Volume 5 Number 9 September 1978 Nucleic Acids Research

Dephosphorylation of histones H1 and H3 during the isolation of metaphase chromosomes

J.A.D'Anna, L.R.Gurley and L.L.Deaven

Cellular and Molecular Biology Group, Los Alamos Scientific Laboratory, University of California, Los Alamos, NM 87545, USA

Received 26 June 1978

ABSTRACT

Histones have been extracted from isolated metaphase chromosomes prepared by the method of Wray and Stubblefield [Exp. Cell Res. 59, 469-478 (1970)] and by a Nonidet P-40 detergent procedure based on the method of Wigler and Axel [Nucleic Acids Res. 3, 1463-1471 (1976)]. Analysis of the densitometer profiles of long polyacrylamide gels shows that the mitotic phosphorylations of histone H1 ($H1_M$) and histone H3 are extensively depleted during chromosome isolation. These data indicate that CHO metaphase chromosomes prepared by standard methodologies do not represent in vivo chromosomes with respect to their histone phosphorylations; therefore, current chemical and structural studies of isolated metaphase chromosomes may require further clarification.

INTRODUCTION

During the last two years, several laboratories have employed micrococcal nuclease (1-4) or micrococcal nuclease and electron microscopy (5) as probes of chromatin structure in isolated metaphase chromosomes. These studies indicate that interphase nucleosome structures are preserved in mitotic chromosomes and that the access of micrococcal nuclease to inter- or intranucleosomal cleavage sites in chromosomes is the same as in interphase chromatin. During the time of these studies, our Laboratory has been investigating H1 superphosphorylation (hereafter referred to as $H1_M$) and H3 phosphorylation during the cell cycle of Chinese hamster (line CHO) cells (6-8). These studies (8) have shown that (a) $H1_M$ and H3 phosphorylations are restricted to those stages of mitosis when chromosomes are maximally condensed (i.e., prophase, metaphase, and anaphase) and (b) $H1_M$ and H3 are dephosphorylated as chromosomes begin to unravel in telophase.

If H1 were superphosphorylated and H3 were phosphorylated in the chromosomes employed for micrococcal nuclease digestion studies (1-5), then those results would suggest that $H1_M$ and H3 phosphorylations did not affect the accessibility of inter- and intranucleosomal DNA to micrococcal nuclease

(1-5). However, the investigators were not concerned with histone phosphorylations at that time and did not consider it in their analyses. Previous studies from this Laboratory (6) have shown that histones H1 and H3 are rapidly dephosphorylated during chromatin isolation, even at 4°C, when sodium bisulfite is omitted during histone isolation (6). While chromosomes (1,9-11) are not isolated by the same procedures used for isolating chromatin (6,8), H1 and H3 nevertheless may be dephosphorylated during chromosome preparation, since sodium bisulfite is not included in chromosome isolation buffers (1,9-11). Because of the possible importance of histone phosphorylation to interpreting past and future studies of chromosome structure, we have quantified the extent of $H1_M$ and H3 phosphorylations in isolated metaphase chromosomes obtained from CHO cells. Chromosomes have been isolated by the method of Wray and Stubblefield (9) and by a NP-40 detergent procedure based on the method of Wigler and Axel (1). These or similar procedures had been used previously to isolate metaphase chromosomes from CHO (5) and from other mammalian cells (1,2) for micrococcal nuclease digestion studies. Our data show that $H1_M$ and H3 phosphorylations are extensively depleted in the isolated chromosomes; therefore, CHO chromosomes prepared by these standard methodologies do not represent *in vivo* chromosomes with respect to their histone phosphorylations.

