° for 3 min to inactivate its endogenous protein kinase activity. The dotted line represents control levels, while the bars represent the effects of cyclic AMP with each protein kinase fraction (Roman numerals refer to fractions obtained as shown in fig. 5). Note that both stimulatory and inhibitory effects of the cyclic nucleotide are observed.[2]

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ent with the results of others (Teng et al., '71; Rickwood et al., '73), show that in contrast to the histones, the non-histone phosphoproteins are highly heterogeneous and exhibit patterns which are specific for different tissues.

Changes in non-histone phosphorylation and gene activity

The hypothesis that the phosphorylation of non-histone proteins is related to the control of gene expression also leads to the obvious prediction that changes in the phosphorylation of these proteins ought to correlate with changes in gene activity. One of the first tests of this prediction was made in human lymphocytes stimulated by the addition of phytohemagglutinin, where it was observed that an increased rate of nuclear protein phosphorylation occurred within the first hour of activation, prior to a major increase in RNA synthesis (Kleinsmith et al., '66b). A similar correlation was found in studies on the maturation of avian red blood cells (Gershey and Kleinsmith, '69b), where it was found that young cells with dispersed, active chromatin exhibited faster rates of non-histone protein phosphorylation and contained more protein-bound phosphate and protein kinase than mature cells with compact, inactive chromatin (table 2). Another example of striking changes in the pattern of non-histone protein phosphorylation is to be found in the recent observations of D. Pumo, G. Stein, and myself on SV-40 transformed fibroblasts (fig. 3). The dramatic increase in phosphorylation of high molecular weight non-histone proteins which we have seen in these transformed cells is of special interest because of its possible relationship to the altered control of cell proliferation which characterizes the malignant state.

Other examples of correlations between non-histone protein phosphorylation and cellular and/or gene activity have now been reported in a large number of different systems, including testosterone stimulated prostate cells (Ahmed and Ishida, '71; Schauder et al., '74), mammary glands stimulated by prolactin (Turkington and Riddle, '69), ovaries stimulated by chorionic

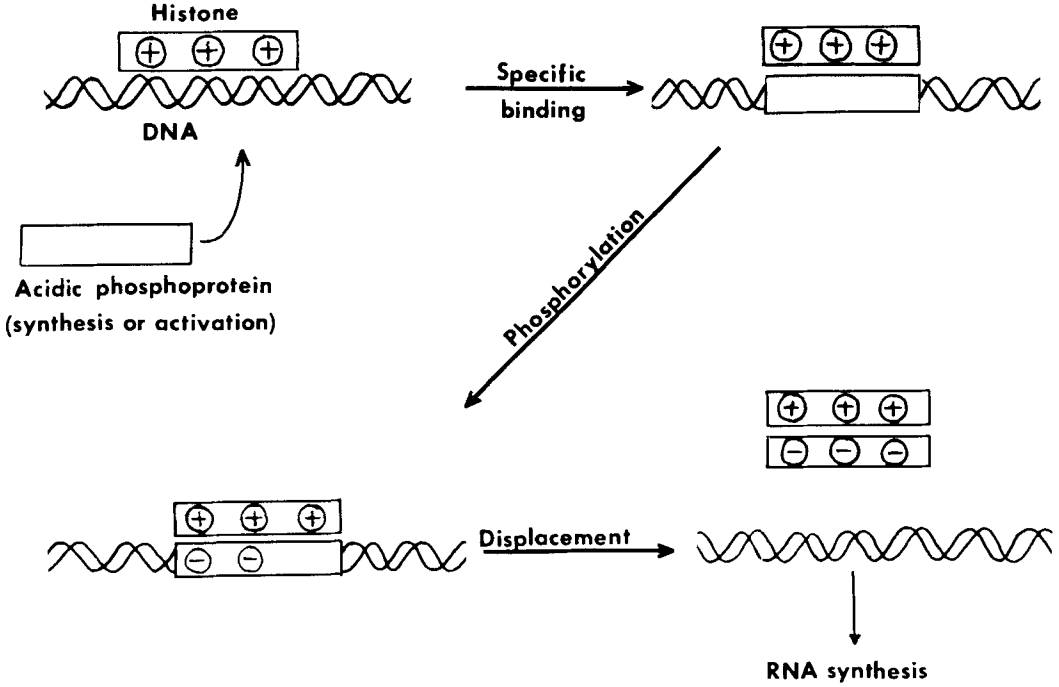

Fig. 8 One model of gene activation incorporating some of the properties of non-histone phosphoproteins. The model is based on the specific binding of non-histone proteins to DNA, their subsequent phosphorylation, and finally histone displacement. The resulting naked DNA is then capable of synthesizing RNA.

