CELL-CYCLE-DEPENDENT PHOSPHORYLATION OF SERINE AND THREONINE IN CHINESE HAMSTER CELL F1 HISTONES

Philip Hohmann, Robert A. Tobey, and Lawrence R. Gurley

Cellular and Molecular Radiobiology Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, New Mexico 87544

Received December 26,1974

SUMMARY: The phosphorylation of serine and threonine was studied in chromatographically purified f1 histones from CHO cells. Two unphosphorylated parental f1 histone fractions were observed in G_1-arrested cells: a major (87%) and a minor (13%) fraction. An additional f1 fraction, a phosphorylated form of the major parent, was chromatographically resolved from exponentially growing cultures. This form of the f1 histone incorporated [^{32}P]phosphate into serine at a rate approximately 5 times greater than into threonine. In mitotic cells, virtually all of the f1 histone chromatographed as a distinct, highly phosphorylated form. This form of the f1 histone incorporated [^{32}P]phosphate more rapidly into threonine than into serine. The greatly enhanced rate of threonine phosphorylation in the f1 histones of mitotic CHO cells was further confirmed by the isolation of a phosphothreonine-containing tryptic peptide virtually absent in exponentially growing cells.

INTRODUCTION

Previous studies concerning the phosphorylation of the lysine-rich (f1) histones of the Chinese hamster cell line CHO have revealed a sequence of phosphorylation events which begins in late G_1, continues at an increased rate throughout S phase, and culminates in a superphosphorylation event attendant with chromosome condensation (1-5). Other reports support the notion of distinct interphase and mitotic phosphorylation events occurring on f1 histones in several cell lines (6-8). Of particular interest to us was the report of Lake (7) who found different tryptic phosphopeptides when the f1 histones from interphase and mitotic Chinese hamster cells (line V79) were compared. These studies were performed before the recent report that both serine and threonine are phosphorylated in growing Ehrlich ascites cells and H-35 rat hepatoma cells (9). Therefore, we investigated the phosphorylation of serine and threonine in CHO cell f1 histones and studied the cell-cycle dependency of the two processes. Using chromatographic fractionation to purify individual f1 histone species, we observed a greatly enhanced rate of threonine phosphorylation in mitotic cells. In addition, we have isolated at least one strongly labeled phosphothreonine-containing tryptic peptide from the f1 histones of mitotic CHO cells.

METHODS

Growth of Cells

Cells were grown in F-10 medium as suspension or monolayer cultures, as described previously (2,3,10). Suspension cultures were synchronized by arresting the cells in early G_1 using isoleucine deprivation (11,12). Exponentially growing suspension cultures were used at a cell density of approximately 3 x 10^5/ml. Mitotic cells were selectively detached from monolayer cultures treated with Colcemid for 4 hr (3).

Labeling of f1 histones was accomplished in F-10 medium containing a normal concentration of phosphate. As previously described (2,3), f1 histone was labeled with [^{3}H]lysine (New England Nuclear, 20 Ci/mg) over several generations using 50 μCi/liter of culture. The f1 histones of G_1-arrested and exponentially growing cells were also labeled with [^{32}P]phosphate (New England Nuclear, carrier-free) over a 2-hr period using 50 mCi/liter of culture. After selective detachment of mitotic cells from monolayer cultures, these cells were suspended in Colcemid-containing medium to maintain them in metaphase and also labeled over a 2-hr period with [^{32}P]phosphate at a concentration of 50 mCi/liter.

Extraction and Chromatography of f1 Histones

The f1 histones were isolated either by direct extraction of frozen-thawed cells with 0.4 N H_2SO_4 and 5% trichloroacetic acid (13) or by the procedure of Johns (14) adapted to ^{4}CHO cells (15). Calf thymus f1 histone carrier was isolated by the procedure of deNooij and Westenbrink (16), and approximately 3 mg of carrier was added to the CHO f1 as an internal standard to which the radioactive fractions could be related after chromatographic fractionation.

