EVIDENCE FROM TRITON X-100 POLYACRYLAMIDE GEL ELECTROPHORESIS THAT HISTONE f2a2, NOT f2b, IS PHOSPHORYLATED IN CHINESE HAMSTER CELLS

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Received October 16,1973

SUMMARY: Chinese hamster cells (line CHO) were labeled in suspension culture with ^{3}H-lysine and ^{32}P-H_3PO_4. Preparative polyacrylamide gel electrophoresis of histone fractions from these cells was performed in the presence of 8 M urea, 6 mM Triton X-100, and 0.9 N acetic acid. This method separates histones f2a2 and f2b by a large distance, thus making it possible to resolve the controversy concerning which histone -- f2b or f2a2 -- is phosphorylated. It is shown that the two most highly phosphorylated histones in interphase CHO cells are f1 and f2a2. Histones f2b and f3 are shown to contain no significant incorporation of $^{32}PO_4$ in interphase cells, while histone f2a1 contains a small but detectable amount of incorporated $^{32}PO_4$. Binding of the nonionic detergent Triton X-100 to hydrophobic centers appears to be greatest for histones f2a2 and f3, thus significantly retarding the mobility of these two histones during electrophoresis.

INTRODUCTION

Interest in histone phosphorylation has recently been stimulated by hypotheses which propose a role for this protein modification in control of DNA activity (review, ref. 1). The phosphate in histones has been shown to occur as O-phosphoserine (2,3). Of the five histone fractions known to exist in somatic interphase mammalian cells, two of these histones are phosphorylated to a much greater extent than the other three. One of them is histone f1 whose phosphorylation is cell-cycle-dependent, occurring in late G_1, S, G_2, and M (4-6). The identity of the other major phosphorylated histone has been the subject of some controversy (7), some laboratories identifying it as f2b and others as f2a2 (1). In addition to these two phosphorylated histones, histone f3, which is phosphorylated very little in interphase, has been shown to be highly phosphorylated when cells condense their chromatin into chromosomes at the time they enter mitosis (6). Histone f2a1 appears to be phosphorylated only to a limited extent at all times in the cell cycle (4-6).

One reason for the persisting confusion concerning the identity of phosphorylated f2b-f2a2 is that histone preparations are often contaminated with phosphorylated nonhistone material (8-10). The preparations must be purified to identify which histones are phosphorylated. Unfortunately, polyacrylamide gel electrophoresis, the method often used for purification, does not resolve

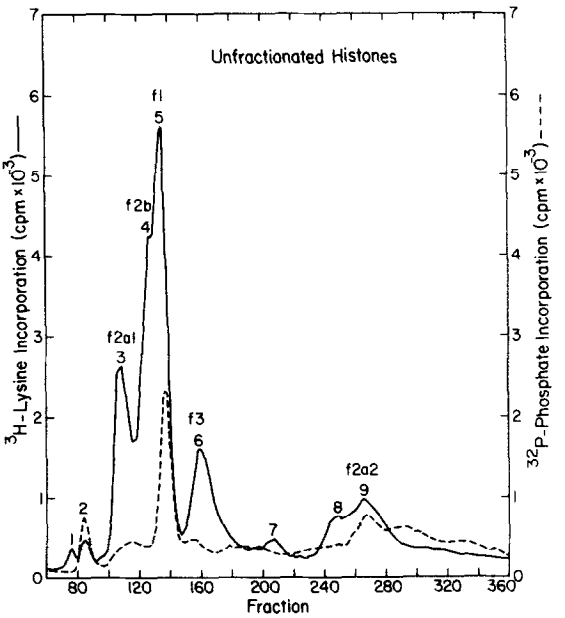

Fig. 1. Preparative polyacrylamide gel electrophoresis of unfractionated histones in the presence of 8 M urea, 6 mM Triton X-100, and 0.9 N acetic acid. Proteins were eluted continuously from the bottom of the gel during electrophoresis and were collected as 2-ml fractions. Individual histone fractions are indicated by the ^{3}H-lysine long-term incorporation (———), and the degree of phosphorylation of each fraction is indicated by the ^{32}P-phosphate 2-hr pulse incorporation (-----).

