

CELL CYCLE-SPECIFIC CHANGES IN HISTONE PHOSPHORYLATION ASSOCIATED WITH CELL PROLIFERATION AND CHROMOSOME CONDENSATION

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ABSTRACT

Preparative polyacrylamide gel electrophoresis was used to examine histone phosphorylation in synchronized Chinese hamster cells (line CHO). Results showed that histone f1 phosphorylation, absent in G₁-arrested and early G₁-traversing cells, commences 2 h before entry of traversing cells into the S phase. It is concluded that f1 phosphorylation is one of the earliest biochemical events associated with conversion of nonproliferating cells to proliferating cells occurring on old f1 before synthesis of new f1 during the S phase. Results also showed that f3 and a subfraction of f1 were rapidly phosphorylated only during the time when cells were crossing the G₂/M boundary and traversing prophase. Since these phosphorylation events do not occur in G₁, S, or G₂ and are reduced greatly in metaphase, it is concluded that these two specific phosphorylation events are involved with condensation of interphase chromatin into mitotic chromosomes. This conclusion is supported by loss of prelabeled ³²PO₄ from those specific histone fractions during transition of metaphase cells into interphase G₁ cells. A model of the relationship of histone phosphorylation to the cell cycle is presented which suggests involvement of f1 phosphorylation in chromatin structural changes associated with a continuous interphase "chromosome cycle" which culminates at mitosis with an f3 and f1 phosphorylation-mediated chromosome condensation.

INTRODUCTION

A currently attractive concept suggests that reversible chemical modifications of histones are involved in modulating the physical state of chromatin, resulting in control of biological activity (1, 2). This concept suggests that changes in histone modification patterns might be expected to occur as cells undergo major transition in their metabolic states, such as when nonproliferating (G₀) cells are converted to proliferating (G₁) cells, when G₁ cells enter the DNA synthetic

period (S), or when traversing cells condense their interphase chromatin into chromosomes at mitosis (M). We recently have been studying the metabolism of histones in synchronized cultures of line CHO Chinese hamster cells (3-10) and have found striking differences in patterns of phosphorylation of individual histones at various stages of the cell cycle (11-13). In this report, we have extended these studies to determine the precise timing of different histone phosphorylation events

and have attempted to correlate histone phosphorylation patterns with specific cell cycle processes. Results indicate that phosphorylation of histone f1 is one of the earliest occurring biochemical events during conversion of nonproliferating cells into cell cycle-traversing cells, commencing in G₁ significantly before initiation of DNA replication. It will also be shown that phosphorylation of f3 and a subfraction of f1 occurs only during the time of chromosome condensation at the beginning of mitosis.

MATERIALS AND METHODS

Cell Culture and Synchronization

Chinese hamster cells (line CHO) were maintained free of pleuropneumonia-like organisms (PPLO) in F-10 medium supplemented with 10% calf and 5% fetal calf sera, penicillin, and streptomycin (14, 15). Total labeling of cellular histones was accomplished by continuous exposure of cells to 50 μ Ci [³H]lysine (sp act 20 mCi/mg) per liter of culture for 52 h before synchronization. Cells in suspension culture were synchronized and accumulated in G₁ arrest after maintenance in isoleucine-deficient medium containing [³H]lysine for 36 h, as described previously (8, 12, 13, 16). Resumption of synchronous cell cycle traverse was accomplished by addition of twice the normal F-10 concentration of isoleucine (15).

Histone Isolation and Purification by Preparative Electrophoresis

Histone fractions were prepared from labeled cells by the first method of Johns (17), as previously described for cultured cells by Gurley and Hardin (3), except that 0.14 M 2-mercaptoethanol was present in all solvents and solutions used for extraction and recovery of arginine-rich histones to prevent dimerization of histone f3 (18). This method separates histones into three fractions: the very lysine-rich histone f1, the arginine-rich histone complex containing f2a1, f2a2, and f3, and the lysine-rich histone f2b. This partial fractionation was performed to separate f2b from f2a2 and f3 before electrophoresis, since band curvature makes it difficult to separate adequately histones f2a2, f2b, and f3 by preparative electrophoresis (11). The arginine-rich histone complex (f2a1, f2a2, and f3) was subjected to preparative polyacrylamide gel electrophoresis as previously described by Gurley and Walters (11), using the method of Panyim and Chalkley (19) adapted for use with a Canalco Prep-Disc apparatus (Canalco, Inc., Rockville, Md.). Histones f2b and f1 were mixed together and subjected to prepara-

tive electrophoresis in a different gel. Purified histone fractions were removed from the bottom of the gel by a cross-flow of buffer and collected in 2-ml fractions for liquid scintillation counting, as described previously (11-13). Individual histone fractions were located from the patterns of incorporated [³H]lysine (for example see Figs. 2-5).