Chromatography of the f1 histones was carried out on a 1 x 20-cm column of BioRex-70 resin using a gradient of 7% to 14% guanidinium chloride (Mann, ultra pure) (total volume = 150 ml) in 0.1 M sodium phosphate buffer, pH 6.8 (13). Calf thymus carrier protein in the chromatographic fractions was determined by absorbancy at 218 nm. Radioactivity incorporated into CHO f1 histone was determined in the chromatographic fractions using PCS liquid scintillation fluid (Amersham/Searle), the ^{3}H and ^{32}P being counted simultaneously by pulse-height analysis in a Packard Tri-Carb spectrometer.

Analysis of Phosphoserine, Phosphothreonine, and Tryptic Phosphopeptides

Phosphoserine and phosphothreonine were analyzed after partial acid hydrolysis of chromatographically purified f1 histone using 6 N HCl at 85° for 4 hr. We assumed that the relative rate of release of a given phosphoamino acid was the same during the hydrolysis of f1 histone from exponential or mitotic cells. While it has been reported that phosphoserine is destroyed more rapidly than phosphothreonine during acid hydrolysis (17), we assumed that changes in the *ratio* of phosphoserine-to-phosphothreonine released reflected a change in their relative content in the f1 histone rather than a selective effect on the destruction of either amino acid. Our conditions of hydrolysis also employ a lower temperature than reported (17). The final separation of phosphoserine from phosphothreonine was accomplished by high-voltage paper electrophoresis (Savant, flat plate) at pH 1.9. The plate was cooled to a constant temperature of 10°, and electrophoresis was carried out for 45 min at 3500 volts (78 volts/cm) on Schleicher and Schuell #589 green ribbon paper. The radioactive amino acids were located by autoradiography using Kodak Blue Brand X-ray film, while the standard phosphoamino acids (Mann) were located with ninhydrin spray (Mann, Spraytec). Areas corresponding to the position of the radioactive amino acid spots were cut from the paper and counted in PCS scintillation fluid.

Chromatographically purified f1 histone was digested with trypsin (Worthington,

TPCK-treated) in 0.1 M NH_4HCO_3, pH 7.9, for 1 hr at 37° (histone-to-trypsin = 50:1). Tryptic phosphopeptides were partially purified by two sequential high-voltage electrophoretic steps on paper (cf. 18). The first step, which immediately followed completion of the tryptic digestion, was carried out at pH 7.9 for 35 min and 3000 volts (68 volts/cm). The radioactive bands were located by autoradiography, cut from the paper, eluted with water, and subjected to a second electrophoretic fractionation on paper at pH 1.9 for 40 min at 3000 volts (68 volts/cm). After fractionation at pH 1.9, some radioactive bands were again eluted with water, and the presence of phosphoserine or phosphothreonine was determined as described above.

