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Production of High Levels of Phosphorylated F_1 Histone by Zinc Chloride†

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ABSTRACT: Methods have been sought to perturb the level of phosphohistones. $ZnCl_2$ (10 mM) exhibits histone phosphate phosphatase in vivo in HTC cells and leads to hyperphysiological levels of F_1 phosphohistone. Treatment of tissue culture cells with this concentration of $ZnCl_2$ leads to a reduction in medium pH to 6.4. Control experiments have indicated that HTC cells grow efficiently at this pH and that the reduction of pH does not produce the hyperphosphorylated state per se. The optimum conditions for the $ZnCl_2$ effect are described. That the effect of $ZnCl_2$ on the heterogeneity of F_1 histone is due to an effect on phosphorylation was demonstrated by the observation that the entire effect is abolished by treatment with alkaline phosphatase. The site of phosphorylation is in the carboxy-terminal end of the F_1 molecule. The inhibitory effect of $ZnCl_2$ on F_3 phosphorylation in metaphase cells is also described.

Both the rate of F_1 phosphorylation and the amount of the phosphorylated forms of the F_1 histone are much higher in dividing cells than in nondividing cells (Balhorn et al., 1971, 1972a–c). There is a rapid phosphorylation of newly synthesized F_1 histone in S phase coupled with a lower rate of phosphorylation of older F_1 histone (Tanphaichitr et al., 1974; Jackson et al., 1976). Yet another burst of F_1 phosphorylation occurs in metaphase cells occurring to some degree at different sites (Balhorn et al., 1975). The F_3 is also phosphorylated in metaphase cells (Balhorn et al., 1975). Three major proposals have been considered for the function of histone phosphorylation in cell division. These are histone deposition (Tanphaichitr et al., 1974; Dixon et al., 1973), extension of the chromosome prior to replication (Adler et al., 1971, 1972; Stevely and Stocken, 1966, 1968), and condensation of the chromosome in mitosis (Bradbury et al., 1973, 1974; Gurley et al., 1974; Lake, 1973a,b). Recent results (Tanphaichitr et al., 1974; Jackson et al., 1976) provide some support for the first proposal.

On the other hand, a considerable amount of circumstantial evidence has indicated that the processes of chromosome condensation and decondensation are coincidental with the attainment of metaphase F_1 phosphorylation and its subsequent dephosphorylation (Bradbury et al., 1973,

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1974; Gurley et al., 1974; Lake, 1973a,b). It has been proposed (Bradbury et al., 1973; Gurley et al., 1974; Lake, 1973a,b) that metaphase F_1 phosphorylation is involved in chromosome condensation. However, the evidence obtained to date does not indicate any more than a temporal correlation between the two events.

To further test these ideas we have sought to perturb the amount of phosphorylated F_1 histone. Decreased levels of phosphorylated F_1 histone have already been produced (Tanphaichitr et al., 1974) by treating HTC cells with cycloheximide and we wanted to develop a method of obtaining higher levels of interphase phosphorylation than normally encountered.

Since F_1 histone phosphorylation is a reversible process (Balhorn et al., 1972d), the production of highly phosphorylated F_1 histone can be achieved in two ways: either by stimulating the activity of F_1 histone kinase or by inhibiting the activity of F_1 phosphate phosphatase. Unfortunately, the authentic (as opposed to nonspecific "histone kinase" and "histone phosphate phosphatase") enzymes have not yet been isolated, purified, and tested for inhibitors in HTC cells. However, the activity of histone phosphatase can be measured in vivo by monitoring the turnover rate of F_1 phosphate (Balhorn et al., 1972d). With this relatively uncomplicated assay, we chose to attempt to produce an elevated level of phosphorylated F_1 species by inhibiting F_1 histone phosphate phosphatase. We will show that zinc chloride can lower the activity of this enzyme about fivefold. This leads to an increase of the bulk level of phosphorylated F_1 species so that 85–90% of the total F_1 molecules are modified. The effect of zinc chloride on histone phosphate phosphatase is similar to its action on the other phosphatases (Delorenzo and Greengard, 1973; Brunel and Cathela, 1973).

