HISTONE PHOSPHORYLATION IN LATE INTERPHASE AND MITOSIS

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SUMMARY: Histone phosphorylation in late interphase has been investigated employing cells synchronized by the isoleucine-deprivation method, followed by resynchronization at the G_1/S boundary using hydroxyurea. Phosphorylation occurred in both f1 and f2a2 as cells synchronously entered S phase following removal of hydroxyurea. The relative rates of phosphorylation of both species of histone increased in G_2-rich and metaphase-rich cultures. A small amount of histone f3 phosphorylation was also observed in M-rich cultures which was not seen in G_1, S, or G_2-rich cultures. It is concluded that f1 phosphorylation is not dependent on continuous DNA replication. These experiments suggest consideration of the concept that f1 phosphorylation is initiated as a preparation for impending cell division.

INTRODUCTION

Numerous experiments have shown that, after histones have been synthesized, some fractions are modified by the phosphorylation of their serine residues (review, ref. 1). Interest in this phenomenon has been stimulated by the hypothesis that phosphorylation may weaken the interaction between DNA and histone, resulting in an activation of the DNA template for RNA synthesis (review, refs. 1,2). However, recently Balhorn *et al.* have questioned whether histone phosphorylation is involved in control of gene activity, since they have found correlations between histone phosphorylation and DNA synthesis in synchronized HTC cells (3). We had previously found that histone f1 phosphorylation could be inhibited by x-irradiation without inhibiting the rate of DNA replication (4). This led us to conclude that f1 phosphorylation was not necessary for the DNA replication process (4). Therefore, to determine the extent to which histone phosphorylation is coordinated with the DNA replication cycle, we have undertaken a study of histone phosphorylation in synchronized Chinese hamster cells.

In an earlier report (5) we showed that histone f2a2 phosphorylation occurs with equal magnitude in both G_1 and S and, therefore, is not dependent on DNA synthesis. In contrast, histone f1 was found to be virtually absent in early G_1 but active in S. In those experiments we could not study histone phosphorylation in G_2 because synchrony decay prevented us from obtaining cultures sufficiently enriched in G_2 cells (6). We have now found that, by

combining the isoleucine-deprivation method of synchronization with hydroxyurea synchronization at the G_1/S boundary, we can considerably improve synchrony in late interphase. Therefore, this report describes the phosphorylation of histones in Chinese hamster cells in S, G_2, and M (mitosis).

METHODS

Chinese hamster cells (line CHO) were grown in suspension culture (4,5,7) for four generations in the presence of ^{3}H-lysine (50 µCi/liter) so that essentially all the histones in the culture were labeled. All subsequent synchronization operations were also performed in the presence of ^{3}H-lysine. Cells were first synchronized in early G_1 by the isoleucine-deficiency method of Tobey and Ley (8,9). These cells were then resynchronized at the G_1/S boundary with 1 mM hydroxyurea, as described by Tobey and Crissman (6). Following removal of hydroxyurea, the cells entered the S phase immediately and began dividing synchronously after 6.5 hr (Fig. 1). The fraction of cells in each phase of the cell cycle was determined by a combination of ^{3}H-thymidine autoradiography, cell concentration determination, mitotic index determination, and flow microfluorometry, as described previously (6,10-12).

Histone phosphorylation was measured at various positions in the cell cycle by treating 1-liter synchronized cultures with 20 mCi of ^{32}P-H_3PO_4 (carrier-free) for 1 hr. Histones were extracted from the cells by the method of Johns (13), as previously described (14), except that 0.14 M 2-mercaptoethanol was present in solutions used for the extraction and recovery of the arginine-rich histones to prevent dimerization of histone f3 (15). Final fractionation and purification of the histones were accomplished by preparative electrophoresis on a Canalco unit, as previously described (4,16).

RESULTS

Growth kinetics for synchronized cultures were determined from unlabeled synchronized cultures (Fig. 1). Similar cultures prelabeled with ^{3}H-lysine were pulse-labeled with ^{32}P-phosphate from 1.5 to 2.5 hr and from 5 to 6 hr after removing hydroxyurea to measure histone phosphorylation in S-rich and G_2-rich populations, respectively. A mitotic-rich culture was obtained by adding Colcemid (to 0.12 µg/ml) at 5 hr after hydroxyurea removal, followed by pulse-labeling with ^{32}P-phosphate from 8 to 9 hr, at which time a large fraction of the cells had entered mitosis (Fig. 1). The cell-cycle distribution of each experimental culture was calculated from an unlabeled parallel synchronized culture (Table I), as described in Methods. Use of separate cultures for such determinations was found acceptable due to exceptionally good reproducibility of the growth kinetics of replica-synchronized cultures (Fig. 1).

Preparative electrophoresis of histones isolated from these cultures revealed that histones f1 and f2a2 were both phosphorylated in S phase (Fig. 2). This was in agreement with our earlier report (5). Phosphorylation of these two histones was observed to increase in G_2-rich and M-rich cultures (Fig. 2). When the cells went from G_2 into mitosis, two qualitative changes were observed in histone phosphorylation. Histone f1 acquired a slowly migrating shoulder in its phosphorylation pattern in M-rich cultures which had not been seen in

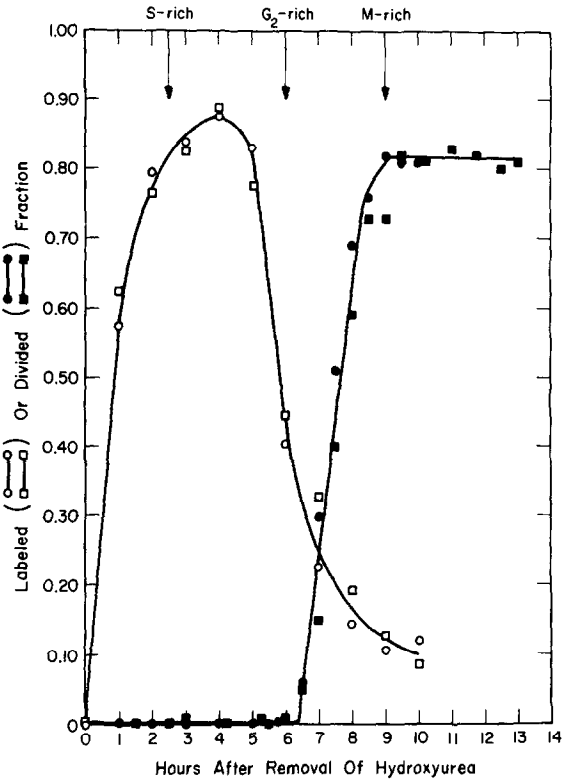

Fig. 1. