A REDUCTION IN THE DEGREE OF H4 ACETYLATION DURING MITOSIS IN CHINESE HAMSTER CELLS

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SUMMARY: Long polyacrylamide gels and curve-resolving methods were used to separate and quantitate histone H4 acetylated subfractions from synchronized line CHO Chinese hamster cells. The proportion of internally acetylated H4 molecules was found to be highest during interphase. A reduction in the proportion of acetylated H4 occurred as cells entered mitosis, and a minimum was measured during prophase and metaphase, the periods of maximal chromosomal condensation. A rapid increase in the proportion of acetylated H4 was observed as chromosomes became dispersed in telophase. These observations in proliferating cells are in accord with prior observations in differentiating systems and support the concept that H4 acetylation is minimal when chromatin is highly condensed and RNA synthesis is minimal.

INTRODUCTION

Histones undergo a variety of post-synthetic modifications including acetylation, phosphorylation, methylation, and ADP-ribosylation (1-3). Previous studies with differentiated and developing systems have demonstrated that acetylation, more than any other histone modification, is associated with transcriptional activity and the relative diffuseness of chromatin (1, 4-11).

Two types of H4 acetylation have been observed. Acetylation of the α-amino group of the NH_2-terminal serine is an early event in histone synthesis. It occurs on all H4 molecules and is irreversible (1,2,4,12). In contrast, nuclear acetylation on internal lysines occurs on only a portion of the molecules, and the acetyl groups turn over (1,4,7,12). It is the proportion of internally acetylated histones which has been correlated with modulation of transcriptional activity and the degree of chromatin compactness (4,6,7,11).

While the above studies have focused primarily on acetylation patterns between differentiated states, little attention has been given to continuously proliferating cells in which changes in chromatin structure (13,14) and RNA synthesis (15,16) are also observed during the cell cycle. Therefore, we have examined H4 acetylation during the cell cycle of CHO cells. Our cell-cycle results indicate that, as has been observed in developing systems, the degree of H4 acetylation is minimal at times when chromatin structure is highly condensed.

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MATERIALS AND METHODS

Cells and Cell Synchronization.--Line CHO Chinese hamster cells were synchronized near the G_1/S boundary by a combination of isoleucine deprivation (17) and hydroxyurea methods (17,18). Four hours following release from hydroxyurea blockade, Colcemid was added (0.06 mg/ml) so that cells progressing into mitosis became arrested in metaphase. Aliquots of 150, 10, and 5 ml of culture (285,000 cells/ml) were withdrawn at half-hour intervals beginning 4 hr after hydroxyurea release and continuing thereafter until 9 hr. The 150-ml aliquot was used to isolate histones, the 10-ml aliquot was used for electron microscopy, and the 5-ml aliquot was used for light microscopy. These and the following aliquots were poured over crushed, frozen F-10 medium to arrest cell-cycle progression.

To follow cell progression from mitosis into interphase, cells were collected by mitotic selection and held on ice (about 2 hr) until 3×10^7 mitotic cells were accumulated (15). The cells were then centrifuged in the cold and resuspended in warm F-10 medium. A series of cultures was harvested at 10, 20, and 30 min and at 1, 3, 6, and 9 hr after release from mitotic selection. Aliquots of 110, 10, and 5 ml were used for histone preparation, electron microscopy, and light microscopy.

Electron Microscopy.--Cells were prepared for electron microscopy as described previously (19,20). Thin-sections were cut on an LKB Ultrotome 1 and examined with a Phillips EM 200 transmission electron microscope at 80 kV. More than 300 cells from each sample were examined. The cells were scored as being in interphase or various phases of mitosis (prophase, metaphase, anaphase, and telophase).

Histone Isolation.--Histones were isolated by the first procedure of Johns (21) as adapted to CHO cells by Gurley and Hardin (22); however, the originally reported extraction volumes were reduced to maintain the proper proportion of volume to the number of cells in the sample. Sodium bisulfite and mercaptoethanol were also used in the appropriate solutions (18).

Electrophoresis.--The arginine-rich histones were separated by electrophoresis on long (0.6 x 25-cm) 12% polyacrylamide gels containing 6 M urea. The gels, a variation of the system developed by Panyim and Chalkley (23), had the same formulation as those used by Alfageme et al. (24) except that Triton DF-16 was omitted. The lyophilized arginine histone sample was dissolved in 6 M urea, 5% acetic acid, 4% 2-mercaptoethanol. About 38 µg of arginine-rich histone was applied to the pre-electrophoresed gels. Electrophoresis was performed at a constant voltage of 200 V for 26 hr. The gels were stained with alizarin black (25) and destained by diffusion. Absorbance profiles of the stained cells were measured with a Gilford Model 240 spectrophotometer equipped with a gel linear transport attachment. Histone H2A served as an internal mobility marker. The different H4 bands were resolved and quantitated with a DuPont Model 310 curve resolver (6,23).