Materials and Methods

Assay for the Amount of Phosphorylated Histone. After histone was isolated as described by Panyim and Chalkley (1969), this assay was performed using high-resolution polyacrylamide gel electrophoresis as described by Balhorn et al. (1972b).

Treatment of HTC Cells with $ZnCl_2$. Zinc chloride (2 M) was dissolved in water and the pH of the solution was adjusted to 4.4. An aliquot of this solution (5 ml) was added to 1 l. of exponentially growing HTC cells, in S-77-S medium. The final concentration of zinc chloride was 10 mM and the pH was 6.4. The pH was adjusted on occasion to 7.4 using 1 N NaOH. However, the cells grew well at the lower pH. At the end of 2.5, 4.5, and 8 h, an aliquot of cells (300 ml) was collected by centrifugation and frozen.

Cells treated with HCl were prepared by adding 1 N HCl until the tissue culture medium had a pH of 6.4. At the end of the eighth hour cells were sedimented and frozen.

Labeling of $ZnCl_2$-Treated HTC Cells with [^{32}P]Phosphate and the Turnover of F_1 Phosphate. HTC cells grown in suspension culture were treated with $ZnCl_2$ and [^{32}P]phosphate (5 mCi/l.) added simultaneously. In both experiments, an aliquot of 300 ml of cells was collected at appropriate times in the chase period and frozen.

Treatment of Phosphorylated F_1 Histone with Escherichia coli Alkaline Phosphatase. F_1 histone was isolated from histones prepared from the $ZnCl_2$-treated, HTC cells using the method of Johns (1964). The F_1 histone (0.2 mg) was dissolved in 0.5 ml of 0.01 M Tris-HCl (pH 8.2) and treated with *E. coli* alkaline phosphatase as described previously (Balhorn et al., 1972b).

Table I: Cell Survival in $ZnCl_2$-Treated Cell Cultures.

h after Addition of 10 mM $ZnCl_2$	Control[a]		$ZnCl_2$-Treated Cells[a,b]	
	No. of Live Cells ($\times10^{-3}$)	% Dead Cells	No. of Live Cells ($\times10^{-3}$)	% Dead Cells
0	448 ± 24	4	448 ± 24	4
9	672 ± 50	9	634 ± 33	7
13	ND[c]	ND[c]	617 ± 43	15
16	824 ± 16	4	111 ± 12	82
33	1080 ± 28	5	0	100

[a] Data are presented with standard deviation from four cell counts. [b] Equal numbers of HTC cells were subcultured at zero time either in the presence or absence of $ZnCl_2$ (10 mM). At the times indicated samples of each population were counted on a hemocytometer. Determination of the dead cells was as described in the text. [c] Not determined.

N-Bromosuccinimide Cleavage of F_1 Histone. F_1 histone (0.2 mg) prepared from ^{32}P-labeled $ZnCl_2$-treated HTC cells as described above was dissolved in 0.4 ml of 0.9 N acetic acid and treated with *N*-bromosuccinimide as described previously (Sherod et al., 1975).

Assay for Dead Cells. Five parts of the cell suspension were mixed with one part of trypan blue (0.5%) and allowed to incubate at room temperature for 5 min. Dead cells were identified as those that absorbed the dye and they were counted in a hemocytometer.

Treatment of Metaphase HTC Cells with Zinc Chloride. HTC cells were trapped in metaphase by colcemid as described by Balhorn et al. (1972a). After 4 h metaphase cells were shaken from the synchrony bottles and transferred into suspension culture. Zinc chloride (10 mM final concentration) and [^{32}P]phosphate (10 mCi) were added to these cells. After 4 h of $ZnCl_2$ treatment, cells were collected and frozen. Histones were isolated as described above. A control experiment was performed similarly but without $ZnCl_2$.

Results

Viability of HTC Cells in the Presence of $ZnCl_2$. The number of 10 mM $ZnCl_2$-treated cells increased at the same rate as that of the untreated control HTC cells for up to 9 h of $ZnCl_2$ treatment (Table I). During this time period the number of dead cells as determined by the absorption of trypan blue of these cells was no larger.