RESULTS

Timing of Phosphorylation and Synthesis of Histone f1

Cells were synchronized in a state of G₁ arrest by growth in isoleucine-deficient medium. Such cells have several features in common with "G₀"-arrested cells in vivo (20), the most notable of which is arrest in the pre-DNA synthetic phase of the cell cycle for prolonged periods under conditions in which biosynthetic capacities remain at high levels without causing cells to enter a state of gross biochemical imbalance (21). Reversal of arrest was accomplished by restoration of isoleucine to the culture. Histone f1 phosphorylation rates after resumption of cycle progression were determined by pulse labeling 500-ml cultures (300,000 cells/ml) for 1 h periods with 10 mCi of carrier-free H₃³²PO₄, after which histones were isolated and purified by gel electrophoresis (Fig. 1 A). Histone synthetic rates were measured by pulse labeling similar 500-ml cultures for 1 h with 125 μ Ci [¹⁴C]lysine (310 mCi/mmol), followed by histone isolation and gel electrophoresis (Fig. 1 B). In parallel with each of the above pulse-labeled cultures, an unlabeled synchronized replica culture was prepared from which aliquots were taken and pulse labeled with [³H]thymidine for 1 h periods after isoleucine restoration. The rate of entry into S phase for the cultures in Fig. 1 A and 1 B was obtained from the fraction of cells labeled autoradiographically with [³H]thymidine in these replica cultures (12, 22). From the results in Fig. 1 A, it is apparent that the f1 phosphorylation rate was very low in early G₁ but increased steadily before entry into S phase. Extrapolation of the phosphorylation rate curve to zero indicated that f1 phosphorylation preceded entry into the DNA synthetic phase of the cell cycle by 2 h. In contrast, newly synthesized f1 appeared on chromatin simultaneously with entry of the cells into the S phase (Fig. 1 B). The plateau in the f1 phosphorylation curve between 4 and 5 h after reversal of G₁ arrest (Fig. 1 A) results from the sudden increase in [³H]lysine incorporation