MATERIALS AND METHODS

Cells and Cell Synchrony

Line CHO Chinese hamster cells were grown as a monolayer in Blake bottles containing F-10 medium supplemented with 15% newborn calf serum, streptomycin, and penicillin (12). After 36 to 44 hr of exponential growth, loosely attached cells were removed by mechanical agitation and discarded by decantation (13); fresh medium containing 0.06 µg/ml of Colcemid was then added. This procedure was repeated 1 hr later. At 3.5 hr after the second decantation, metaphase cells were harvested by selective mechanical detachment from the monolayer (13). An aliquot of the cells was treated with hypotonic sucrose and fixed in 3:1 methanol:acetic acid for phase contrast microscopy (8). Examination of the fixed cells on air-dried slides showed that the mitotically selected cells were typically 90% metaphase. Interphase cells remaining attached to the Blake bottles after mitotic selection were washed once with ice-cold F-10 medium, scraped from the bottles with a rubber policeman, and recovered by washing the bottles twice with ice-cold F-10 medium. These cells were greater than 99% interphase, determined from

phase contrast microscopy of fixed cells. G_1-Arrested cells were obtained in suspension culture by the isoleucine deprivation method of Tobey and Ley (14).

Preparation of Chromosomes by the Method of Wray and Stubblefield

Mitotically selected cells in Colcemid-containing medium were centrifuged, resuspended in 8 ml of the same medium, and placed on ice. After 30 to 40 min at 0°C, the cells were pelleted at low speed in a clinical centrifuge and washed once with cold chromosome isolation buffer (CIB) according to Wray and Stubblefield (9). CIB is 0.5 m$\underline{M}$ $CaCl_2$, 0.1 m$\underline{M}$ PIPES (pH 6.7), and 1 $\underline{M}$ hexylene glycol. The cell pellet was quickly resuspended in 37°C CIB to a concentration of 7×10^6 cells/ml and transferred to a plastic centrifuge tube. Since chromosomes adhere to glass (5), plastic tubes and pipettes were used hereafter during chromosome isolation. After 6 to 8 min at 37°C, the cells were syringed repeatedly through a 22-gauge needle until the chromosomes were free in solution (9). At this point, the chromosome suspension was quickly chilled on ice. The suspension was then layered over a 3.5-cm deep solution of 20% sucrose in CIB and centrifuged for 12 min (4°C) at 5250 rpm (4500 x g) on a Sorval HB-4 swinging bucket rotor (15). The chromosome pellet was resuspended in CIB and transferred to a 12-ml Corex tube for histone isolation. Chromosomes resuspended in CIB were photographed using phase contrast microscopy.

Preparation of Chromosomes by a Detergent Method

Mitotically selected cells in Colcemid-containing medium were quickly pelleted by centrifugation, resuspended in 8 ml of the same medium, and placed on ice. All subsequent procedures were carried out at 0 to 4°C. After 30 to 40 min on ice, the cells were pelleted by low-speed centrifugation and washed once with cold Tris-calcium buffer (TCB). TCB is 15 m$\underline{M}$ Tris-HCl, 3 m$\underline{M}$ $CaCl_2$ (pH 7.2) (1,11). The cells were resuspended in ice-cold TCB to 5×10^6 cells/ml to allow the cells to swell. After 6 to 10 min, 2.5% Nonidet P-40 (NP-40, a nonionic detergent manufactured by Shell Chemical Company) in TCB was added, while vortexing, to make the solution 0.2% in NP-40. Cells were homogenized in a Dounce homogenizer (Kontes Glassware) with a tight B pestle for 6 to 10 strokes or until the cells were broken (1).

The homogenate was transferred to a plastic tube in which the chromosomes were pelleted at 1140 x g for 5 min in a clinical centrifuge. The chromosomes were subsequently washed once with TCB containing 0.2% NP-40

and twice with TCB without NP-40 (1); the chromosomes were recovered each time by centrifugation at 1140 x g. Pelleted chromosomes were resuspended with a plastic-tipped pipette in TCB and transferred to a 12-ml Corex glass tube for histone isolation. The resuspended chromosomes were photographed through a phase contrast microscope.