When the time of $ZnCl_2$ treatment was extended beyond 13 h, the number of dead cells increased steadily. Finally, after 24 h all of the cells lysed and only debris could be observed (Table I). Hence, all experiments performed in vivo using $ZnCl_2$ were not extended beyond 13 h. However, even in 9-h $ZnCl_2$-treated cells there was a measure of damage. Cells pretreated with $ZnCl_2$ for 9 h and then resuspended in fresh medium without $ZnCl_2$ survived for an extended time period (10 h) but were unable to reinitiate cell division (data not shown).

Effect of $ZnCl_2$ on the Turnover Rate and the Total Amount of Phosphorylated F_1 Histone. In untreated HTC cells, the half-life of F_1 phosphate is 4.5–5 h (Balhorn et al., 1972d). On the other hand, the half-life of F_1 phosphate in $ZnCl_2$-treated cells is 20 h as shown in Figure 1a. In this experiment the cells were labeled with [^{32}P]phosphate in the presence of $ZnCl_2$ (10 mM) for 8 h before initiating the

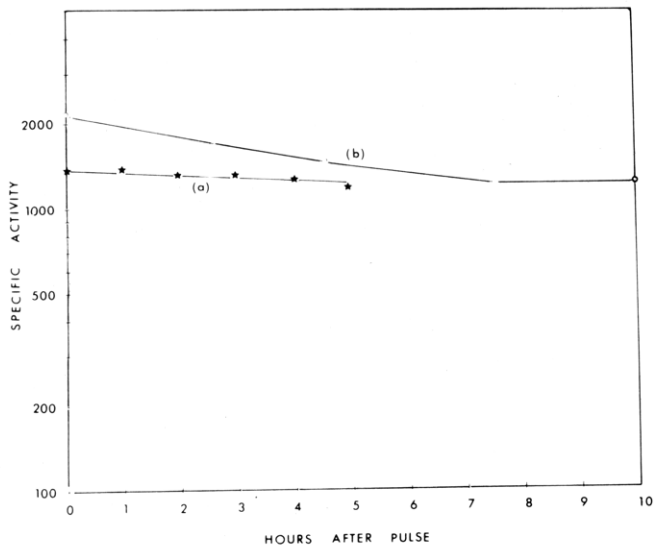

FIGURE 1: Turnover of F_1 phosphate in $ZnCl_2$-treated cells. Two experiments were performed: (a) $ZnCl_2$ was present both during the pulse and the chase period; (b) $ZnCl_2$ was present only in the chase period. Specific activity was measured as counts per minute of ^{32}P/milligram of assayed histone.

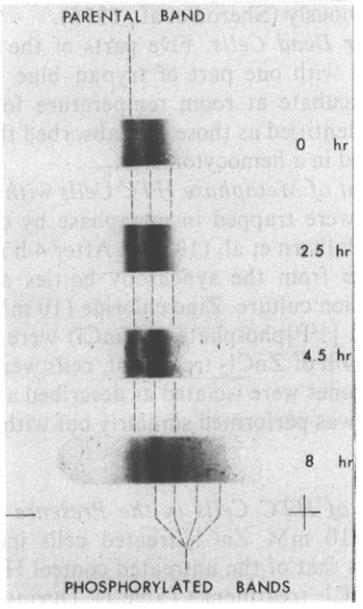

FIGURE 2: Amount of phosphorylated F_1 histone as a function of time of treatment with $ZnCl_2$.