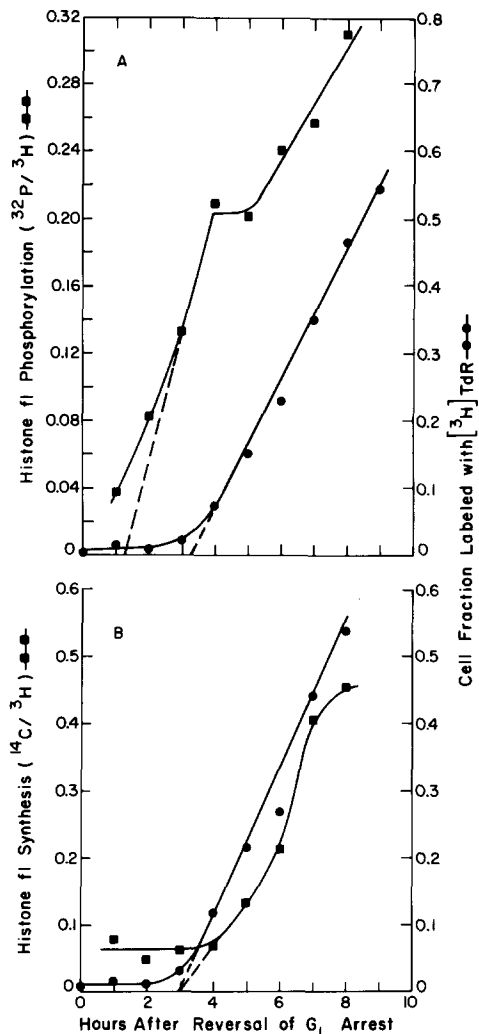


FIGURE 1 Relationship of histone f1 metabolism to the entry of synchronized CHO cells into S phase. Kinetics of entry of each culture into the S phase were measured by determining the fraction of cells labeled autoradiographically with [³H]thymidine, as described in the text (—●—). Replica cultures were exposed to [³H]lysine before synchronization, during synchronization by isoleucine deficiency, and after release from G₁ arrest as described in the text. (A) Phosphorylation of f1 (—■—) was measured by the 1 h incorporation of ³²P₄ into the electrophoretic f1 fraction. (B) Synthesis of f1 (—■—) was measured by the 1 h incorporation of [¹⁴C]lysine into the electrophoretic f1 fraction. The amount of long-term [³H]lysine incorporation into each electrophoretic histone peak has been shown to be proportional to the amount of protein in that peak (11). Therefore, the respective ³²P/³H and ¹⁴C/³H ratios indicate the rate of phos-

phorylation and synthesis relative to the amount of histone f1 recovered in the electrophoretic peak (i.e., relative specific activity).

Histone Phosphorylation Related to Mitosis

In a previous report, we demonstrated that CHO cultures rich in mitotic cells preferentially phosphorylated f3 and a subfraction of f1 (13), in addition to phosphorylation of f1 and f2a2 normally seen in interphase cells (11–13). Lake et al. (23) have also observed a preferential phosphorylation of these histones in metaphase cells. These two additional phosphorylated histones were not detected in G₂-rich cultures devoid of mitotic cells (13) or in exponentially growing cultures (11, 23). To determine whether those f3 and f1 subfraction phosphorylation events were related to conversion of dispersed interphase chromatin into condensed mitotic chromosomes, histone phosphorylation was measured during the transition of cells from G₂ to metaphase in the following manner. Exponential cultures, pre-labeled with [³H]lysine and grown as monolayers, were treated with Colcemid (0.12 μg/ml), and after 2 h the accumulated cells arrested in metaphase were dislodged selectively from monolayers with a mechanical shaker (24). The detached cells were resuspended in Colcemid-containing medium (to maintain metaphase arrest) and exposed to 20 μCi H₃³²PO₄ per ml of medium for 2 h, after which histones were isolated and purified by gel electrophoresis. The pattern of phosphorylation of histones which occurred only during metaphase (i.e., M-rich cells) was determined from this culture.

After detachment of metaphase cells, described above, medium containing H₃³²PO₄ and Colcemid was added back to the monolayers, and after an additional 2 h metaphase cells were shaken off and histones were extracted immediately and purified. All metaphase-arrested cells accumulated in this sample had been exposed to ³²PO₄ as they moved

from G_2 into M (i.e., $G_2 \rightarrow M$ cells). Since G_2 is 3 h under our culture conditions (25), no cells were collected from the monolayer which had phosphorylated their histones in G_1 , S, or early G_2 .

To measure the dephosphorylation of histones during the transition of cells from metaphase to G_1 , metaphase cells which had been labeled with $^{32}\text{PO}_4$ during a $G_2 \rightarrow M$ transition were collected for 2 h in the presence of Colcemid. These metaphase cells were then dislodged by shaking, washed, and resuspended in fresh medium free of both Colcemid and $^{32}\text{PO}_4$. After 2 h, 90% of the cells had reentered G_1 , and at that time histones were extracted and analyzed. These cells were exposed to labeled phosphate only during the $G_2 \rightarrow M$ transition, and the histone phosphorylation pattern was determined after the cells had reentered G_1 (i.e., $M \rightarrow G_1$ cells).