Histone Isolation

All histone isolation procedures were performed in a cold room (4°C). Chromosomes suspended in CIB or TCB in a Corex tube were pelleted at full speed for 5 min in an International clinical centrifuge. The chromosomes were then suspended once in isotonic saline containing 50 mM sodium bisulfite and repelleted. Chromosomes that adhered to the sides of the tube were pushed to the bottom with a glass rod. Histones were extracted directly from chromosomes using the histone extraction reagents employed in the Johns procedure (16,17) plus sodium bisulfite and mercaptoethanol in the appropriate extractants (6). The previously reported extraction volumes (17) were scaled down in proportion to the number of cells used for chromosome isolation (8). All extraction times (17), except for overnight extraction, were tripled because we were extracting directly from intact chromosomes rather than from finely dispersed chromatin.

Histones were also isolated directly from blended mitotic cells, interphase cells, and G_1-arrested cells by the first method of Johns (16,17). Again, sodium bisulfite and mercaptoethanol were employed in the appropriate extraction solutions as previously described (6,8). This method (16) separates histones into three fractions: (a) the H1 histones; (b) a mixture containing H2A, H3, and H4; and (c) histone H2B. Each of these fractions was dissolved in water and lyophilized to dryness in preparation for electrophoresis.

Electrophoresis and Analysis of Histone Phosphorylation

Histones were separated by high-resolution electrophoresis on long (0.6 x 25 cm) acetic acid:urea polyacrylamide gels according to the method of Panyim and Chalkley (18). These procedures have been described previously in detail (8). Histone H1 electrophoresis was performed in 2.5 M urea:0.9 M acetic acid:15% polyacrylamide gels (8,18) with calf thymus H1 (purified by BioRex 70 chromatography) serving as a mobility marker. After preelectrophoresis and prior to loading a CHO H1 sample, the calf thymus H1 mobility marker was loaded on the gel (7). After electrophoresis for 4 hr, the CHO H1 sample was then loaded on the gel, and electrophoresis was

continued (8). Electrophoresis of the histone fraction containing H2A, H3, and H4 was performed in 6 M urea:0.9 M acetic acid:12% polyacrylamide gels (8). H2A and H4 served as internal mobility markers for H3 analysis. All gels were stained with 0.2% amido black 10B (BioRad Laboratories) in 9% acetic acid:30% methanol and destained by diffusion in the same solvent without amido black.

Absorbance profiles of the electrophoretic gels were measured at 630 nm with a Gilford Model 240 spectrophotometer equipped with a gel linear transport attachment. The mobilities of the various phosphorylated H1 bands resolved in the gel were determined relative to the position of the internal calf thymus H1 mobility marker. The number of phosphate groups per H1 molecule was determined from the reduced mobility of phosphorylated H1 relative to that of unphosphorylated H1 by the method of Chalkley et al. (19) as previously described (7,8). The mobilities of the different H3 bands similarly were determined using histones H2A and H4 as mobility markers.

The overlapping bands of each histone in densitometer profiles of the gels were resolved electronically and their band areas quantified using a DuPont Model 310 curve resolver (20). The relative mass of each phosphorylated histone band was then calculated as a percentage of the total mass of histone H1 or histone H3. Relative quantities of H3, H4, and H1 for a given chromosome or chromatin preparation were estimated from the integrated absorbance of each histone in the densitometer profiles and from the relative volumes of electrophoresis buffer added to each Johns fraction.

RESULTS

Metaphase Chromosomes

Phase contrast micrographs of isolated CHO chromosomes prepared by the method of Wray and Stubblefield are shown in Fig. 1. Chromosomes prepared in this manner exhibited good morphology (Fig. 1), and they were suspended individually. There were very few stretched chromosomes (9). When left at 0 to 4°C, the chromosomes retained their morphology for several days. Histones for analysis were extracted immediately after chromosome isolation.

Chromosomes prepared by the NP-40 procedure (Fig. 2) did not exhibit as good a morphology as those shown in Fig. 1 and contained more stretched chromosomes. These chromosomes also exhibited a tendency to aggregate and were often suspended as a group of chromosomes that resembled the mitotic apparatus from a single cell.