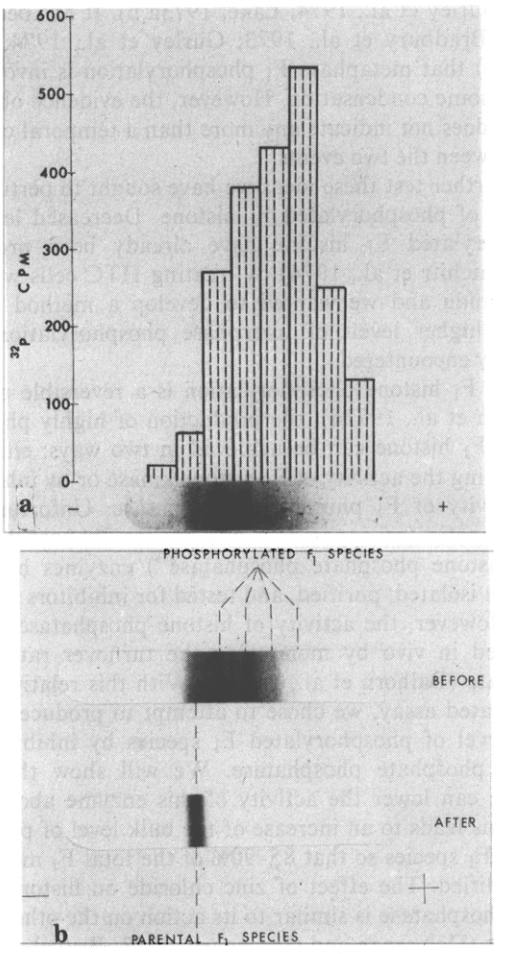

FIGURE 3: (a) Incorporation of [^{32}P]phosphate in the slower moving bands of F_1 histone isolated from ^{32}P-labeled, $ZnCl_2$-treated cells. (b) Electrophoretic microheterogeneity pattern of F_1 histone isolated from $ZnCl_2$-treated cells, before and after treatment with *E. coli* alkaline phosphatase.

(5 h) chase period. The inhibitory effect of $ZnCl_2$ on histone phosphate phosphatase was apparent immediately upon initiating the chase. However, if the ^{32}P labeling was performed in the absence of $ZnCl_2$, which was then added at the start of the chase period, then the half-life of F_1 phosphate did not abruptly change (Figure 1b). In the first 4–7 h of the chase period of the second experiment, the half-life of F_1 phosphate was increased to about 9 h. Only in the eighth hour did the turnover rate approach that observed in the first experiment. These results suggest that the development of substantial inhibition of the histone phosphate phosphatase is not abrupt, probably due to slow accumulation of zinc chloride within the cell. These observations are supported by data on the total amount of phosphorylated F_1 species as a function of time of treatment with $ZnCl_2$. As shown in Figure 2, 4.5 h after the cells were first treated with $ZnCl_2$, the degree of phosphorylation has not greatly changed from that of the untreated exponentially growing HTC cells. As such about 65% of the total F_1 molecules are in phosphorylated form. However, at the end of the eighth hour of $ZnCl_2$ treatment, the phosphorylated form amounts to around 85–90% of the total F_1 histone. At this time, the activity of F_1 phosphate phosphatase was also substantially inhibited (Figure 1b).

The addition of $ZnCl_2$ to the medium decreased the pH to 6.4. It was possible that the high levels of phosphorylated F_1 species might be due to the low pH. To test this idea, the pH of the cell suspension was lowered to 6.4 by adding HCl. After 8 h at this pH the histones were isolated. Cells grown at low pH show the same degree of phosphorylation as untreated exponentially growing HTC cells. Finally, the pH of the $ZnCl_2$-treated cells was adjusted to 7.4 but the level of phosphorylated F_1 species of these cells still remained at 85–90% (data not shown). These results indicate that the high level of phosphorylated F_1 histone is produced by $ZnCl_2$ per se.

Nature of Modified F_1 Histone Isolated from $ZnCl_2$-Treated Cells. We have tested whether the additional microheterogeneity produced in the presence of $ZnCl_2$ is due to phosphorylation. HTC cells were labeled with [^{32}P]phosphate in the presence of $ZnCl_2$ and the F_1 histone was analyzed by high-resolution polyacrylamide gel electrophoresis.