TABLE 2

Phosphorylation of nuclear proteins in avian erythrocytes [1]

	Young reticulocytes	Mature erythrocytes
RNA synthesis	+	−
DNA synthesis	+	−
State of chromatin	Diffuse	Dense
Phosphorylation rate (cpm/30 min)	2850	1120
Turnover rate ($t_{1/2}$)	15 min	40 min
Phosphoprotein kinase (units/mg DNA)	4.6	2.3
$\frac{\text{Phosphoprotein} - P}{\text{DNA} - P}$	.074	.023

[1] Gershey and Kleinsmith, '69b.

gonadotropin (Jungmann and Schweppe, '72), cortisol and corticosterone stimulated liver (Allfrey et al., '73; Bottoms and Jungmann, '73), aldosterone stimulated kidney (Liew et al., '73), synchronously dividing HeLa cells (Platz et al., '73; Karn et al., '74), mitotic cycle of *Physarum polycephalum* (LeStourgeon and Rusch, '71), growth cycle of Yoshida ascites sarcoma cells (Riches et al., '73), sea urchin development (Platz and Hnilica, '73), isoproterenol stimulated salivary glands (Ishida and Ahmed, '73, '74), breast carcinoma (Kadohama and Turkington, '73), azo-dye carcinogenesis (Chiu et al., '73), and phenobarbital treated liver (Blankenship and Bresnick, '74). Thus in a wide variety of different experimental situations the phosphorylation of non-histone proteins has been found to be subject to alteration as the activity of the cell and genome is modified.

Effects of non-histone phosphoproteins on RNA synthesis

If the non-histone phosphoproteins are regulators of gene activity, then one would also predict that they should affect the rate of RNA synthesis in cell-free systems. We have tested this point in a system employing rat DNA as a template with purified rat liver RNA polymerase, and have found that the addition of rat liver phosphoprotein can cause more than a doubling in the synthesis of RNA (Shea and Kleinsmith, '73). Interestingly this effect is blocked by preincubating the phosphoprotein with alkaline phosphatase, suggesting that the protein-bound phosphate groups are critical in this regard. Furthermore the observed stimulatory effect on RNA synthesis appears to be template specific, since the synthesis of RNA from foreign DNA sources was not found to be stimulated by the rat liver phosphoprotein. Stimulatory effects of non-histone phosphoproteins on RNA synthesis have also been observed in other laboratories (Langan, '67; Kamiyama et al., '71, '72; Kostraba and Wang, '72a,b; Teng et al., '71; Rickwood et al., '72).

DNA-binding of phosphorylated non-histone proteins

Since specific genetic regulatory molecules purified from microbial systems have turned out to be allosteric proteins which recognize and bind to specific sites on DNA, it might be expected that specific gene regulators in eukaryotic cells would also be able to bind to DNA in a specific fashion. In order to explore this question experiments were performed employing DNA-cellulose chromotography to test the DNA-binding properties of the phosphorylated non-histone proteins (Kleinsmith, '73). It was found that at physiological ionic strength, about 1% of this protein fraction prepared from rat liver will bind to columns of rat DNA. Both the percentage of binding observed and the binding constant for the protein-DNA interaction were found to depend on the ionic strength. Treatment with various enzymes demonstrated the protein nature of the bound material. At physiological ionic strength, binding sites on the DNA were found to be saturated at a value approximately 1 μg of phosphorylated protein per 100 μg of DNA. The material which bound to the DNA was observed on SDS-acrylamide gel electrophoresis to be a heterogeneous family of proteins in the molecular weight range of 30,000 to 70,000. Most interesting was the observation that these proteins bound preferentially to homologous DNA, and exhibited a decreasing affinity for DNAs as they become more distant evolutionarily (fig. 4).

Nuclear protein kinases: heterogeneity and effects of cyclic AMP

If the phosphorylation of non-histone proteins is involved in gene control, then one would expect this protein modification reaction to be itself subject to external regulation. An obvious candidate for a regulator of this reaction is of course cyclic

AMP, which is known to be an activator of protein phosphorylation reactions. Many of the early studies on protein phosphorylation in intact nuclei or in non-histone protein fractions, however, failed to show any significant effects of cyclic AMP (Kaplowitz et al., '71; Ahmed, '71; Kamiyama and Dastugue, '71). But when the protein kinase activities associated with the purified non-histone phosphoprotein fraction are separated via phosphocellulose chromatography, a very different picture emerges (Kish and Kleinsmith, '74). It turns out that a heterogeneous family of nuclear protein kinases exist which phosphorylate the non-histone proteins in different ways and which differ in their regulation via cyclic AMP (figs. 5–7). Some of these protein kinases are stimulated via cyclic AMP, while the phosphorylation reactions catalyzed by others are inhibited. Thus, the earlier failures to observe effects of cyclic