f2b from f2a2 by a distance sufficient to indicate convincingly which of these two histones is phosphorylated. This problem results from the fact that histones f2b and f2a2 migrate next to one another during polyacrylamide gel electrophoresis (9,11). When whole histone preparations labeled with $^{32}PO_4$ are fractionated by this method, most $^{32}PO_4$ is coincident with fractions f1 and f2b (7). However, we have recently shown that, if histones are first fractionated by differential extraction from chromatin and these fractions are then subjected to electrophoresis, the bulk of $^{32}PO_4$ is associated with f1 and f2a2 and that only a small amount of $^{32}PO_4$ is associated with f2b (9). This led us to propose that the negative charge of the phosphates produces a reduction in the net positive charge of phosphorylated f2a2 to a value lower than that of unphosphorylated f2a2, causing the phosphorylated f2a2 to run slower in electrophoresis gels than the unphosphorylated f2a2. The result of this "phosphate shift" in mobility was that phosphorylated f2a2 migrated coincidentally with histone f2b (9). Balhorn *et al.* (12) reached the same conclusion from experiments in which they found that varying the urea concentration in polyacrylamide gels caused a shift in f2b mobility but caused no shift in incorporated $^{32}PO_4$ coincident with the f2b peak.

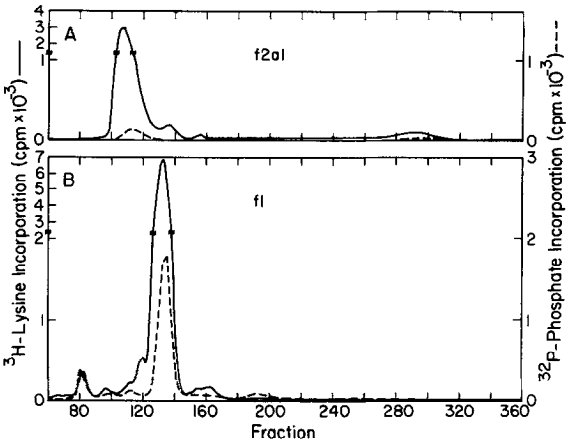

Fig. 2. Preparative polyacrylamide gel electrophoresis of histone fractions in the presence of 8 M urea, 6 mM Triton X-100, and 0.9 N acetic acid: (A) histone f2a1 and (B) histone f1; (———) ^{3}H-lysine long-term incorporation and (-----) ^{32}P-phosphate 2-hr pulse incorporation.

It has been argued that the phosphorylation of f2b might reduce the strength of its binding to DNA so that phosphorylated f2b could be ethanol-extracted with f2a2 during differential extraction procedures (9,13,14). If this occurred, it could account for the observation described above, i.e., the appearance of phosphorylated f2b in the f2a2 fraction (9). One way to test this proposal would be to subject histones to an electrophoretic system which separates f2b from f2a2 by a large distance. Under such conditions, the small shift in mobility resulting from phosphorylation of the histones would not be sufficient to result in confusion in identifying which histone is phosphorylated. Zweidler *et al.* (15-17) have developed a method for separating proteins on the basis of their hydrophobic character which separates f2b and f2a2 by such a large distance. We have used this method to identify which histone -- f2b or f2a2 -- is phosphorylated.

METHODS

Chinese hamster cells (line CHO) were grown exponentially in suspension culture (18) for three generations in the presence of ^{3}H-lysine (50 μCi/liter) so that essentially all histones in the cells were labeled. Incorporation of $^{32}PO_4$ into the histones of these cells was accomplished by treating 1-liter cultures with 20 mCi of ^{32}P-H_3PO_4 (carrier-free) for 2 hr just prior to harvest. Histone fractions f1, f2b, f3, f2a1, and f2a2 were obtained from the chromatin of cells by the first method of Johns (13) and of Phillips and Johns (19), as previously described for cultured cells by Gurley and Hardin (14), except that 0.14 M 2-mercaptoethanol was present in all solutions used for extraction and recovery of arginine-rich histones to prevent dimerization of histone f3 (20). Unfractionated histones were extracted from chromatin with 0.4 N H_2SO_4 and were recovered by precipitation with ten volumes of acetone. All histones were dissolved in water and lyophilized to dryness for storage prior to electrophoresis.