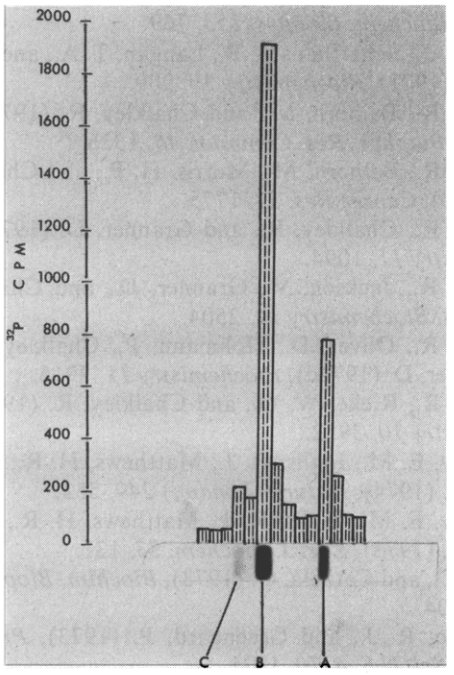

FIGURE 4: ^{32}P content of electrophoresed, *N*-bromosuccinimide-treated F_1 fragments from ^{32}P-labeled, $ZnCl_2$-treated cells. Three fragments were obtained: (A) the uncleaved F_1 histone; (B) the C-terminal fragment; (C) the N-terminal fragment.

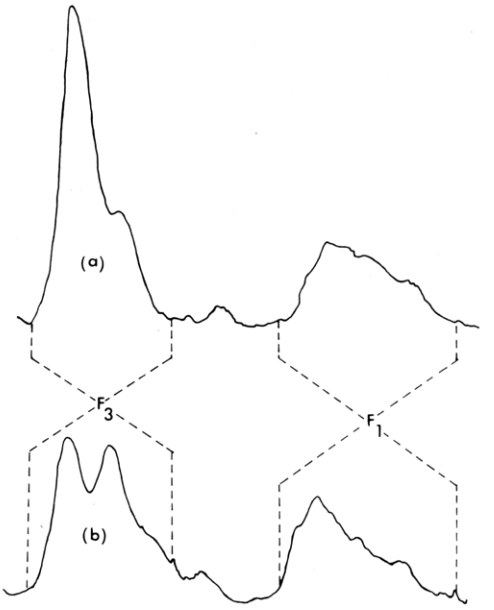

FIGURE 5: Electrophoretic microheterogeneity of F_3 histone isolated from: (a) untreated metaphase cells; (b) $ZnCl_2$-treated metaphase cells.

Table II: Specific Activity (cpm/mg) of ^{32}P Incorporated into F_1, F_3, and F_{2a2} in vivo.

	F_1	F_3	F_{2a2}
Untreated exponentially growing cells	5005		ND[a]
$ZnCl_2$-treated exponentially growing cells	4466		ND
Untreated metaphase cells	5467	3471	893
$ZnCl_2$-treated metaphase cells	5525	327	879

[a] Not determined.

The results are shown in Figure 3a. Four modified bands of the F_1 histone can be seen by standard staining. These four bands all contained ^{32}P counts. In addition a slice from the gel immediately above the tetraphosphorylated species also contained some label. This indicates that in the presence of $ZnCl_2$, at least 5 out of the 12 serine residues of the F_1 molecules can be phosphorylated. In untreated, exponentially growing HTC cells (Balhorn et al., 1972b) two of them are normally so modified.

The result of a second experiment which supports the idea that the modified bands are phosphorylated protein is shown in Figure 3b. Lysine-rich histone was isolated from $ZnCl_2$-treated cells. After treatment with *Escherichia coli* alkaline phosphatase, the microheterogeneity disappears, leaving one main parental band. This behavior is similar to the phosphatase-treated lysine-rich histone isolated from untreated exponentially growing HTC cells. Moreover, if the $ZnCl_2$-treated cells were labeled in vivo with [^{32}P]phosphate, all the ^{32}P counts diminished to background level after the treatment of the F_1 phosphate with alkaline phosphatase (data not shown).