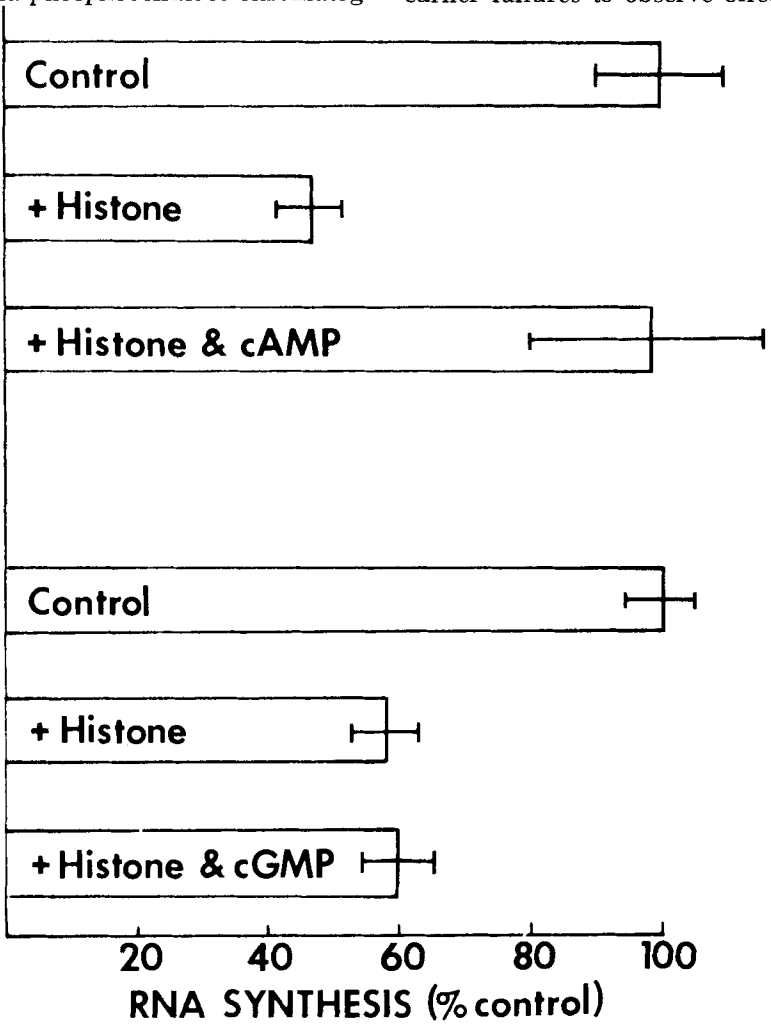

Fig. 9 Effects of histone and cyclic nucleotides on RNA synthesis in vitro. The reaction volume of 0.75 ml contained 45 μmoles Tris-HCl, pH 8.0; 2.25 μmoles NaF; 15 μmoles $MnCl_2$; 2.25 μmoles spermine tetrahydrochloride; 0.3 μmoles CTP, GTP, and ATP; 100 μg calf thymus DNA; 1 μC ^{3}H-UTP; 0.4 mg total rat liver RNA polymerase (Roeder and Rutter, '70); and where indicated, 100 μg f_1 histone and 10^{-5} M cyclic AMP or cyclic GMP. Incubation was performed 20 min at 37°, and incorporation of radioactivity into acid-insoluble material determined by precipitation and washing in 10% TCA – 2% pyrophosphate. Note that cyclic AMP, but not cyclic GMP, stimulates RNA synthesis in the presence of histone.

AMP on non-histone protein phosphorylation may have been due to the masking of these complex and divergent effects which occurs when analyses are performed on less purified preparations.

A similar problem has occurred in attempts to observe effects of cyclic AMP on non-histone protein phosphorylation in intact animals. Although the phosphorylation of total non-histone proteins has been reported to be unaffected by injections of cyclic AMP (Byvoet, '71), analyses of the phosphorylation of individual components by SDS-acrylamide gel electrophoresis indicates a small stimulation of some specific components (Johnson and Allfrey, '72).

Effects of histones on non-histone protein phosphorylation

In addition to cyclic AMP, another factor which has been found to influence the phosphorylation of non-histone proteins is the presence of histones. In view of the fact that histones are generally viewed as inhibitors of enzymatic reactions, it was rather surprising to find that the addition of histones to the purified non-histone phosphoprotein fraction greatly enhanced the endogenous rate of phosphorylation (Kaplowitz et al., '71). The stimulatory effect of histones is quite specific, since other small basic proteins like cytochrome c were not found to exert a comparable effect. The evidence suggested that the histones are not being phosphorylated themselves, but rather act to make more sites available for phosphorylation in the phosphoprotein itself.

