Pineal Protein Kinase: Effect of Enzymic Phosphorylation on Actinomycin D Binding by, and Template Activity of, Chromatin

(calf thymus/histones/cyclic AMP/bovine kinase/DNA)

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ABSTRACT The ability of protein kinase from bovine-pineal gland to phosphorylate calf-thymus chromatin and thereby to alter the association between chromatin DNA and histones was investigated. Phosphorylation of calf-thymus chromatin by pineal protein kinase results in an apparent decreased binding between the histones and DNA in chromatin, as indicated by (i) an increase in actinomycin D-binding sites after phosphorylation and (ii) an increase in the template capacity of the calf-thymus chromatin after phosphorylation. F₁ histone and F₃ histone are the major histone classes in the chromatin that are phosphorylated by the protein kinase. These results support the hypothesis that pineal protein kinase may function at the transcriptional level.

Melatonin levels and synthesis in mammalian-pineal glands exhibit a pronounced diurnal rhythm (1). This rhythm, controlled by sympathetic nerves arising from the superior cervical ganglion, is the result of dramatic changes in serotonin *N*-acetyltransferase (2-4). The events that translate neuronal activity into enzyme changes are not known, but it appears that norepinephrine released from the nerves activates adenylate cyclase, and that cAMP may be a mediator in the sequence of events leading to increased enzyme activity. The work of Berg and Klein (5) shows that treatment of the gland in organ culture with either cycloheximide or actinomycin D prevents both the subsequent norepinephrine-induced and dibutyryl-cAMP-induced stimulation of melatonin production and the associated increases in the level of the enzyme serotonin *N*-acetyltransferase. These results imply that, in the pineal gland, cAMP may exert its action at the transcriptional level.

The means by which cAMP might affect the transcriptional process in the pineal gland remain unknown. Recently, however, we found a highly active cAMP-dependent protein kinase in bovine-pineal glands (6). Arginine-rich histone and lysine-rich histone serve as excellent substrates for the enzyme. Therefore, we speculated that cAMP may influence the levels of the various pineal enzymes through its effect on the pineal protein kinase. Stimulation of the protein kinase by high concentrations of cAMP could result in increased phosphorylation of certain regions of the chromatin, resulting in relaxation of the tight association between DNA and histone and, thus, increased transcription of various portions of the DNA. In order to test this hypothesis, the ability of the pineal cAMP-dependent protein kinase to phosphorylate chromatin and thereby to alter the association between its DNA and histones was determined. Because of the great difficulty involved in preparing large amounts of

pineal chromatin, and because it is widely held that histones and their binding to chromatin do not differ much from one tissue to the next, calf-thymus chromatin was used.

EXPERIMENTAL PROCEDURE

The cAMP-dependent protein kinase used in these studies was bovine; it was about 95% pure (6). Calf-thymus chromatin was prepared according to the method of Clark and Felsenfeld (7). [γ -³²P]ATP and [³H]UTP were obtained from New England Nuclear Corp. [³H]Actinomycin D was purchased from Schwarz-Mann and unlabeled actinomycin D was obtained from Calbiochem. *Escherichia coli* B RNA polymerase was prepared from mid-log-phase cells (Miles Laboratories) by the method of Bonner *et al.* (8), and was assayed by the method of Berg, Barrett, and Chamberlain (9).

Incorporation of ³²P into Protein was determined by the method of Kabat (10).

Protein and DNA Were Determined by the methods of Lowry *et al.* (11) and Dische (12), respectively.

Binding of [³H]Actinomycin D to Phosphorylated Chromatin. Calf-thymus chromatin equivalent to 7.3 μ g of DNA was phosphorylated for 10 min at 37° in a 200- μ l reaction mixture containing 10 mM Tris·HCl (pH 8.0), 1 mM MgCl₂, 2.5 μ M ATP, and 7 μ g of pineal protein kinase. Phosphorylation was conducted in the presence and absence of 5 μ M cAMP. Immediately after phosphorylation of the chromatin, the reaction tubes were placed in an ice bath and 200 μ l of 10 mM Tris·HCl (pH 8.0) was added to each reaction tube, followed by the addition of 4.12 nmol of [³H]actinomycin D (1 μ Ci) in 100 μ l of 10 mM Tris·HCl (pH 8.0). Actinomycin D binding to the chromatin was then determined (13).