Cleavage of ^{32}P-Labeled F_1 with N-Bromosuccinimide. Treatment of F_1 histone with *N*-bromosuccinimide gives two peptide fragments following cleavage at tyrosine-72. In untreated exponentially growing HTC cells, the C-terminal peptide contains the serine phosphate groups, whereas the N-terminal peptide has essentially none of these esters (Sherod et al., 1975). When $ZnCl_2$-treated cells were labeled with [^{32}P]phosphate in vivo, F_1 histone isolated from these cells gave an *N*-bromosuccinimide cleavage pattern identical with that of the control system with the phosphoserine groups restricted to the carboxy-terminal peptide and uncleaved material as shown in Figure 4. The specific activity of the amino-terminal fragment is misleading as this small peptide stains inefficiently; however, amino- and carboxy-terminal fragments are produced in equimolar amounts as a result of *N*-bromosuccinimide cleavage and thus the ^{32}P counts per minute associated with each fragment are a direct measure of the extent of modification of a given peptide. Assuming that the F_1 histone of HTC cells (derived from a rat cell) has a similar amino acid composition to that of rabbit thymus (Rall and Cole, 1971; Jones et al., 1974) we conclude that at least 5 out of 7 of the serine residues in the carboxy-terminal half of the molecule are capable of being phosphorylated.

Effect of $ZnCl_2$ Treatment on Phosphorylation of Other Histones. Histones F_1 and F_3 are massively phosphorylated in metaphase cells (Lake, 1973a). During an experiment designed to measure the turnover of metaphase-labeled F_1 phosphate we noted that $ZnCl_2$ severely inhibited the formation of phosphorylated forms of F_3 and ^{32}P incorporation into the F_3 histone. This is reflected in a decreased extent of modification of F_3 from that typical of metaphase to that characteristic of interphase as shown in Figure 5 and Table II. We note that $ZnCl_2$ is also most likely exerting an effect on the kinase responsible for phosphorylating F_1 since the ^{32}P incorporation is not significantly greater than that seen in controls despite the major inhibition of phosphatase activity. Analysis of the $ZnCl_2$-treated metaphase cells in the light and electron microscope revealed normal mitotic chromosomes (Tanphaichitr et al., 1976).

Discussion

We have shown that $ZnCl_2$ produces abnormally elevat-

ed levels of phosphorylated F_1 histone by inhibiting the activity of F_1 histone phosphate phosphatase. This effect in HTC cells in vivo is similar to that described in vitro in other systems. Delorenzo and Greengard (1973) reported the total inhibition of membrane-bound alkaline phosphatase by $ZnCl_2$ in toad bladder. Brunel and Cathela (1973) also have described a similar effect in alkaline phosphatase isolated from bovine brain. In addition, these workers have documented that the mechanism of inhibition by $ZnCl_2$ is noncompetitive. In untreated exponentially growing HTC cells, the half-life of F_1 phosphate is 4.5 to 5 h, whereas in $ZnCl_2$-treated cells, it is about 25 h. Consequently by the eighth hour of $ZnCl_2$ treatment, the total amount of the phosphorylated form of F_1 histone is about 90% instead of the 65% found in the normal system. Although $ZnCl_2$ can produce high levels of phosphorylated F_1 histone by inhibiting the capacity of histone phosphate phosphatase, it does not significantly modify the capacity of F_1 histone kinase (Table II) though evidently F_3 histone kinase is inhibited dramatically (Table II). Whether this is an effect on the enzyme or substrate is not known. The data from the *N*-bromosuccinimide cleavage indicate that the phosphorylation of F_1 histone in the presence of $ZnCl_2$ is in the same general region as in the exponentially growing cells, namely in the carboxy-terminal peptide.

The use of $ZnCl_2$ and its varied effects on histone phosphorylation provide us with a useful tool for the study of the role of phosphorylation in chromosome function. It is possible to produce massively phosphorylated interphase F_1 histone and we can assay for the effect of such a modification on chromosome structure. Furthermore, since we can inhibit the F_1 phosphate hydrolysis this provides us with a means of extending typical metaphase F_1 phosphate modification into the G_1 phase and we can thus probe its effect on chromosome decondensation at this time. Finally we have identified a means of inhibiting F_3 phosphorylation in metaphase. Curiously enough no obvious physiological result was attendant upon this inhibition and we wonder if the phosphorylation of F_3 arose by a fortuitous exposure to cytoplasmic kinase enzymes as a result of nuclear membrane breakdown.

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