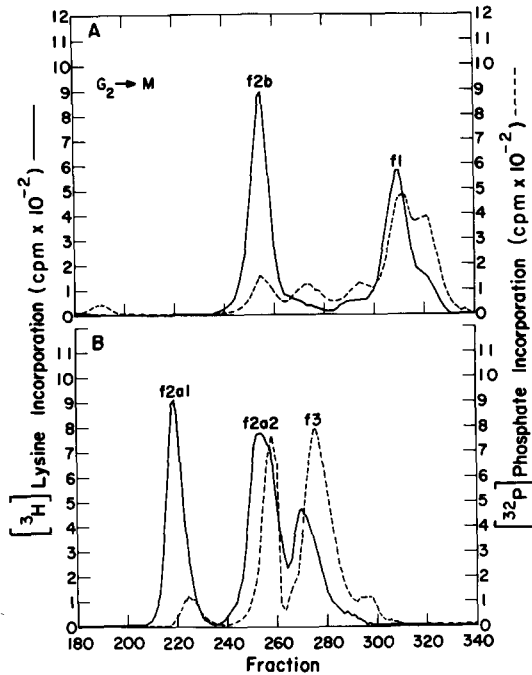


FIGURE 2 Preparative electrophoresis profile of histone phosphorylation in cells traversing from G_2 , through prophase, and collected in metaphase: (A) histones f1 and f2b, and (B) histones f2a1, f2a2, and f3. Individual histone fractions are indicated by 52 h incorporation of $[^3\text{H}]$ lysine (—). Phosphorylation of each fraction is indicated by incorporation of $^{32}\text{PO}_4$ (---) for 2 h, as described in the text. Electrophoretic migration proceeds from right to left.

We have shown in a previous report (13) that there is no detectable phosphorylation of f3, nor is there any phosphorylation of the electrophoretically slow-migrating f1 subfraction in G_2 -rich cultures. In Fig. 2, representing phosphorylation patterns in cells traversing from G_2 into M, histone f3 and the slow-migrating f1 subfraction were phosphorylated at a high rate; however, in cells already in metaphase at time of exposure to $^{32}\text{PO}_4$, f3 and the slow-migrating f1 subfraction were phosphorylated at a much slower rate (Fig. 3). In cells pre-labeled with $^{32}\text{PO}_4$ specifically during traverse from G_2 into M and then allowed to progress into G_1 , there was a nearly total dephosphorylation of histone f3 and the slow-migrating f1 subfraction, whereas pre-labeled phosphate in histones f1 and f2a2 was lost at a much lower rate (Fig. 4). The data in Figs. 2–4, combined with previous data on G_2 phosphorylation (13), indicate that histone f3 and the slow-migrating f1 subfraction are phosphorylated at

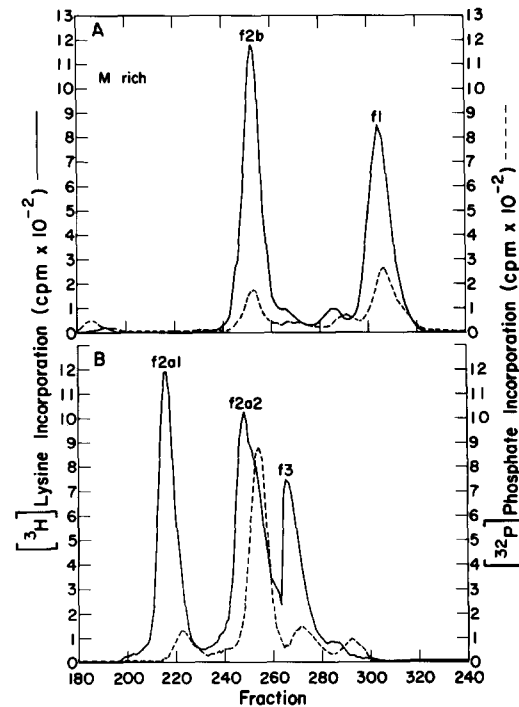


FIGURE 3 Preparative electrophoresis profile of histone phosphorylation in metaphase cells. The description of incorporation patterns is the same as in Fig. 2. (Note: This culture was obtained from the same monolayer culture as the $G_2 \rightarrow M$ cells in Fig. 2.)