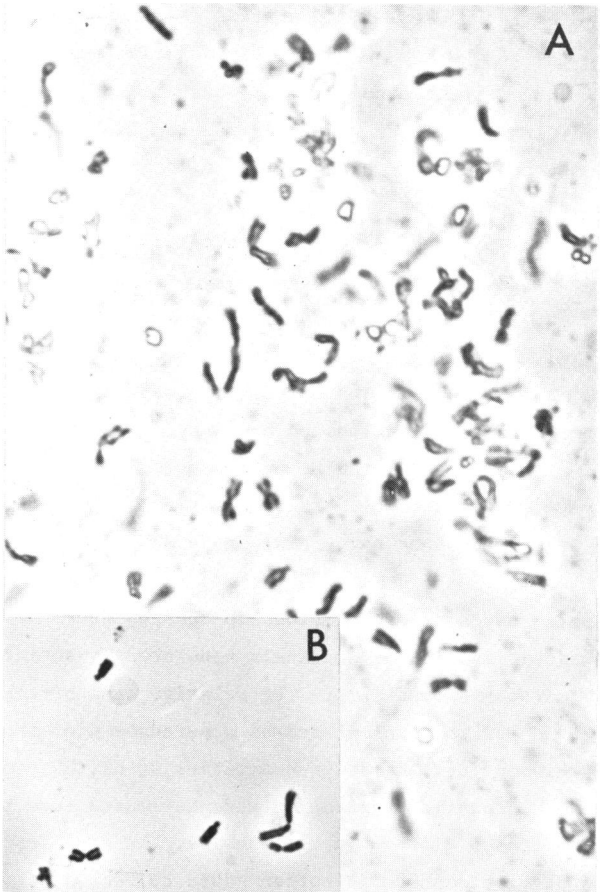

Fig. 1. Phase contrast light micrographs of chromosomes prepared by the procedure of Wray and Stubblefield (9): (A) before and (B) after sedimentation through 20% sucrose in the chromosome isolation buffer. In both cases, the chromosomes were suspended in chromosome isolation buffer (9).

H1 Phosphorylation in Isolated Chromosomes

Previous studies (8) have shown that H1 is most highly phosphorylated during prophase, metaphase, and anaphase, while it is unphosphorylated in G_1-arrested cells (6,7). In those studies, 50 mM sodium bisulfite was present (6-8) during the isolation of histones by the first method of Johns (16). That procedure has been used in this study to isolate histones from cells mitotically selected in the presence of Colcemid and from cells arrested in G_1 by isoleucine deprivation. These respective H1 preparations serve as superphosphorylated and unphosphorylated references (Fig. 3).