These findings indicate that histone-phosphoprotein interactions may result in

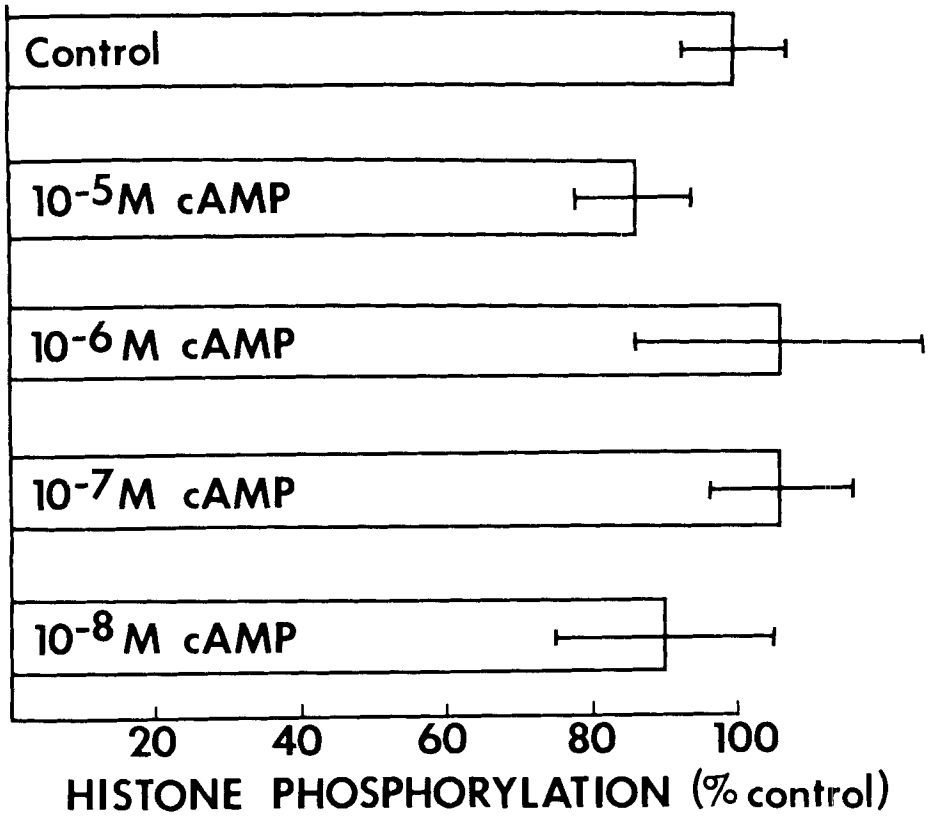

Fig. 10 Effects of cyclic AMP on f_1 histone phosphorylation employing RNA polymerase as the source of protein kinase. The reaction volume of 0.5 ml contained 25 μmoles Tris-HCl, pH 7.4; 12.5 μmoles $MgCl_2$; 1 μC [γ-^{32}P] ATP; 0.3 mg rat liver RNA polymerase (Roeder and Rutter, '70); 100 μg f_1 histone, and varying concentrations of cyclic AMP. After incubation for 10 min at 30°, incorporation of radioactivity into acid-insoluble material was determined by precipitation and washing in 10% TCA – 2% pyrophosphate. Note that cyclic AMP has no significant effect on the phosphorylation of f_1 histone in this system.

significant changes in the properties of the phosphoprotein, 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. Since some of the non-histone phosphoproteins have the ability to recognize and bind to spe-

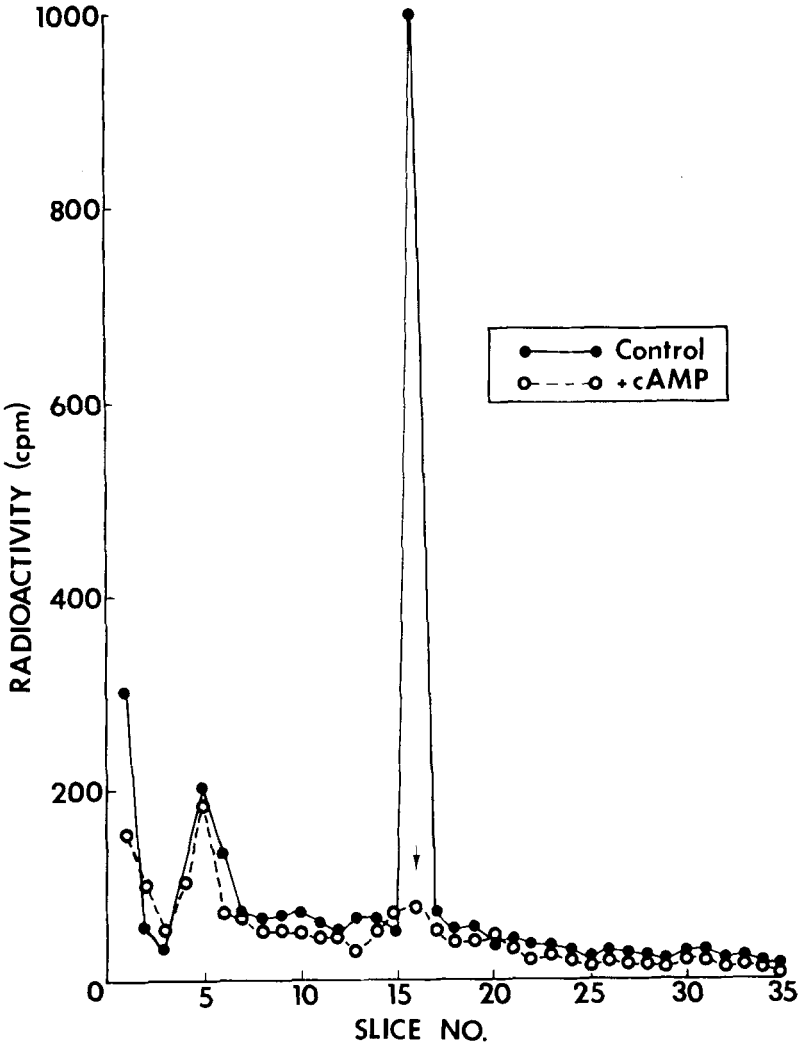

Fig. 11 Effect of cyclic AMP on the phosphorylation of endogenous proteins of rat liver RNA polymerase. Incubation conditions were as described in the legend to figure 10, except histone was omitted. Reactions were stopped by addition of solid urea to a final concentration of 4 M. Samples were dialyzed against 0.01 M sodium phosphate (pH 7.0) – 0.1% SDS – 0.1% β-mercaptoethanol, and electrophoresis performed in 10% polyacrylamide gels containing 0.1% SDS. Radioactivity in one mm gel slices is plotted. Note the peak of radioactivity which is inhibited in the presence of 10^{-6} M cyclic AMP (arrow).

cific DNA sequences, an overall model of gene control can thus be envisioned which involves an initial specific binding of these proteins to certain regions of DNA, followed by enhanced non-histone protein phosphorylation and histone displacement as just described (fig. 8).