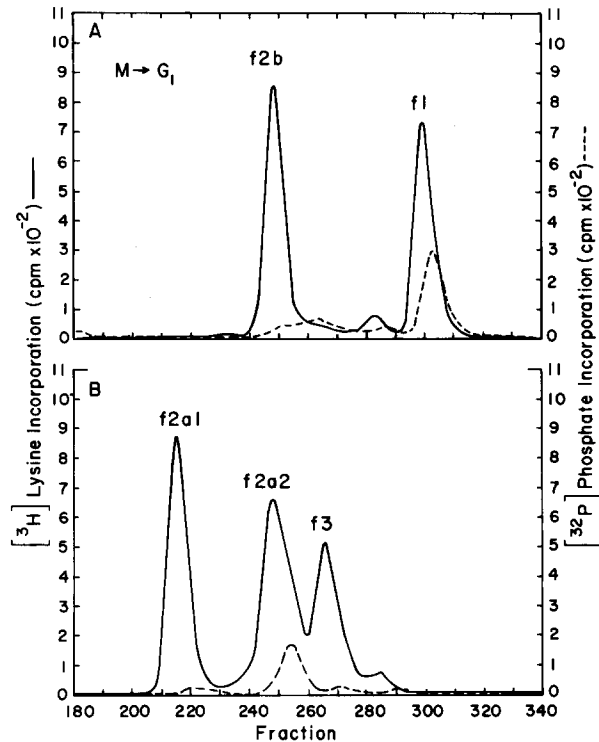


FIGURE 4 Dephosphorylation of $^{32}\text{PO}_4$ -prelabeled histones during transition of metaphase cells to interphase G_1 cells. Cells were labeled with $^{32}\text{PO}_4$ during transition from G_2 into metaphase in the presence of Colcemid, as in Fig. 2. Colcemid and $^{32}\text{PO}_4$ were then removed, and metaphase cells were allowed to enter G_1 for 2 h. Histones were then prepared and subjected to preparative electrophoresis. The description of the profile of incorporated $^{32}\text{PO}_4$ remaining in G_1 cells is the same as in Fig. 2.

the time cells enter mitosis and are dephosphorylated as cells move out of mitosis into G_1 .

To ensure that Colcemid did not adversely affect histone phosphorylation, exponentially growing monolayer cultures were treated with Colcemid and $\text{H}_3^{32}\text{PO}_4$ for 2 h, and mitotic cells (such as those shown in Fig. 2) were dislodged selectively and discarded. Interphase cells (G_1 , S, and G_2) remaining in the monolayer were then removed with EDTA, and the histone phosphorylation pattern was determined (Fig. 5). It is readily apparent that f1 and f2a2 were phosphorylated in these Colcemid-treated interphase cells, while histone f3 and the slow-migrating f1 subfraction were not. An essentially identical histone phosphorylation pattern was obtained in interphase cells not treated with Colcemid (13). Therefore, it appears that the Colcemid treatment for 2 h used in these experiments had no adverse effects on histone phosphorylation and does not

nonspecifically stimulate histone f3 and slow-migrating f1 phosphorylation.

DISCUSSION

By combining the data in this report with results obtained previously (11-13), we may obtain a view of the relationship between histone phosphorylation and the CHO cell cycle. This relationship is illustrated in Fig. 6 and is discussed below. Histone f2a2 phosphorylation was observed to occur in G_1 -arrested cultures as well as in all stages of the cell cycle in proliferating cells (12, 13). Therefore, it is impossible at this time to link phosphorylation of this histone to specific cell cycle events such as DNA replication, mitosis, etc. However, note that f2a2 phosphorylation could represent a "necessary but not sufficient" requirement for cell cycle traverse, since the phosphorylation rate of this histone does increase as cells traverse from G_1 to M (12, 13).