Densitometer tracings of H1 extracted from metaphase cells, G_1-arrested

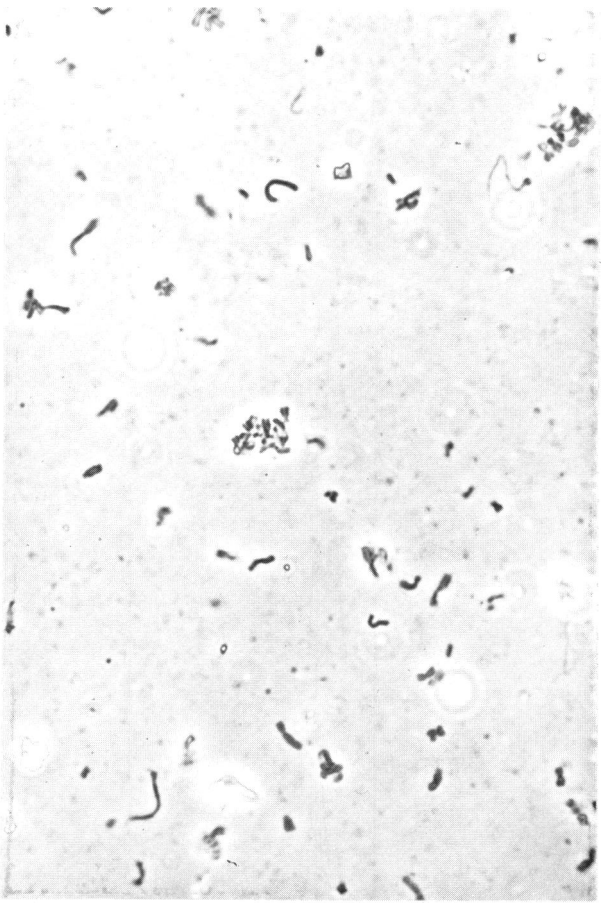

Fig. 2. Phase contrast light micrographs of chromosomes prepared by the NP-40 detergent method described in the text.

cells, and isolated metaphase chromosomes are shown in Fig. 3. There clearly are differences in band patterns of H1 from isolated chromosomes, compared with that of H1 isolated from metaphase cells. H1 from metaphase cells is found almost exclusively in bands 4 through 8, while most of the H1 from isolated chromosomes is found in bands 1 through 3. By correcting for the overlap of bands arising from H1 primary structure subfractions [details of this analysis have been previously reported (8)], we can quantify H1 into three categories (8): (a) $H1_M$ which has 4 to 6 phosphates per molecule (7); (b) $H1_I$ which has 1 to 3 phosphates per molecule (7); and (c) $H1_0$ which is unphosphorylated (6). Classification and quantitation of H1 from the densitometer tracings of Fig. 3 are given in Table I. The data show that the level of superphosphorylated $H1_M$ is very low in isolated

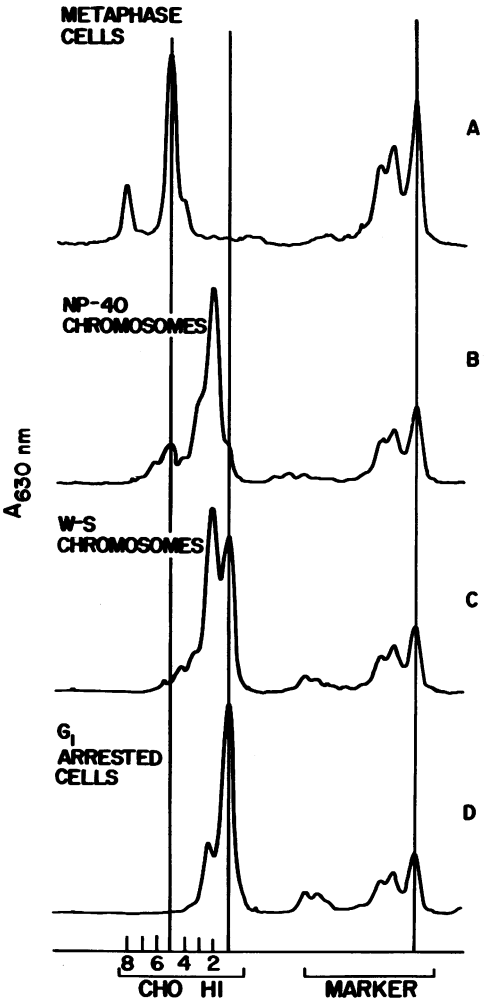

Fig. 3. Electropherograms of CHO H1 isolated from (A) Colcemid-arrested metaphase cells; (B) chromosomes prepared by the NP-40 detergent method; (C) chromosomes prepared by the procedure of Wray and Stubblefield; and (D) cells arrested in G_1 by isoleucine deprivation. Calf thymus H1, loaded 4 hr prior to CHO H1, is the mobility marker on the right. The direction of migration is from left to right; hence, the most highly phosphorylated H1 is found to the left of the electropherograms. The numbers in the abscissa simply identify the different H1 bands (see ref. 7).