Effects of cyclic AMP on RNA synthesis and phosphorylation of RNA polymerase-associated proteins

One of the many limitations of the above model is that it does not provide any explanation for the decrease in protein phosphorylation observed when cyclic AMP is added to some of the nuclear protein kinase fractions. Recent studies carried out in our laboratory by L. Dokas and D. Rittschof on the effects of cyclic AMP on RNA synthesis and protein phosphorylation in cell-free systems suggest that one possible site for this effect is the enzyme RNA polymerase. It has been found in these studies that 10^{-6} M cyclic AMP will stimulate RNA synthesis in a system containing purified rat DNA, rat liver RNA polymerase, and histone. This stimulatory effect is not observed in the absence of histone, nor is it produced by substituting cyclic GMP for cyclic AMP (fig. 9). Since the RNA polymerase fraction employed contains endogenous protein kinase activity, it seemed possible that the presence of cyclic AMP was affecting a protein phosphorylation reaction. Studies on the effects of cyclic AMP on histone phosphorylation in this system showed no alteration in the presence of the cyclic nucleotide (fig. 10). Analysis of the phosphorylation of proteins associated with the RNA polymerase fraction by SDS-acrylamide gel electrophoresis, however, showed

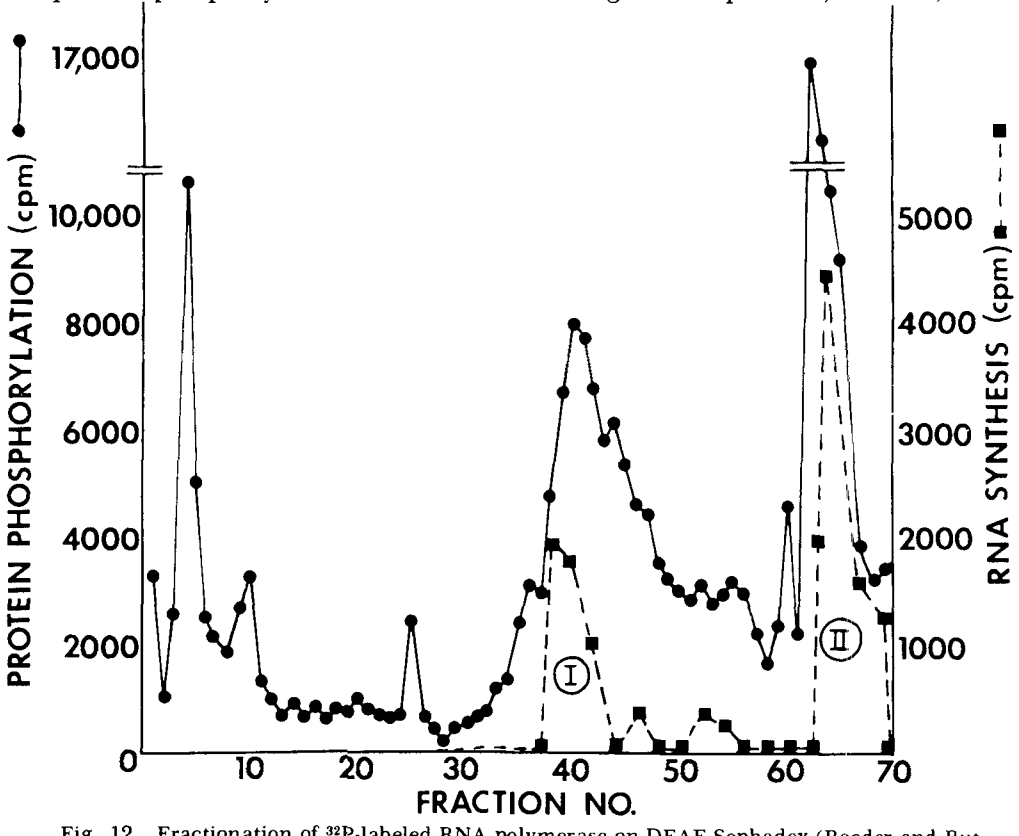

Fig. 12 Fractionation of ^{32}P-labeled RNA polymerase on DEAE-Sephadex (Roeder and Rutter, '70). Labeling was performed as described in the legend to figure 11, while RNA polymerase activity was measured as described in the legend to figure 9. Note that phosphorylated proteins appear in the void volume, as well as in the regions where RNA polymerases I and II are eluted.

that cyclic AMP was specifically inhibiting the phosphorylation of one protein component (fig. 11).