RESULTS

H4 Band Assignments.--Electrophoresis of histone H4 on long polyacrylamide gels (e.g., Fig. 1) gives rise to a number of bands (4,6,11,12,23). Isotopic labeling and sequence studies in other laboratories indicate that the slower migrating bands arise primarily from acetylation of the ε-amino group of specific internal lysines (1,2,4,7,11). Phosphorylation also reduces H4 mobility but appears to be only a minor contributor to the mass of slower

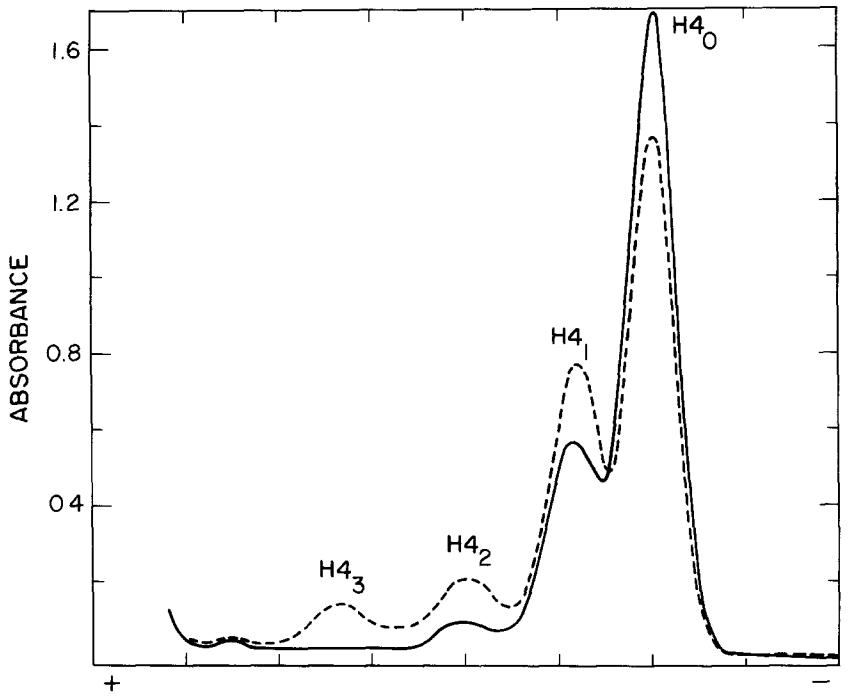

Fig. 1. Electropherograms of H4 isolated from exponentially growing cells (-----) or from cells prepared by mitotic selection in the absence of Colcemid (———). The direction of migration is from left to right.

TABLE I

Quantitation of the H4 Subfractions from Exponential and Mitotic Cells

Cell Origin	$H4_0$	$H4_1$	$H4_2$	$H4_3$
Exponential	50.6 ± 1.0	34.1 ± 2.0	11.0 ± 0.6	4.4 ± 1.4
Mitotic selection	68.2 ± 1.7	25.4 ± 1.6	5.1 ± 0.6	1.3 ± 0.5
Mitotic selection + Colcemid	69.2 ± 2.6	23.6 ± 2.1	5.5 ± 1.7	1.7 ± 0.7

migrating bands (4). Thus, in accordance with others, the slower migrating bands of H4 are assigned as reflecting primarily changes in degree of acetylation (4,6,7,11). The bands are labeled $H4_0$, $H4_1$, $H4_2$, and $H4_3$ in order of decreasing mobility; the subscripts approximate the number of internal acetylations per molecule.

Exponential vs Mitotic Cells.--The first indication that H4 is deacetylated in mitotic cells came from casual observation. Suspension cultures