metaphase chromosomes; however, both the NP-40 chromosomes and the Wray-Stubblefield chromosomes have a large portion of their H1 phosphorylated at the interphase level of 1 to 3 phosphates per molecule ($H1_I$). We consequently conclude that $H1_M$ is dephosphorylated to $H1_I$ and $H1_0$ during the isolation of metaphase chromosomes. Dephosphorylation is more severe in

Table I. Quantitation of H1 Phosphorylation

H1 Source	Mitotic Fraction	% of Total H1 $H1_0$	$H1_I$	$H1_M$
Wray-Stubblefield Chromosomes*	0.92	48	50	2
NP-40 Chromosomes+	0.95	13	71	16
Metaphase Cells	0.92	3	4	93

*Chromosomes prepared by the method of Wray and Stubblefield, as described in the text.

+Chromosomes prepared by the detergent NP-40 procedure, as described in the text.

the Wray-Stubblefield chromosomes; this may arise during the 37°C incubation period of the procedure.

H3 Phosphorylation in Isolated Chromosomes

Densitometer tracings of H2A and H3 from metaphase cells, from interphase cells, and from isolated metaphase chromosomes are shown in Fig. 4. As with histone H1, the electrophoretic patterns of H3 from isolated chromosomes are very different from that of H3 from metaphase cells. Indeed, the electrophoretic pattern of H3 from the Wray-Stubblefield chromosomes is essentially the same as that of the interphase control (Fig. 4).

Interphase H3 has a characteristic electrophoretic band pattern that is shown in Fig. 4D. During mitosis, however, essentially all H3 molecules become phosphorylated (8). Phosphorylation reduces the mobility of each H3 band and results in a shift in the electrophoretic band pattern to that of Fig. 4A. This shift in band pattern has been employed previously (8) to quantify the extent of H3 phosphorylation during entrance into and during exit from mitosis. Using the same method of analysis (8), the quantity of unphosphorylated H3 ($H3_U$) and the quantity of phosphorylated H3 ($H3_M$) were estimated. These estimates (Table II) indicate that all H3 from chromosomes prepared by the Wray-Stubblefield procedure is totally dephosphorylated, while 70% of the H3 molecules are dephosphorylated in the NP-40 chromosomes. We conclude, therefore, that H3, like H1, is dephosphorylated during chromosome isolation. Again, dephosphorylation is more severe in chromosomes prepared by the procedure of Wray and Stubblefield than in chromosomes prepared by the NP-40 detergent method.