Determination of Template Capacity of Phosphorylated Calf-Thymus Chromatin. Calf-thymus chromatin equivalent to 7.3 μ g of DNA was phosphorylated for 10 min at 37° in a 50- μ l reaction mixture containing 80 mM Tris·HCl (pH 8.0), 4 mM MgCl₂, 10 μ M ATP, and 7 μ g of pineal protein kinase. The phosphorylation was conducted in the presence and absence of 5 μ M cAMP. Immediately after phosphorylation of the chromatin, 0.1 μ mol each of ATP, CTP, GTP, and [³H]UTP (2.5 μ Ci) were added, followed by 6-8 units of *E. coli* RNA polymerase. Sufficient *E. coli* RNA polymerase was added so that the calf-thymus chromatin was limiting.

TABLE 1. *Phosphorylation of calf-thymus chromatin by the cAMP-dependent pineal protein kinase*

Additions	pmol of ^{32}P incorporated per 10 μg of DNA*
None	3.1 $\pm$ 0.2
5 μM cAMP	23.5 $\pm$ 2.4

Calf-thymus chromatin equivalent to 7.3 μg of DNA was phosphorylated for 10 min at 37° in a 200- μl reaction mixture containing 10 mM Tris·HCl (pH 8.0), 1 mM MgCl_2 , 2.5 μM [γ - ^{32}P]ATP, and 7 μg of pineal protein kinase.

* Values are the average and standard deviation from four experiments.

Transcription was allowed to proceed for 10 min at 37°, and the RNA synthesized was determined (9).

Preparation and Electrophoresis of [^{32}P]Histones. Calf-thymus chromatin was phosphorylated as described above, but with [γ - ^{32}P]ATP as the phosphate donor. After phosphorylation of the chromatin, the histones were extracted with 0.2 M H_2SO_4 and purified as described by Benjamin (14). The ^{32}P -labeled histones were electrophoresed on 7.5-cm 15% polyacrylamide gels (15).

RESULTS

Phosphorylation of Calf-Thymus Chromatin by cAMP-Dependent Protein Kinase from Pineal Glands. The ability of the cAMP-dependent protein kinase to phosphorylate calf-thymus chromatin is demonstrated in Table 1. The addition of 5 μM cAMP results in an about 7-fold stimulation of phosphorylation of calf-thymus chromatin.

Effect of Phosphorylation of Calf-Thymus Chromatin on Actinomycin D Binding. Actinomycin D binding to chromatin, measured as described by Beato, Seifart, and Sekeris (13), was used to determine the effect of phosphorylation on DNA-histone interactions. As indicated by Clark and Felsenfeld (7), about half of the DNA in chromatin is exposed and is thus readily susceptible to actinomycin D binding. The exposure of new genes on the DNA by specific interaction of an agent with its chromatin receptor has been associated with an about 15% increase in actinomycin D binding, as

TABLE 2. *The effect of phosphorylation of calf-thymus chromatin on actinomycin D binding*

Additions	pmol of [^3H]Actinomycin D bound per μg of DNA*
None	9.0 (8.0–9.9)
cAMP	9.3 (8.7–9.8)
ATP	9.3 (9.1–10.1)
ATP + cAMP	9.6 (9.2–10.0)
ATP + protein kinase	12.2 (11.6–12.6)
ATP + protein kinase + cAMP	12.7 (12.2–13.4)

Calf-thymus chromatin equivalent to 7.3 μg of DNA was phosphorylated, and actinomycin D binding was determined.

* Each value represents the mean of four experiments with duplicate determinations. The range of values is given in parentheses.

demonstrated (13) in the binding of cortisol to its chromatin receptor. As shown in Table 2, phosphorylation of calf-thymus chromatin by the protein kinase resulted in a significant increase in actinomycin D binding to the calf-thymus chromatin. This increase in binding was indicative of a relaxation in the association between DNA and histone. The addition of cAMP to the protein kinase-chromatin incubation mixture did not result in a further increase in actinomycin D binding, as would be expected from the increased phosphorylation of the chromatin in the presence of cAMP.

The Effect of Phosphorylation of Calf-Thymus Chromatin on Its Template Capacity. As a second indicator of the effect of chromatin phosphorylation on DNA-histone association, the template capacity of calf-thymus chromatin for *E. coli* RNA polymerase was investigated. As shown in Table 3, phosphorylation of calf-thymus chromatin by protein kinase resulted in an about 25% increase in template capacity, indicating relaxation of DNA-histone association and exposure of new regions of DNA to the RNA polymerase. There appeared, however, to be no stimulation of this increased template effect by cAMP.

Identification of the Phosphorylated Histone Classes. Various classes of histones have been reported to be phosphorylated *in vivo* (16–22). Therefore, it was of interest to determine the histone classes phosphorylated by pineal protein kinase. Chromatin was phosphorylated by pineal protein kinase with [γ - ^{32}P]ATP as the phosphate donor. The labeled histones were extracted and purified as described in *Methods*. Although significantly greater phosphorylation of the chromatin occurs in the presence of cAMP (Fig. 1), the major histone classes phosphorylated by protein kinase both in the presence and absence of 5 μM cAMP are the F_1 and F_2 histones. Phosphorylation of F_{2b} histones is also observed in either the presence or absence of cAMP.