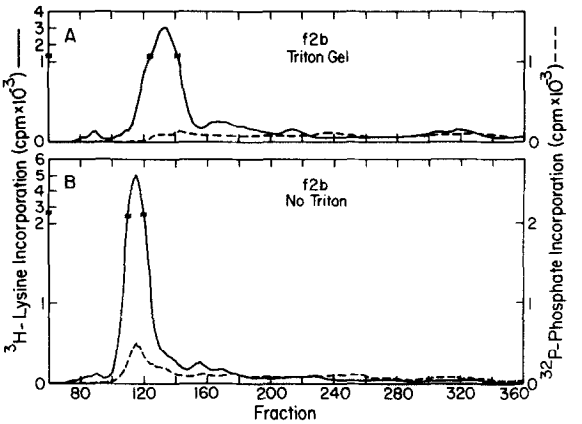

Fig. 3. Preparative polyacrylamide gel electrophoresis of histone f2b: (A) electrophoresis in the presence of 8 M urea, 6 mM Triton X-100, and 0.9 N acetic acid and (B) electrophoresis conditions the same as (A) except that Triton X-100 was absent; (———) ^{3}H-lysine long-term incorporation and (-----) ^{32}P-phosphate 2-hr pulse incorporation.

Histones from 3 x 10^8 cells were subjected to preparative disc electrophoresis by the method of Zweidler *et al.* (15) using a 2-cm long, 15% polyacrylamide gel containing 8 M urea, 6 mM Triton X-100, and 0.9 N acetic acid. The gel was cast in a Canalco Prep-Disc apparatus designed for the continuous removal of protein from the bottom of the gel by a cross-flow of buffer. Using 0.9 N acetic acid in both electrode baths and the eluting cross-flow, electrophoresis was accomplished at 190 volts and 12.5 milliamperes without pre-electrophoresis. Collection of the eluting cross-flow was made at a rate of 2-ml fractions every 2 min. ^{3}H and ^{32}P in these fractions were counted simultaneously in a Packard Tri-Carb spectrometer using Aquasol liquid scintillation fluid from New England Nuclear Corp. (9).

RESULTS AND DISCUSSION

The electrophoresis of whole histone preparations in 8 M urea, 6 mM Triton X-100, and 0.9 N acetic acid reproducibly produced nine peaks (Fig. 1). Electrophoresis of the various histone fractions by the same method (Figs. 2A, 2B, 3A, 4A, and 4B) identified the major peaks (3, 4, 5, 6, and 9 in Fig. 1) as f2a1, f2b, f1, f3, and f2a2, respectively. In addition, peak 2 was associated with f1 and may represent a presumptive f1 degradation product often seen in other electrophoretic patterns of f1 (4-6,9). Peak 8 was associated with f3. When histone f3 was purified by redissolving it in aqueous 0.14 M 2-mercaptoethanol and reprecipitating it by the method of Johns (13,14), peak 8 remained with f3, while peak 9 (f2a2) was removed (Fig. 4C). The identity of peaks 1 and 7 could not be positively identified in any of the fractionated histones.

Without Triton X-100 present during electrophoresis, histones are separated in the following order of decreasing mobility: f2a1, f2a2, f2b, f3, and