It is unlikely that the differences between the histone electrophoretic

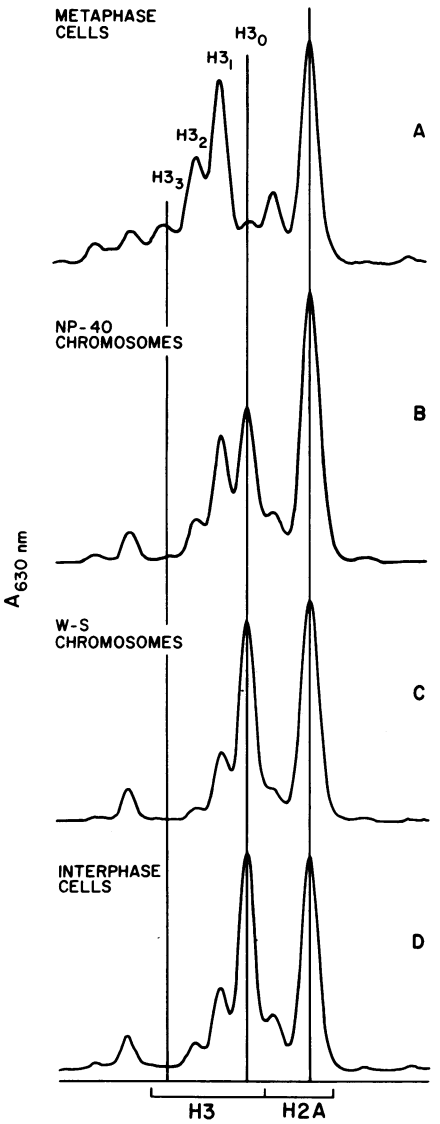

Fig. 4. Electropherograms of histones H2A and H3 from the H2A + H3 + H4 extracts. The histones were obtained from (A) Colcemid-arrested metaphase cells; (B) chromosomes prepared by the NP-40 detergent method; (C) chromosomes prepared by the method of Wray and Stubblefield; and (D) interphase cells remaining after mitotic selection in the presence of Colcemid. The subscripts of H3 serve only to identify different H3 bands.

patterns of isolated chromosomes and metaphase cells arise from selective loss of phosphorylated species during chromosome isolation. If selective histone loss were to account for the differences in the electropherograms,

Table II. Extent of H3 Phosphorylation

H3 Source	Mitotic Fraction	% $H3_U$	% $H3_M$
Wray-Stubblefield Chromosomes*	0.92	100	0
NP-40 Chromosomes+	0.95	70	30
Metaphase Cells	0.92	15	85

*Chromosomes prepared by the method of Wray and Stubblefield, as described in the text.

+Chromosomes prepared by the NP-40 detergent procedure, as described in the text.

then 65-100% of the phosphorylated H3 and 83-98% of the superphosphorylated $H1_M$ would have to be lost from isolated chromosomes. These losses would correspond to 55-85% of total H3 and 77-90% of total H1. From the electropherograms, the ratio of integrated absorbance of extracted histone H3 to that of extracted histone H4 is 0.87 for NP-40 chromosomes, 1.04 for Wray-Stubblefield chromosomes, and 1.05 for controls. In addition, the ratio of integrated absorbance of extracted H1 to that of extracted H3 plus extracted H4 is 0.22 for NP-40 chromosomes, 0.18 for Wray-Stubblefield chromosomes, and 0.18 $\pm$ 0.4 for controls. Hence, while it is possible that small quantities of phosphorylated histones could be lost during chromosome isolation, it is highly improbable that selective histone loss could account for the data in Tables I and II.

DISCUSSION

These data show that $H1_M$ and H3 phosphorylations are extensively depleted during the isolation of CHO metaphase chromosomes by standard procedures. Dephosphorylation is more severe in chromosomes isolated by the procedure of Wray and Stubblefield which employs several minutes of incubation at 37°C. Yet these same chromosomes remain as discrete entities in suspension, are stable for several days at 4°C, and are the most commonly prepared chromosomes for structural studies (5,21,22). Superphosphorylated $H1_M$ is not totally dephosphorylated to unphosphorylated $H1_0$; rather, large portions of H1 remain that contain 1 to 3 phosphates per molecule. This degree of phosphorylation is similar to that observed for $H1_I$ interphase phosphorylation (8). If H1 remained as $H1_I$ in the chromosomes used for nuclease digestion studies (1-5), then the data would indicate that $H1_I$

phosphorylation does not affect the accessibility of inter- and intra-nucleosomal DNA to micrococcal nuclease. We note, however, that the published nuclease digestions employed a 37°C incubation which could provide further opportunity for $H1_I$ to be dephosphorylated to $H1_0$.

We emphasize that these experiments were performed on line CHO Chinese hamster cells. Besides CHO cells (5), other cell lines including HeLa cells (1), the kangaroo rat Dipodomys ordii (2), and the slime mold Physarum polycephalum (3,4) have been used for nuclease digestion of chromosomes. We do not know if $H1_M$ and H3 are easily dephosphorylated in all cell lines or if CHO cells possess exceptionally active phosphatases. It is clear, however, that H1 and H3 phosphorylation must be measured in each chromosome preparation before the chemical and physical properties of the isolated chromosomes can be related to histone phosphorylation.