DISCUSSION

These experiments clearly indicate that phosphorylation of chromatin by pineal protein kinase can alter the DNA-histone interaction in chromatin, resulting in apparent decreased binding between the histones and DNA. This diminished binding and simultaneous exposure of new regions of the DNA is indicated by (i) the increase in actinomycin

TABLE 3. *The effect of phosphorylation of calf-thymus chromatin on template capacity*

Additions	pmol of [^3H]UMP incorporated per μg of DNA*
None	18.1 (16.5–19.8)
cAMP	17.5 (16.3–17.5)
ATP	20.7 (19.7–21.3)
ATP + cAMP	20.0 (19.2–20.4)
ATP + protein kinase	24.4 (23.1–25.8)
ATP + protein kinase + cAMP	23.3 (22.6–24.6)

Calf-thymus chromatin equivalent to 7.3 μg of DNA was phosphorylated and the template capacity was determined.

* Each value represents the mean of three experiments with duplicate determinations. The range of values is given in parentheses.

D-binding sites after phosphorylation, and (ii) the increase in the template capacity of calf-thymus chromatin after phosphorylation. Thus, these experiments indicate a possible regulatory role for protein kinase in the transcriptional process, although the possibility of the enzyme functioning in another capacity is by no means eliminated.

Kamiyama and Dastugue (23) initially reported a direct correlation between the chromatin-associated protein kinase activity and the stimulation of chromatin template capacity. These investigators later concluded that the suppression by nonhistone protein of the histone inhibition of RNA synthesis on a DNA template is not directly related to the protein kinase activity associated with chromatin, but rather to the nonhistone phosphoproteins associated with chromatin (24). The findings in the present report demonstrate that phosphorylation of calf-thymus chromatin by purified pineal protein kinase results in the release of template restriction. It is possible that the pineal protein kinase is phosphorylating both histone and nonhistone proteins and that these resultant phosphoproteins are acting as polyanions that release the histone inhibition of template activity. This conclusion is partially substantiated by the work of Brown and Coffey (25), who demonstrated that specific polyanions can cause a release of nuclear template restrictions.

F_1 and F_3 are the major histone classes phosphorylated by pineal protein kinase when chromatin is used as the substrate. Stocken and Ord (26) have observed that phosphorylation of histone F_1 occurs at the time of new DNA synthesis. Balhorn *et al.* (27, 28) have also reported that histones F_1 and F_{2a2} are phosphorylated only at a time when new DNA is synthesized, and these investigators have concluded that F_1 phosphorylation is an obligatory step in DNA synthesis or cell replication rather than in specific gene activation. Langan (17), however, has found that the administration of glucagon to rats results in a marked increase in the phosphorylation of a specific serine residue in the F_1 histone of liver. This F_1 histone phosphorylation, which Langan feels is the mechanism by which glucagon induces RNA synthesis in its target tissues, appears to be catalyzed by a cAMP-dependent histone kinase in liver. Thus, the increase in template capacity that results from chromatin phosphorylation by pineal protein kinase may be due to specific phosphorylation of the F_1 histone, although this still remains a point of conjecture.

The failure of cAMP to enhance the ability of the protein kinase to alter the DNA-histone association in chromatin by means of phosphorylation of the histone is difficult to explain. Although marked stimulation of chromatin phosphorylation by the pineal protein kinase occurred when cAMP was added (Table 1), no increase in either the number of actinomycin D-binding sites or the template capacity of the chromatin occurred beyond that produced by the pineal protein kinase alone (Tables 2 and 3). Several explanations can be offered for this lack of stimulation by cAMP. First, minimal phosphorylation of chromatin may be sufficient to obtain maximal dissociation of histone-chromatin. Thus, under the *in vitro* experimental conditions, sufficient phosphorylation of chromatin may be obtained both in the presence and absence of cAMP. Examination of this possibility proved to be difficult. The use of lower amounts of protein kinase gave equivocal results, probably because the maximal stimulation of actinomycin D binding and template activity was only about 20–30% and intermediate levels of

stimulation are difficult to quantitate. Second, Greengard and his colleagues (29) demonstrated that the addition of histones to cAMP-dependent protein kinase can partially dissociate