RESULTS

The number of f1 histone species resolved chromatographically from CHO

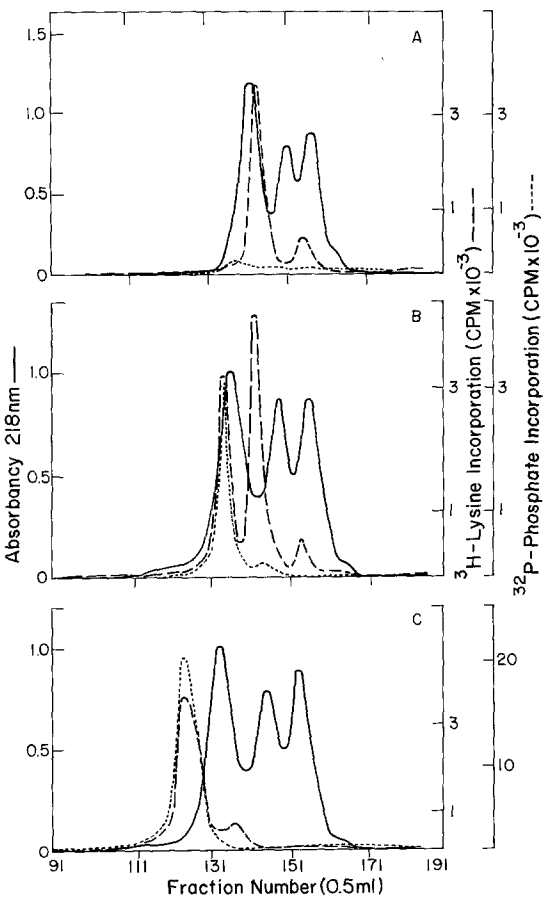
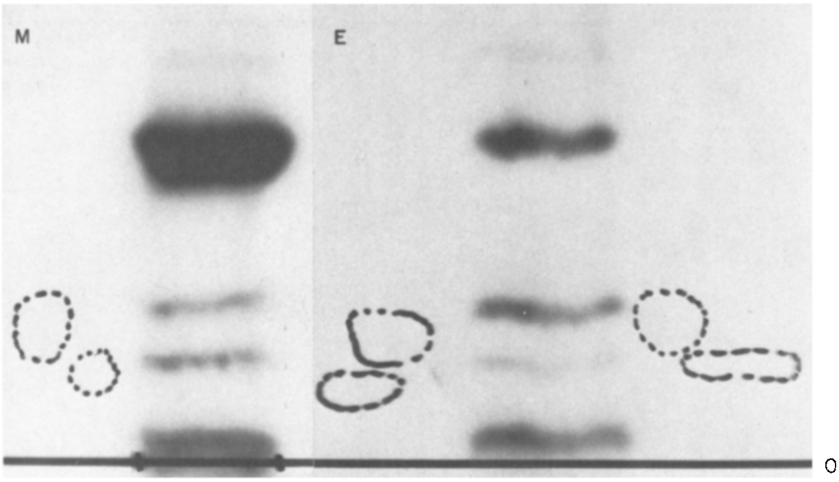


Fig. 1. Chromatography of f1 histones. Radioactive CHO cell f1 histones were chromatographed in the presence of calf thymus f1 carrier, as described in Methods: (———) absorbancy of calf thymus f1 carrier at 218 nm; (— —) incorporation of [^{3}H]lysine into CHO f1 histone; and (-----) incorporation of [^{32}P]phosphate into CHO f1 histone. The flow rate was 1 ml/hr, and fractions were collected at 30-min intervals. (A) CHO f1 histone from cells arrested in early G_1 by isoleucine deprivation; (B) CHO f1 histone from exponentially growing cells; and (C) CHO f1 histone from metaphase-arrested cells.



CPM / Amino Acid

	Mitotic	Exponential
Ser-P	703	2096
Thr-P	822	472

Fig. 2. Autoradiograph depicting the electrophoretic separation of [^{32}P]-labeled phosphoserine and phosphothreonine from CHO f1 histones: (E) exponential cultures; (M) mitotic cultures; and (O) origin, anode at top. The fastest migrating radioactive substance is inorganic [^{32}P]phosphate. Migration of the standards indicated by dotted circles on film (phosphoserine faster migrating, phosphothreonine slower migrating). Material at origin undefined (probably unhydrolyzed protein). The film was exposed for 5 days. Values at bottom represent the total cpm for the phosphoamino acids shown.