Recent electron microscopy and sedimentation studies indicate that H1 is necessary for the formation of supercoils or solenoids from nucleosomes (23) or the 200 to 300 Å chromatin fiber (24,25). It is not known if H1 and H3 phosphorylations play a role in forming or stabilizing these higher orders of chromatin structure (8,26). The development of a method that will allow the isolation and manipulation of chromosomes which retain their $H1_M$ and H3 phosphorylations could be important to answering this question. Experiments similar to those of Renz et al. (24) and Finch and Klug (23) might be performed on phosphorylated oligonucleosomes isolated from mitotic chromosomes. Until such methods are demonstrated, however, model studies of phosphorylated histones, chromatin dissociation-reassociation procedures, and enzymic phosphorylation of chromatin appear to be the available approaches to determining the roles of $H1_M$ and H3 phosphorylations in chromatin structure and function.

ACKNOWLEDGMENTS

The authors thank Mr. Joseph G. Valdez and Ms. Linda Ferzoco for their assistance in performing these experiments. This work was performed under the auspices of the United States Department of Energy.

REFERENCES

1 Wigler, M. H. and Axel, R. (1976) Nucleic Acids Res. 3, 1463-1471.
2 Bostock, C. J., Christie, S., and Hatch, F. T. (1976) Nature 262, 516-519.
3 Vogt, V. M. and Braun, R. (1976) FEBS Lett. 64, 190-192.
4 Hozier, J. C. and Kaus, R. (1976) Chromosoma (Berl.) 57, 95-102.
5 Compton, J. L., Hancock, R., Oudet, P., and Chambon, P. (1976) Eur. J. Biochem. 70, 555-568.
6 Gurley, L. R., Walters, R. A., and Tobey, R. A. (1975) J. Biol. Chem.

250, 3936-3944.
7 Hohmann, P., Tobey, R. A., and Gurley, L. R. (1976) J. Biol. Chem. 251, 3685-3692.
8 Gurley, L. R., D'Anna, J. A., Barham, S. S., Deaven, L. L., and Tobey, R. A. (1978) Eur. J. Biochem. 84, 1-15.
9 Wray, W. and Stubblefield, E. (1970) Exp. Cell Res. 59, 469-478.
10 Maio, J. J. and Schildkraut, C. L. (1967) J. Mol. Biol. 24, 29-39.
11 Willecke, K. and Ruddle, F. H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 1792-1796.
12 Tobey, R. A., Petersen, D. F., Anderson, E. C., and Puck, T. T. (1966) Biophys. J. 6, 567-581.
13 Tobey, R. A., Anderson, E. C., and Petersen, D. F. (1967) J. Cell. Physiol. 70, 63-68.
14 Tobey, R. A. and Ley, K. D. (1971) Cancer Res. 31, 46-51.
15 Stubblefield, E., Cram, S., and Deaven, L. (1975) Exp. Cell Res. 94, 464-468.
16 Johns, E. W. (1964) Biochem. J. 92, 55-59.
17 Gurley, L. R. and Hardin, J. M. (1968) Arch. Biochem. Biophys. 128, 283-292.
18 Panyim, S. and Chalkley, R. (1969) Arch. Biochem. Biophys. 130, 337-346.
19 Chalkley, R., Balhorn, R., Oliver, D., and Granner, D. (1973) in Protein Phosphorylation Control Mechanisms, Huijing, F. and Lee, E. Y. C., Eds., pp. 251-277, Academic Press, New York.
20 Panyim, S. and Chalkley, R. (1969) Biochemistry 8, 3972-3974.
21 Stubblefield, E. and Wray, W. (1971) Chromosoma (Berl.) 32, 262-294.
22 Bak, A. L., Zeuthen, J., and Crick, F. H. C. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1595-1599.
23 Finch, J. T. and Klug, A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1897-1901.
24 Renz, M., Nehls, P., and Hozier, J. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1879-1883.
25 Thoma, F. and Koller, Th. (1977) Cell 12, 101-107.
26 Worcel, A. and Benyajati, C. (1977) Cell 12, 83-100.