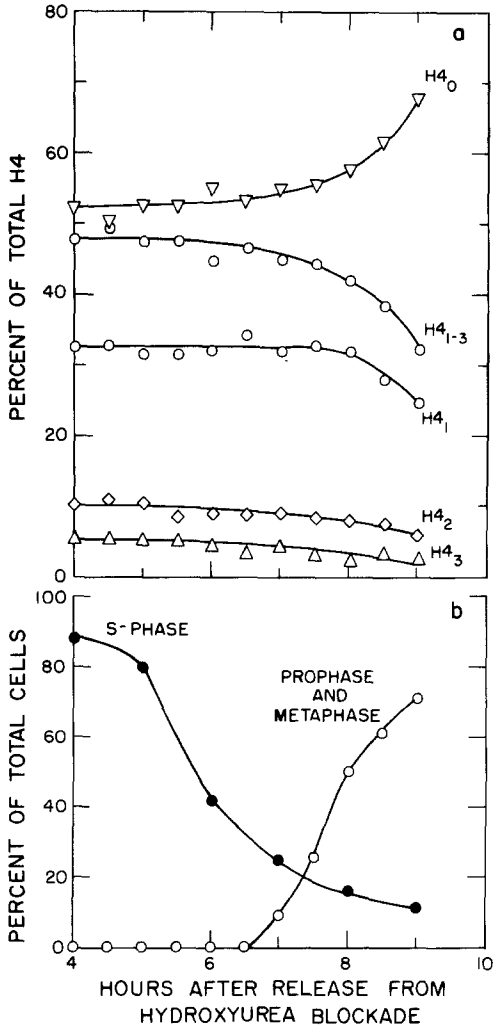

Fig. 2. (a) Distribution of H4 subfractions during entrance into mitosis. (b) Distribution of cells in prophase and metaphase during entrance into mitosis. These values were determined from electron micrographs. The points for S-phase cells represent typical values for release from hydroxyurea blockade (17).

synchronously enriched in metaphase cells were found to contain a greater percentage of $H4_0$ than exponentially growing cells. When histones were isolated from mitotically selected cells without Colcemid or with Colcemid, similar results were obtained (Fig. 1, Table I). These data indicate that the level of acetylated H4 is reduced in mitotic samples compared to the level in interphase cells of exponential cultures. Additionally, the reduced H4 acetyla tion in mitosis cannot be attributed to Colcemid treatment (Table I).

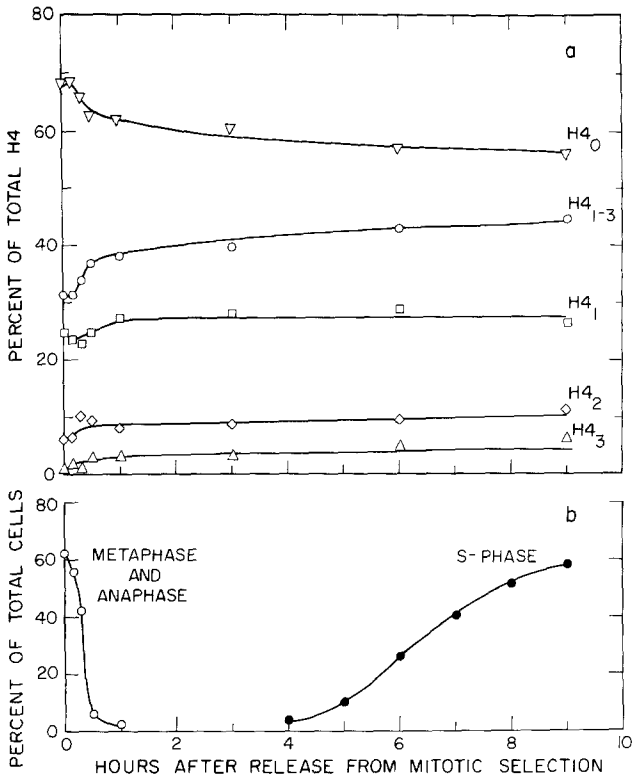

Fig. 3. (a) Distribution of H4 subfractions during exit from mitosis. (b) Distribution of cells in metaphase and anaphase during exit from mitosis. The numbers of cells in metaphase and anaphase, telophase, and G_1 were scored from electron micrographs. The "percentage of cells in metaphase and anaphase" has been corrected for changes in scoring as the cells progress from anaphase to telophase and G_1. Two telophase or G_1 cells, as scored from thin-sections, are equivalent in histone and DNA content to one metaphase or one anaphase cell. Therefore, to correct for differences in histone mass, the scored numbers of telophase and G_1 cells have been divided by two. The points for S-phase cells represent typical values for this type of experiment (29).

Entrance into Mitosis.--Although H4 exhibits a lesser degree of acetylation in mitotic cells, it was not known when, during the cell cycle, H4 became deacetylated. Therefore, cells were synchronized in suspension culture so that their progression into mitosis could be followed. Analysis of the isolated histones (Fig. 2a) shows that the percentage of unmodified $H4_0$ increases slightly ($\sim$1.5%) between t = 4.0 and t = 6.5 hr but that the larger increase (13.5%) occurs between t = 6.5 and t = 9.0 hr. The latter period marks the condensation of chromatin into chromosomes during prophase and metaphase (Fig. 2b). Therefore, essentially all net deacetylation occurs during chromosomal condensation.