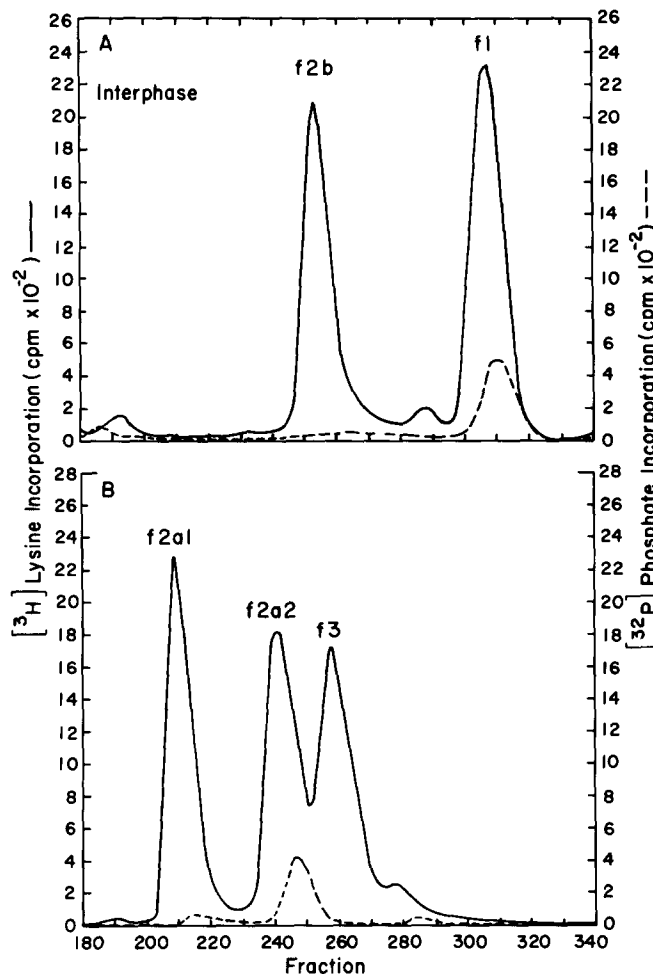


FIGURE 5 Preparative electrophoresis profile of histone phosphorylation in interphase cells growing in monolayer in the presence of Colcemid. After a 2 h incorporation of $^{32}\text{PO}_4$ into exponentially growing monolayer CHO cells in the presence of Colcemid, metaphase cells such as those in Fig. 2 were shaken off and discarded. The remaining interphase cells in the monolayer were harvested by rinsing them three times with saline-GM (the balanced salt and glucose solution G minus MgSO_4 and CaCl_2 of Puck et al. [26]) to remove excess medium, followed by shaking the culture bottles with saline-GM containing 0.5 mM EDTA to detach interphase cells. Histones were then prepared and subjected to preparative electrophoresis. The description of the profile of incorporated $^{32}\text{PO}_4$ in these Colcemid-treated interphase control cells is as presented in Fig. 2.

Histone f1 phosphorylation was absent in G_1 -arrested cells and in traversing cells in early G_1 (12) but commenced at 2 h before initiation of DNA replication (Fig. 1) and increased in rate throughout late interphase and into mitosis (13). Stevely and Stocken have also observed f1 phosphorylation preceding DNA synthesis *in vivo* using regenerating rat liver (27). These observations indicate that phosphorylation of histone f1 is not

linked solely to DNA synthesis. In studies with Chinese hamster V79 cells, Lake and Salzman (28) also have concluded that f1 phosphorylation is not coordinated directly with DNA synthesis. We have been unable to confirm the suggestion by Balhorn et al. (29), derived from studies of synchronized HTC cells, that histones f1 and f2a2 are phosphorylated strictly coincidentally with DNA synthesis.

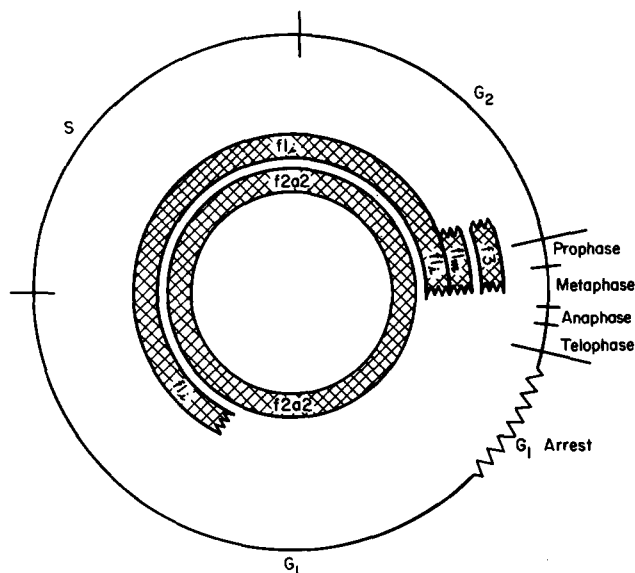


FIGURE 6 Relationship of histone phosphorylation to the cell cycle of line CHO Chinese hamster cells. The 16.5 h generation time of these cells in F-10 medium may be divided into a 9 h G₁, 4 h S, 3 h G₂, and 0.5 h M (25). The periods in which histones f1, f2a2, and f3 are phosphorylated are indicated by the shaded bands. F1_i denotes f1 phosphorylation observed in interphase. F1_m denotes phosphorylation observed in the slower-migrating f1 subfraction of cells entering mitosis (Fig. 2).