---

cells depends on the cell-cycle position. As shown in Fig. 1A, there are two unphosphorylated parental forms in G_1-arrested cells, and these elute in a characteristic position relative to the carrier. In exponentially growing cells (Fig. 1B), a third f1 fraction (a phosphorylated form of the major parent) elutes ahead of and in addition to the parental forms. In mitotic cells (see Fig. 1C), virtually all of the f1 histone elutes as a distinct phosphorylated species well ahead of the positions occupied by the parental and phosphorylated forms of Fig. 1B. Similar observations on the effect of phosphorylation on the chromatographic behavior of CHO f1 histones were recently made by Gurley *et al.* (5) using a modified system of chromatography. We have confirmed that the phosphorylated forms of the f1 histone seen in Figs. 1B and 1C are not degradation products and can be related to the major parent by alkaline phosphatase digestion and electrophoresis in long polyacrylamide gels (data not shown). The major parent electrophoreses as a single band on gels.

We exploited the ability of the chromatographic system to resolve the

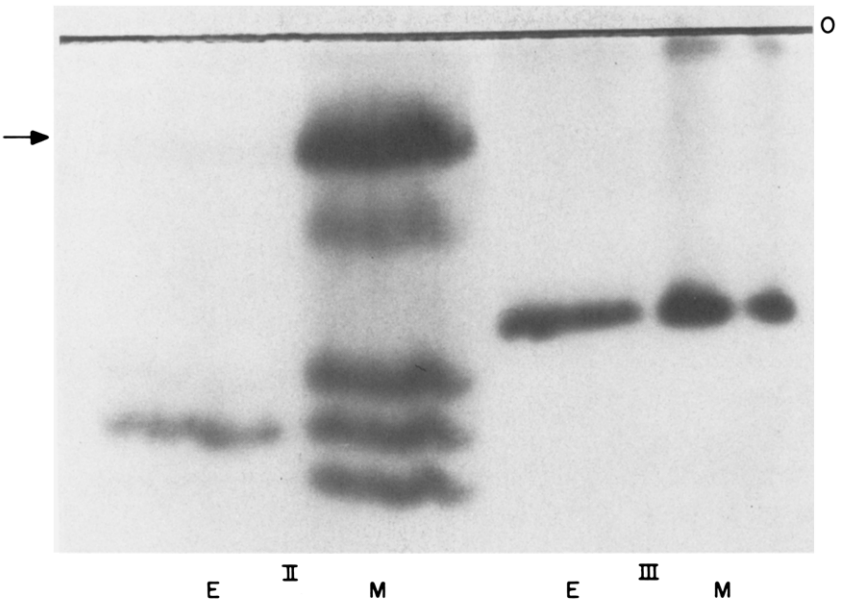


Fig. 3. Autoradiograph depicting the tryptic phosphopeptides resolved by high-voltage paper electrophoresis at pH 1.9 from the f1 histones of mitotic (M) and exponentially growing (E) CHO cells. The conditions of electrophoresis are described in Methods; (O) represents origin, anode at top. The tryptic digest had been subjected to a prior electrophoretic fractionation at pH 7.9, and the bands designated II and III were selected for fractionation at pH 1.9. Band III contains only phosphoserine in M and E cells. The arrow indicates the phosphothreonine-containing peptide found in band II of M cells. This peptide could not be studied in E cells due to its trace amount. The film was exposed for 7 days.

phosphorylated forms of CHO f1 histones to study the rate of serine and threonine phosphorylation in exponential and mitotic cultures. The conditions used to extract the f1 histones precluded any studies on the possible existence of acid-labile phosphate (19).

An autoradiograph depicting the separation of phosphoserine from phosphothreonine is shown in Fig. 2. The data in this figure also show the relative rate of phosphorylation of serine to threonine in 1 of 3 experiments utilizing exponentially growing cells (E) and 1 of 2 experiments utilizing mitotic cells (M). The data of Fig. 2 clearly demonstrate that the ratio of phosphothreonine-to-phosphoserine is greatly enhanced in mitotic cultures. Since the data in Fig. 2 were obtained from two separate experiments, the relative *quantities* of radiophosphate in the E and M cell preparations should not be compared. In the experiment depicted, the mitotic population was 74%, the remainder of the cells having escaped the Colcemid blockade into G_1, a time when f1 histone is rapidly

dephosphorylated (3,5). In a second experiment, the mitotic population was 90%, and the rate of threonine phosphorylation approached 2 times that of serine phosphorylation. In the three closely agreeing experiments utilizing exponential cultures, the rate of serine phosphorylation was approximately 5 times that of threonine phosphorylation. Thus, there is about a 10-fold increase in the relative rate of threonine phosphorylation in the f1 histones of mitotic CHO cells when a 2-hr labeling period was studied. A detailed study of the kinetics of phosphorylation of these two amino acids in relation to the complete cell cycle is presently in progress.