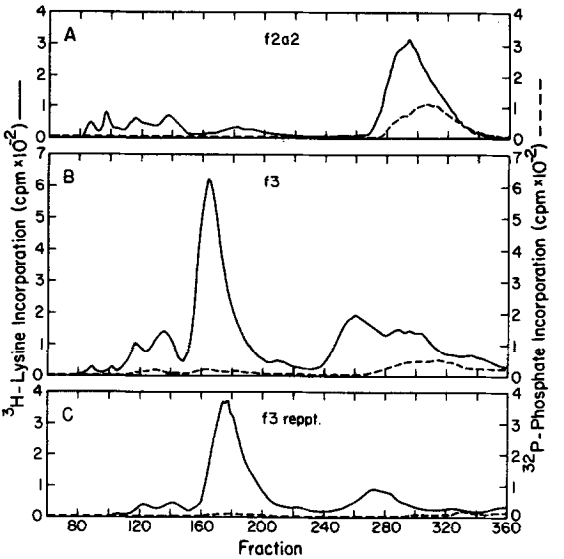

Fig. 4. Preparative polyacrylamide gel electrophoresis of histone fractions in the presence of 8 M urea, 6 mM Triton X-100, and 0.9 N acetic acid (A) histone f2a2, (B) histone f3, and (C) histone f3 purified by reprecipitation; (———) ^{3}H-lysine long-term incorporation and (-----) ^{32}P-phosphate 2-hr pulse incorporation.

f1 (9,11). It can be seen in Fig. 1 that Triton causes a greater reduction in the mobility of f3 and f2a2 than in the mobility of f2a1, f2b, and f1. As a result, the order of decreasing mobility in Triton gels is f2a1, f2b, f1, f3, and f2a2. Thus, f2a2 and f2b are widely separated by this method, presumably because f2a2 binds considerably more nonionic detergent to hydrophobic centers than does f2b (15-17). Histone f3 also appears to bind more detergent than the other histones with the exception of f2a2.

As a result of the wide separation of f2b and f2a2, it is quite clear that the two major phosphorylated histones in interphase CHO cells are f1 (Fig. 2B) and f2a2 (Fig. 4A). If phosphorylated f2b had been extracted with unphosphorylated f2a2 as a result of phosphorylation weakening the binding between DNA and f2b, as has been argued, one would have expected the peak containing $^{32}PO_4$ to be eluted at the f2b position around fraction number 130 in Fig. 4A. Instead, the $^{32}PO_4$ eluted exactly where one would expect phosphorylated f2a2 to be eluted, around fraction number 310 (i.e., slightly slower than the bulk of f2a2 due to the "phosphate shift" in mobility). This offers strong evidence that f2a2, not f2b, is phosphorylated in CHO cells.

In previous experiments, we have always detected a small amount of incorporated $^{32}PO_4$ in f2b when electrophoresis was performed using non-Triton gels (4-6,9). This phenomenon is illustrated in Fig. 3B. As we have pointed out previously, this phosphate peak migrates exactly concidentally with the f2b

peak, showing no "phosphate shift" (Fig. 3B) such as is seen to occur with the other phosphorylated histones (4). This led us to suggest that $^{32}PO_4$ incorporation observed for f2b was due to a contaminant (probably phosphorylated f2a2) rather than due to the phosphorylation of f2b itself (4). By comparing the results of f2b electrophoresis in Triton and non-Triton gels (Figs. 3A and 3B, respectively), it can be seen that Triton has caused the shift of almost all incorporated $^{32}PO_4$ away from the f2b peak, confirming our previous speculation that $^{32}PO_4$ in f2b is a contaminant (4). Thus, it is concluded that histones f2b (Fig. 3A) and f3 (Fig. 4B) are not phosphorylated in interphase CHO cells. Histone f2a1 (Fig. 2A) contains a very limited but detectable amount of incorporated $^{32}PO_4$.

As mentioned above, we found the mobility of histone f3 to be retarded sufficiently in the presence of Triton to cause it to migrate slower than f1. Zweidler *et al.* did not observe such a pronounced effect of Triton on f3 in their laboratory (15). It is known that different methods of histone preparation can make large differences in the secondary structure of histones (21). It is possible, therefore, that the exposure of hydrophobic groups to nonionic detergent binding may vary considerably, depending on the method of preparation, and result in such variability between laboratories. The reason for this difference will undoubtedly be resolved as more experience with this new system is obtained.

ACKNOWLEDGMENTS

The authors would like to acknowledge the excellent technical assistance of Mr. Joseph G. Valdez and Mr. John L. Hanners in performance of the experiments. This work was performed under the auspices of the U. S. Atomic Energy Commission.

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