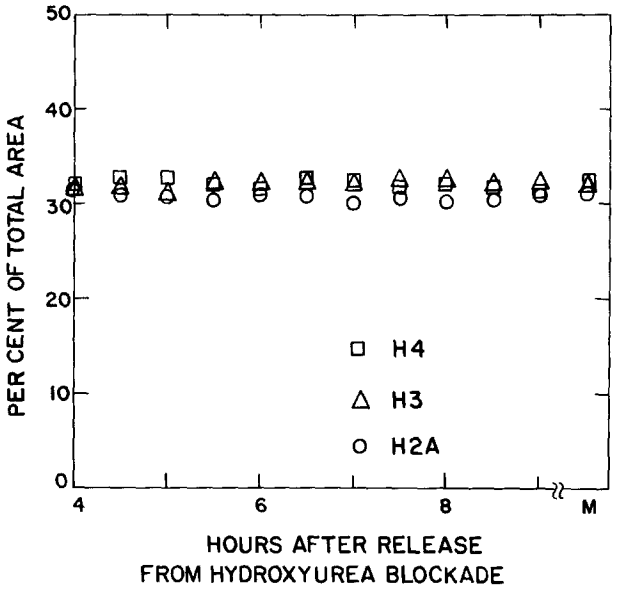

Fig. 4. Distribution of histones H2A (-O-), H3 (-Δ-), and H4 (-□-) in samples used for analysis in Fig. 2a and from cells collected by mitotic selection in the presence of Colcemid (M). The areas were calculated from the electropherograms.

Changes in quantities of $H4_2$ and $H4_3$ are small so that their kinetics are difficult to interpret; nevertheless, there is at least a 50% reduction in each of the bands in going from S-rich cultures at t = 4.0 hr to the metaphase-rich state at t = 9.0 hr.

Entrance into Interphase.--During the first hour after release from mitotic selection (Fig. 3), the percentage of internally acetylated H4 ($H4_{1-3}$) increases from 31 to 38%. This change kinetically parallels the unraveling of anaphase chromosomes as they enter telophase. The percentage of internally acetylated H4 molecules subsequently increases to 44% as the cells progress from early G_1 to S-rich culture. The proportion of each of the acetylated fractions, $H4_1$, $H4_2$, and $H4_3$, increases during progression.

Extraction Artifact?--There is always concern that a variation in quantity of modified histones may result from selective histone extraction or incomplete precipitation (26). We find that the relative band areas of the arginine-rich histones are constant from sample-to-sample. In Fig. 4 are shown the percentages of total stained band areas for histones H2A, H3, and H4 from samples used to follow acetylation during progression into metaphase (Fig. 2). The quantitative consistency of the measurements indicates that the modulations in H4 acetylation are unlikely to be extraction artifacts.

DISCUSSION

Previous studies of differentiated and developing systems have shown that decreased levels of histone acetylation can be related to reduced rates of RNA synthesis and to increased condensation of chromatin (4-11). Our data now demonstrate that, during the cell cycle of proliferating cells, minimum levels of H4 acetylation can be temporally correlated with chromosomal condensation during mitosis, a time in animal cells at which most nuclear RNA synthesis is inhibited (16).

The variation in extent of H4 acetylation during the cell cycle in CHO cells is very similar to that observed by Ruiz-Carrillo et al. in developing erythrocytes (6). In CHO cells, the fraction of internally acetylated H4 molecules drops from 47% in S-rich cultures to 31% in mitotically selected cells. In developing erythrocytes, the level drops from 37% in erythroblasts to 24% in mature erythrocytes which have highly condensed nuclei and reduced RNA synthetic rates (6,7). Although the variations in the fraction of internally acetylated H4 are similar for CHO and duck erythrocytes, the magnitudes of the acetylated fraction are different. Whether or not these differences are related to the extent of genetic activity in the two cell types or to other differences between the types of cells is unknown.

In developing erythrocytes, Ruiz-Carrillo et al. (6) also observed a reduction in the degree of H3 acetylation as cells progressed from the erythroblast to the mature erythrocyte. We were unable to follow H3 acetylation during mitosis because extensive H3 phosphorylation becomes superimposed on acetylation during mitosis and causes a shift in the H3 electrophoretic pattern (20).

Although in many cases H3 and H4 acetylation can be temporally correlated with RNA synthesis and the diffuseness of chromatin, acetylation remains a perplexing phenomenon: (a) There does not appear to be a direct quantitative relationship between the degree of histone acetylation and rate of RNA synthesis (7). (b) Histone acetylation, like histone phosphorylation, is a dynamic process exhibiting rapid turnover (7,12). (c) There are reports in which correlations of histone acetylation and RNA synthesis are not observed (see ref. 26). (d) There is no unequivocal evidence as to how in vivo histone acetylation modulates chromosomal structure and function (27,28).

We have shown recently that the onset and kinetics of H3 phosphorylation and H1 superphosphorylation are tightly coupled with condensation of chromatin into metaphase chromosomes (20); furthermore, the disappearance of the phosphorylated forms coincides with unraveling of chromosomes in telophase. This report now shows that the degree of H4 acetylation is minimal during mitosis. These data suggest that a number of variables must be considered in molecular structural studies of mitotic chromatin.

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