Further evidence to support the enhanced rate of threonine phosphorylation in the f1 histones of mitotic CHO cells was gained from a preliminary study of tryptic phosphopeptides. Since the rapid decay of ^{32}P limits the number of sequential analytical steps to which the f1 histones, phosphopeptides, and phosphoamino acids can be subjected, we chose an arbitrary group of peptides which could be easily resolved in two sequential electrophoretic steps (cf. 18). Sequential steps are more versatile than the standard two-dimensional fractionation techniques employed, since the various phosphopeptides are not always resolved equally well in a two-dimensional fractionation. The tryptic digests of the f1 histones of exponential and mitotic CHO cells were first subjected to electrophoretic fractionation at pH 7.9, as described in Methods. Of the several phosphopeptide bands resolved, two (designated II and III) were isolated and further fractionated by a second electrophoretic step at pH 1.9. These bands yielded the patterns shown in Fig. 3. Peptide band III appears as a single major phosphorylated band common to both exponential and mitotic cell f1 histone. We have positively identified phosphoserine in this band; phosphothreonine was not detected. Peptide band II is complex in mitotic cell f1 histone. One peptide band appears strongly in the f1 histone from exponential cells, but others are weak or absent. The strongly labeled band indicated by the arrow in mitotic cell f1 contains only phosphothreonine. This phosphorylated band is virtually undetectable in exponential cells and, therefore, could not be studied for its phosphoamino acid content. We have not as yet identified the phosphoamino acids in the other peptides of band II. We cannot claim that any of the bands shown in Fig. 3 are pure peptides, nor do we know the location of the serine or threonine residues in the primary structure of the f1 histone. Our preliminary evidence, however, does support the notion that the phosphorylation of at least one threonine residue is greatly enhanced in the f1 histone of mitotic CHO cells.

DISCUSSION

The present and other (5) studies show that the chromatographic behavior

of the f1 histones of CHO cells can be influenced by phosphorylation. Other studies have demonstrated that enzymatic phosphorylation of a number of f1 histones at one specific site [serine 37, site A (18)] does not influence chromatographic behavior (20), while enzymatic phosphorylation at another specific serine residue [serine 106, site B (18)] does (21). In view of that observation plus the results obtained in this report, we conclude that the specific site(s) phosphorylated (either enzymatically or *in vivo*) determine whether the chromatographic behavior of f1 histone is altered. Since we do not yet know the specific location of the serine and threonine residues phosphorylated in CHO cells, we cannot say whether these sites are related to sites A and B mentioned above.

Recent studies in this Laboratory have sought to establish the concept of a "chromosome cycle" (3); that is, a sequence of histone modifications which result in specific changes in chromatin structure as cells traverse their life cycle. Therefore, we were gratified that the present studies so clearly showed a difference in the rate of phosphorylation of serine and threonine in the f1 histone of mitotic and exponential cultures. This observation supports the contention that the modifications occurring on f1 histones are specific for certain parts of the cell cycle (cf. 7) since different amino acid residues are involved.