FIG. 1. Electrophoresis of ^{32}P -labeled histones. Calf-thymus chromatin equivalent to 150 μg of DNA was phosphorylated in 1-ml reaction mixtures consisting of 10 mM Tris-HCl (pH 8.0), 1.0 mM MgCl_2 , 2.5 μM [$\gamma\text{-}^{32}\text{P}$]ATP, and 35 μg of pineal protein kinase. The phosphorylation was conducted in the presence (A) or absence (B) of 5 μM cAMP. Identical reaction tubes were then pooled and the ^{32}P -labeled histones were extracted (15).

the protein kinase into its inhibitory and catalytic subunits. Therefore, the lack of stimulation by cAMP may be due to the dissociation of the protein kinase into its cAMP-independent form by those regions of the chromatin that control, by means of their phosphorylation and dephosphorylation, the association between DNA and histone. This possibility, however, should have also been reflected in the ^{32}P incorporation and was not. Finally, it is possible that phosphorylation of some nonhistone protein is responsible for the changes in chromatin properties and that this phosphorylation is cAMP-independent and is not reflected in the overall gross phosphorylation.

In conclusion, while the current experiments fail to provide evidence for a direct role of cyclic AMP in the transcription regulatory process, they do suggest that the cAMP-activated protein kinase may indeed mediate the differential transcription of genes through phosphorylation of specific histone subclasses.

1. Wurtman, R. J., Axelrod, J. & Phillips, L. S. (1963) *Science* **142**, 1071-1073.
2. Axelrod, J., Shein, H. M. & Wurtman, R. J. (1969) *Proc. Nat. Acad. Sci. USA* **62**, 544-549.
3. Klein, D. C. & Rowe, J. (1970) *Mol. Pharmacol.* **6**, 164-171.
4. Klein, D. C., Berg, G. & Weller, J. (1970) *Science* **168**, 979-980.
5. Berg, G. R. & Klein, D. C. (1971) *Endocrinology* **89**, 453-464.
6. Fontana, J. A. & Lovenberg, W. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 2787-2790.
7. Clark, R. J. & Felsenfeld, G. (1971) *Nature* **229**, 101-106.
8. Bonner, J., Chalkley, G. R., Dahmus, M., Fambrough, D., Fujimura, F., Huang, R. C., Huberman, J., Jensen, R., Marushige, K., Ohlenbusch, H., Olivera, B. & Widholm, J. (1968) in *Methods in Enzymology*, eds. Grossman, L. & Mol-dave, K. (Academic Press, New York), Vol. XII, Part B, pp. 3-65.
9. Berg, D., Barrett, K. & Chamberlain, M. (1971) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. XXI, pp. 506-519.
10. Kabat, D. (1971) *Biochemistry* **10**, 197-203.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
12. Dische, Z. (1930) *Mikrochemie* **8**, 4-32.
13. Beato, M., Seifart, K. H. & Sekeris, C. E. (1970) *Arch. Biochem. Biophys.* **138**, 272-284.
14. Benjamin, W. B. (1971) *Nature* **234**, 18-20.
15. Panyim, S. & Chalkley, R. (1969) *Arch. Biochem. Biophys.* **130**, 337-346.
16. Langan, T. A. (1969) *J. Biol. Chem.* **244**, 5763-5765.
17. Langan, T. A. (1969) *Proc. Nat. Acad. Sci. USA* **64**, 1276-1283.
18. Murray, K. & Milstein, C. (1967) *Biochem. J.* **105**, 491-495.
19. Sung, M. & Dixon, G. H. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 1616-1623.
20. Hayashi, T. & Iwaki, K. (1970) *J. Biochem. (Tokyo)* **68**, 415-417.
21. Kleinsmith, L. J., Allfrey, V. G. & Mirsky, A. E. (1966) *Proc. Nat. Acad. Sci. USA* **55**, 1182-1189.
22. Ord, M. G. & Stocken, L. A. (1969) *Biochem. J.* **112**, 81-89.
23. Kamiyama, M. & Dastugue, B. (1971) *Biochem. Biophys. Res. Commun.* **44**, 29-36.
24. Kamiyama, M., Dastugue, B. & Kruh, J. (1971) *Biochem. Biophys. Res. Commun.* **44**, 1345-1350.
25. Brown, D. G. & Coffey, D. S. (1971) *Biochem. Biophys. Res. Commun.* **42**, 326-333.
26. Stocken, L. A. & Ord, M. G. (1969) *Biochem. J.* **114**, 51P.
27. Balhorn, R., Chalkley, R. & Granner, D. (1972) *Biochemistry* **11**, 1094-1097.
28. Balhorn, R., Bordwell, J., Sellers, L., Granner, D. & Chalkley, R. (1972) *Biochem. Biophys. Res. Commun.* **46**, 1326-1333.
29. Miyamoto, E., Petzold, G. L., Harris, J. S. & Greengard, P. (1971) *Biochem. Biophys. Res. Commun.* **44**, 305-312.