In order to determine whether this protein component was associated with RNA polymerase, DEAE-Sephadex column chromatography was performed on the ^{32}P-labeled material. Although some of the phosphorylated proteins came out in the void volume where there is no RNA polymerase activity, a great portion of the radioactivity was found in the regions where RNA polymerases I and II are eluted (fig. 12). Most interesting was the finding that the labeling of the void volume proteins by [γ-^{32}P] ATP is enhanced by cyclic AMP in the classic fashion, but the labeling of the protein in the regions of RNA polymerases I and II is inhibited by cyclic AMP (fig. 13). These results point to the possibility that the enhancement of RNA synthesis by cyclic AMP may involve a decrease in the phosphorylation of a protein component which is closely associated with or part of this enzyme.

CONCLUSION

A number of properties of the phosphorylated non-histone proteins have been described in this paper which are compatable with the idea that the phosphorylation of these proteins is involved in gene control mechanisms. Although none of these lines of evidence is conclusive in itself, the total picture which emerges from all these independent lines of approach is strongly sug-

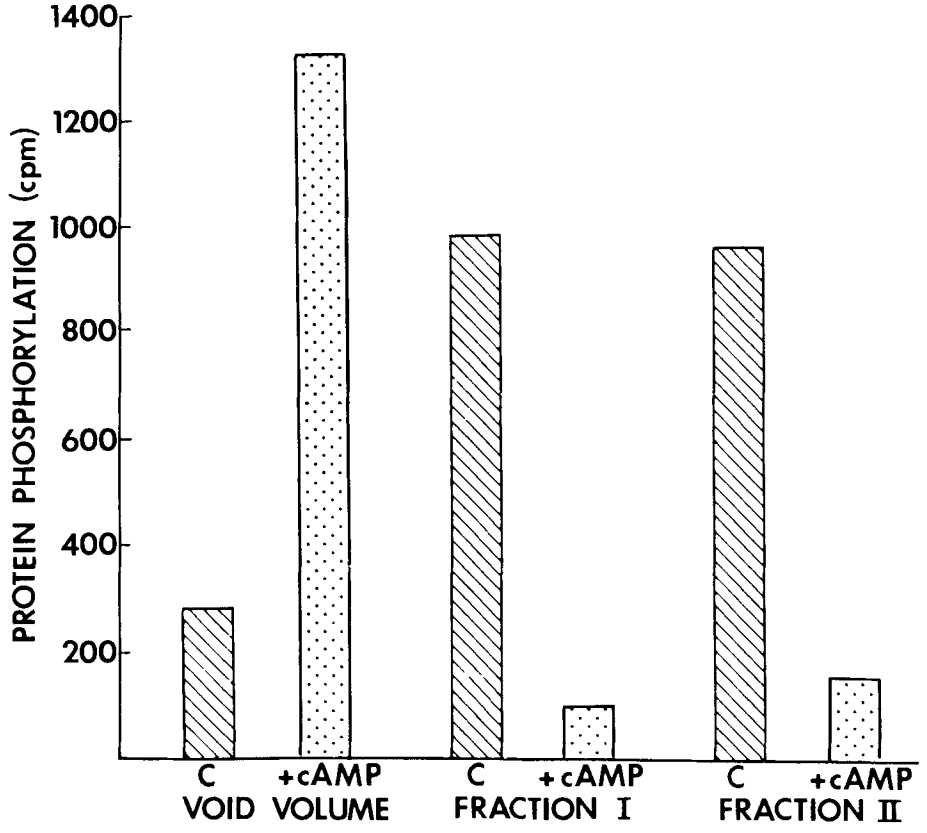

Fig. 13 Effects of cyclic AMP on protein kinase activities associated with total rat liver RNA polymerase. RNA polymerase was fractionated on a column of DEAE-Sephadex and the void volume, RNA polymerase I, and RNA polymerase II collected. Endogeneous protein phosphorylation was measured as described in the legend to figure 10. Note that cyclic AMP (10^{-6} M) stimulates protein phosphorylation in the void volume, but inhibits it in the regions of RNA polymerase I and II.

gestive of some role of these proteins in genetic regulation. However, it also seems likely that no single model is adequate for explaining all the properties of these proteins. Indeed, in view of the large quantity of phosphorylated non-histone proteins present in chromatin, their marked heterogeneity, and the large differences in the relative amounts of the various components, it appears much more reasonable to conclude that this is a mixture of proteins which play a variety of different structural, enzymatic, and regulatory roles. As a result of this complexity, the purification and characterization of individual phosphoprotein components will be required before the exact functions of individual components can be determined, and this task promises to be one of the critical challenges of the future.

LITERATURE CITED

Ahmed, K. 1971 Studies on nuclear phosphoproteins of rat ventral prostate: incorporation of ^{32}P from [γ-^{32}P] ATP. Biochim. Biophys. Acta, 243: 38–48.

Ahmed, K., and H. Ishida 1971 Effect of testosterone on nuclear phosphoproteins of rat ventral prostate. Mol. Pharmacol., 7: 323–327.