While many cells and tissues have four or five major f1 histone subfractions (20,22), CHO cells have but one major subfraction. This fact greatly simplifies the analysis of phosphopeptides. Thus, the CHO cell line appears to be an ideal system for studying the rate of phosphorylation, phosphate turnover, and phosphate content at specific sites in an f1 histone. In addition, the CHO cell line may be easily synchronized in early G_1, late G_1, and M (3-5,11,12). We, therefore, expect to be able to accurately relate the kinetics of phosphorylation of specific sites within the f1 histone to cell-cycle position. We are aware that the conclusions we reach in these studies using CHO cells may not apply exactly to other cell lines. The f1 histones are a species-specific group of proteins with different structures (20,22,23), and there is already one example where a specific phosphorylation site has not been conserved among the f1 histones (20). Therefore, it is possible that some of the sites we are studying in CHO cell f1 histone may not be the same in other cell lines. This could arise from species-specific changes in the structures of the f1 histones leading to different tryptic phosphopeptides, substitution of serine for threonine, or even species-specific phosphorylation sites. In fact, whether species-specific phosphorylation sites exist among f1 histones is a fundamental question, the answer to which will not only broaden our knowledge of the evolution and conservation of f1 histone structure and function but will also make the large number of reports concerning the true molecular function of f1 histone phosphorylation vastly more interpretable.

ACKNOWLEDGMENT

This work was performed under the auspices of the U. S. Atomic Energy Commission.

REFERENCES

1. Gurley, L. R., Walters, R. A., and Tobey, R. A. (1973) Arch. Biochem. 154, 212-218.
2. Gurley, L. R., Walters, R. A., and Tobey, R. A. (1973) Biochem. Biophys. Res Commun. 50, 744-750.
3. Gurley, L. R., Walters, R. A., and Tobey, R. A. (1974) J. Cell Biol. 60, 356-364.
4. Gurley, L. R., Walters, R. A., and Tobey, R. A. (1974) Arch. Biochem. Biophys. 164, 469-477.
5. Gurley, L. R., Walters, R. A., and Tobey, R. A. (1974) J. Biol. Chem., submitted.
6. Marks, D. B., Paik, W. K., and Borun, T. W. (1973) J. Biol. Chem. 248, 5660-5667.
7. Lake, R. S. (1973) J. Cell Biol. 58, 317-331.
8. Balhorn, R., Tanphaichitr, N., Chalkley, R., and Granner, D. (1973) Biochemistry 12, 5146-5150.
9. Langan, T. A., and Hohmann, P. (1974) Federation Proc. 33, 1597 abs.
10. Tobey, R. A., Petersen, D. F., Anderson, E. C., and Puck, T. T. (1966) Biophys. J. 6, 567-581.
11. Tobey, R. A., and Ley, K. D. (1971) Cancer Res. 31, 46-51.
12. Tobey, R. A. (1973) in "Methods in Cell Biology," Vol. 6, Academic Press, Inc., New York, pp. 67-112.
13. Hohmann, P., and Cole, R. D. (1971) J. Mol. Biol. 58, 533-540.
14. Johns, E. W. (1964) Biochem. J. 92, 55-59.
15. Gurley, L. R., and Hardin, J. M. (1968) Arch. Biochem. Biophys. 128, 285-292.
16. deNooij, E. H., and Westenbrink, H. G. (1962) Biochim. Biophys. Acta 62, 608-609.
17. Allerton, S. E., and Perlman, G. E. (1965) J. Biol. Chem. 240, 3892-3898.
18. Langan, T. A. (1971) Ann. N. Y. Acad. Sci. 185, 166-180.
19. Chen, C. C., Smith, P. L., Bruegger, B. B., Holtz, S. L., Halpern, R. M., and Smith, R. A. (1974) Biochemistry 13, 3785-3789.
20. Langan, T. A., Rall, S. C., and Cole, R. D. (1971) J. Biol. Chem. 246, 1942-1944.
21. Hohmann, P., and Langan, T. A., unpublished observations.
22. Bustin, M., and Cole, R. D. (1968) J. Biol. Chem. 243, 4500-4505.
23. Rall, S. C., and Cole, R. D. (1971) J. Biol. Chem. 246, 7175-7190.