Since phosphorylation of f1 preceded DNA and histone synthesis (Fig. 1), it is apparent that f1 phosphorylated 2 h before entry into the S phase was not newly synthesized but, instead, was pre-existing f1 synthesized during a previous proliferation cycle. Newly synthesized f1 is phosphorylated later in the same cell cycle after it appears in the chromatin (30). Therefore, it appears that phosphorylation of old f1 before initiation of DNA synthesis may represent one of the earliest biochemical events indicating that a cell has embarked upon a program of proliferation. Consistent with this notion is the demonstration by Balhorn et al. (31) of a linear correlation between tumor growth rate and extent of f1 phosphorylation. That is, proliferative capacity is related directly to f1 phosphorylation capacity.

From studies on the physical state of chromatin, Bradbury et al. (32) have suggested that histone f1 may play an essential role in chromosome organization and that phosphorylation of this histone may be essential to chromosome condensation. Thus, it is possible that phosphorylation of f1 occurring in late G₁, S, G₂, and M might result in continuous structural alterations in chromatin throughout interphase, culminating in chromosome condensation as cells enter mitosis. In sup-

port of this notion are Pederson's data obtained with synchronized HeLa cells and BSCb green monkey kidney cells (33). He found that accessibility of DNase and actinomycin to DNA varied at different stages of the cell cycle. These findings support the idea that a "chromosome cycle" may exist in eukaryotic cells involving continuous alterations in chromatin structure throughout interphase. This chromosome cycle is interrupted in nonproliferating cells (33), as is f1 phosphorylation (12, 34). We suggest that changes in the physical state of chromatin during such a chromosome cycle may be linked to f1 phosphorylation.

Further suggestive evidence that f1 phosphorylation is involved with cell cycle-specific structural changes in chromatin is provided by the data of Hildebrand and Tobey (35, 36). In studies with CHO cells, they demonstrated an enhanced association of DNA complexed to lipoprotein which commenced approximately 2 h before entry into the S phase and persisted at a high level into the G₂ phase. The timing of enhanced association of DNA with lipoprotein is remarkably similar to the pattern of histone f1 phosphorylation (Fig. 1), suggesting that the two processes may be linked. Although we do not yet know why an apparent chromatin structural alteration should occur

significantly in advance of initiation of DNA synthesis, it is speculated that this structural change associated with fl phosphorylation might represent a prerequisite preparative step for orderly genome replication. For example, structural components of chromatin may undergo a necessary rearrangement during late G₁ to facilitate orderly separation of daughter DNA molecules during its synthesis in the S phase. Such a segregating process would be expected to persist and possibly even to accelerate (as does fl phosphorylation) in G₂ in preparation for final genome separation during mitosis. Numerous additional speculative models may be envisioned.

Histone fl phosphorylation has been demonstrated to be quite complex, involving several levels of phosphorylation on more than one parent fl subspecies (30). Lake and Salzman (28) have shown that phosphorylation occurs on both of the two major fl subspecies in Chinese hamster cells whether the cells are in interphase or metaphase. Therefore, it is possible that the burst of phosphorylation we observed in the slow-migrating fl subfraction during the G₂/M transition (fl_m in Fig. 6) occurs on the same fl molecules phosphorylated earlier in the cell cycle (fl_i in Fig. 6). Such additional phosphorylation would result in a shift in electrophoretic mobility of the faster-migrating phosphorylated fl peak (fl_i) to the slower-migrating phosphorylated fl peak (fl_m) seen in Fig. 2.

In view of the phosphorylation of histone f3 and fl_m specifically as cells enter mitosis and subsequent dephosphorylation of these same fractions as cells exit from M, it seems likely that phosphorylation of these specific histones may play an integral role in chromosome condensation. From studies of *Physarum polycephalum*, Bradbury et al. (37) have similarly speculated that phosphorylation of histone fl may trigger chromosome condensation. Taken together, these data suggest that phosphorylation of f3 and fl_m may represent a final interphase operation, terminating in chromosome condensation during prophase.

Sadgopal and Bonner (38) have shown that the degree of disulfide bond formation in f3 is much higher in metaphase chromosomes than in interphase chromatin, implying that oxidation of f3 is involved in maintenance of chromosome structure during mitosis. The specific phosphorylation of f3, which we observe to occur just before metaphase, suggests the possibility that the function of f3 phosphorylation may be to alter f3 conformation

on DNA so that disulfide bonds can form during chromosome formation.

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