Allfrey, V. G., E. M. Johnson, J. Karn and G. Vidali 1973 Phosphorylation of nuclear proteins at times of gene activation. In: Protein Phosphorylation in Control Mechanisms. F. Huijing and E. Y. C. Lee, eds. Academic Press, New York, pp. 217–249.

Blankenship, J., and E. Bresnick 1974 Effects of phenobarbital on the phosphoryltion of acidic nuclear proteins of rat liver. Biochim. Biophys. Acta, 340: 218–227.

Bottoms, G. D., and R. A. Jungmann 1973 Effect of corticosterone on phosphorylation of rat liver nuclear proteins *in vitro*. Proc. Soc. Exp. Biol. Med., *144*: 83–88.

Byvoet, P. 1971 Uptake of radioactive phosphate into rat liver lysine-rich histones and phosphoproteins. Res. Commun. Chem. Pathol. Pharmacol., 2: 78–86.

Chiu, J.-F., C. Craddock, S. Getz and L. S. Hnilica 1973 Nonhistone chromatin protein phosphorylation during azo-dye carcinogenesis. FEBS Lett., 33: 247–250.

Gershey, E. L., and L. J. Kleinsmith 1969a Phosphoproteins from calf-thymus nuclei: studies on the method of isolation. Biochim. Bioph. Acta, *194*: 331–334.

——— 1969b Phosphorylation of nuclear proteins in avian erythrocytes. Biochim. Biophys. Acta, *194*: 519–525.

Gilbert, W., and B. Mueller-Hill 1967 The lac operator is DNA. Proc. Nat. Acad. Sci. U.S.A., 58: 2415–2421.

Hagen, D. C., and B. Magasanik 1973 Isolation of the self-regulated repressor protein of the *Hut* operons of *Salmonella typhimurium*. Proc. Nat. Acad. Sci. U.S.A., 70: 808–812.

Hnilica, L. S. 1972 The Structure and Biological Functions of Histones. CRC Press, Cleveland.

Ishida, H., and K. Ahmed 1973 Studies on phosphoproteins of submandibular gland nuclei isolated from isoproterenol-treated rats. Exp. Cell Res., 78: 31–40.

——— 1974 Studies on chromatin-associated protein phosphokinase of submandibular gland from isoproterenol-treated rats. Exp. Cell Res., 84: 127–136.

Johnson, E. M., and V. G. Allfrey 1972 Differential effects of cyclic adenosine-3′,5′-monophosphate on phosphorylation of rat liver nuclear acidic proteins. Arch. Biochem. Biophys., *152*: 786–794.

Jungmann, R. A., and J. S. Schweppe 1972 Mechanism of action of gonadotropin. I. Evidence of gonadotropin-induced modifications of ovarian nuclear basic and acidic protein biosynthesis, phosphorylation, and acetylation. J. Biol. Chem., 247: 5535–5542.

Kadohama, N., and R. W. Turkington 1973 Altered populations of acidic chromatin proteins in breast cancer cells. Cancer Res., 33: 1194–1201.

Kamiyama, M., and B. Dastugue 1971 Rat liver non-histone proteins: correlation between protein kinase activity and activation of RNA synthesis. Biochem. Biophys. Res. Commun., *44*: 29–36.

Kamiyama, M., B. Dastugue and J. Kruh 1971 Action of phosphoproteins and protein kinase from rat liver chromatin on RNA synthesis. Biochem. Biophys. Res. Commun., 44: 1345–1350.

Kamiyama, M., B. Dastague, N. Defer and J. Kruh 1972 Liver chromatin non-histone proteins. Parial fractionation and mechanism of action on RNA synthesis. Biochim. Biophys. Acta, 277: 576–583.

Kaplowitz, P. B., R. D. Platz and L. J. Kleinsmith 1971 Nuclear phosphoproteins. III. Increase in phosphorylation during histone-phosphoprotein interaction. Biochim. Biophys. Acta, 229: 739–748.

Karn, J., E. M. Johnson, G. Vidali and V. G. Allfrey 1974 Differential phosphorylation and turnover of nuclear acidic proteins during the cell cycle of synchronized HeLa cells. J. Biol. Chem., 249: 667–677.

Kish, V. M., and L. J. Kleinsmith 1974 Nuclear protein kinases. Evidence for their heterogeneity, tissue specificity, substrate specificities, and differential responses to cyclic adenosine 3′,5′-monophosphate. J. Biol. Chem., 249: 750–760.

Kleinsmith, L. J. 1973 Specific binding of phosphorylated non-histone chromatin proteins to deoxyribonucleic acid. J. Biol. Chem., 248: 5648–5653.

Kleinsmith, L. J., and V. G. Allfrey 1969a Nuclear phosphoproteins. I. Isolation and characterization of a phosphoprotein fraction from calf thymus nuclei. Biochim. Biophys. Acta, *175*: 123–135.

——— 1969b Nuclear phosphoproteins. II. Metabolism of exogenous phosphoprotein by intact nuclei. Biochim. Biophys. Acta, *175*: 136–141.

Kleinsmith, L. J., V. G. Allfrey and A. E. Mirsky 1966a Phosphoprotein metabolism in isolated lymphocyte nuclei. Proc. Nat. Acad. Sci. U.S.A., 55: 1182–1189.

——— 1966b Phosphorylation of nuclear protein early in the course of gene activation in lymphocytes. Science, *154*: 780–781.

Kostraba, N. C., and T. Y. Wang 1972a Differential activation of transcription of chromatin by non-histone fractions. Biochim. Biophys. Acta, 262: 169–180.

——— 1972b Transcriptional transformation of Walker tumor chromatin by nonhistone proteins. Cancer Res., 32: 2348–2352.

Krebs, E. G. 1972 Protein kinases. Curr. Topics Cell Regul., 5: 99–133.

Langan, T. A. 1967 A phosphoprotein preparation from liver nuclei and its effect on the inhibition of RNA synthesis by histones. In: Regulation of Nucleic Acid and Protein Biosynthesis. V. V. Koningsberger and L. Bosch, eds. Elsevier, Amsterdam, pp. 233–242.

——— 1969 Phosphorylation of liver histone following the administration of glucagon and insulin. Proc. Nat. Acad. Sci. U.S.A., 64: 1276–1283.

LeStourgeon, W. M., and H. P. Rusch . 1971 Nuclear acidic protein changes during differentiation in *Physarum polycephalum*. Science, *174*: 1233–1236.

Liew, C. C., D. Suria and A. G. Gornall 1973 Effects of aldosterone on acetylation and phosphorylation of chromosomal proteins. Endocrinology, 93: 1025–1034.

Louie, A. J., and G. H. Dixon 1973 Kinetics of phosphorylation and dephosphorylation of testis histones and their possible role in determining chromosomal structure. Nature New Biol., 243: 164–168.

Nakanishi, S., S. Adhya, M. E. Gottesman and I. Pastan 1973 *In vitro* repression of the transcription of *gal* operon by purified *gal* repressor. Proc. Nat. Acad. Sci. U.S.A., 70: 334–338.

Platz, R. D., and L. S. Hnilica 1973 Phosphorylation of nonhistone chromatin proteins during sea urchin development. Biochem. Biophys. Res. Commun., 54: 222–227.

Platz, R. D., V. M. Kish and L. J. Kleinsmith 1970 Tissue specificity of non-histone chromatin phosphoprotein. FEBS Lett., 12: 38–40.

Platz, R. D., G. S. Stein and L. J. Kleinsmith 1973 Changes in the phosphorylation of non-histone chromatin proteins during the cell cycle of HeLa S_3 cells. Biochem. Biophys. Res. Commun., 51: 735–740.

Ptashne, M. 1967 Specific binding of the λ phage repressor to λ DNA. Nature, 214: 232–234.

Riches, P. G., K. R. Harrap, S. M. Sellwood, D. Rickwood and A. J. MacGillivray 1973 Phosphorylation of nuclear proteins in rodent tissues. Biochem. Soc. Trans., 1: 70–72.

Rickwood, D., G. Threlfall, A. J. MacGillivray and J. Paul 1972 Studies on the phosphorylation of chromatin non-histone proteins and their effect on deoxyribonucleic acid transcription. Biochem. J., 129: 50P–51P.

Rickwood, D., P. G. Riches and A. J. MacGillivray 1973 Studies of the *in vitro* phosphorylation of chromatin non-histone proteins in isolated nuclei. Biochim. Biophys. Acta, 299: 162–171.

Riggs, A. D., G. Reiness and G. Zubay 1971 Purification and DNA-binding properties of the catabolite gene activation protein. Proc. Nat. Acad. Sci. U.S.A., 68: 1222–1225.

Roeder, R. G., and W. J. Rutter 1970 Specific nucleolar and nucleo-plasmic RNA polymerases. Proc. Nat. Acad. Sci. U.S.A., 65: 675–682.

Schauder, P., B. J. Starman and R. H. Williams 1974 Effect of testosterone on phenol-soluble nuclear acidic proteins of rat ventral prostate. Proc. Soc. Exp. Biol. Med., 145: 331–333.

Segal, H. L. 1973 Enzymatic interconversion of active and inactive forms of enzymes. Science, 180: 25–32.

Shea, M., and L. J. Kleinsmith 1973 Template-specific stimulation of RNA synthesis by phosphorylated non-histone chromatin proteins. Biochem. Biophys. Res. Commun., 50: 473–477.

Shimizu, Y., N. Shimizu and M. Hayashi 1973 *In vitro* repression of the tryptophan operon by *trp* repressor. Proc. Nat. Acad. Sci. U.S.A., 70: 1990–1994.

Stein, G. S., T. C. Spelsberg and L. J. Kleinsmith 1974 Nonhistone chromosomal proteins and gene regulation. Science, 183: 817–824.

Teng, C. S., C. T. Teng and V. G. Allfrey 1971 Studies of nuclear acidic proteins. Evidence for their phosphorylation, tissue specificity, selective binding to deoxyribonucleic acid, and stimulatory effects on transcription. J. Biol. Chem., 246: 3597–3609.

Turkington, R. W., and M. Riddle 1969 Hormone-dependent phosphorylation of nuclear proteins during mammary gland differentiation *in vitro*. J. Biol. Chem., 244: 6040–6046.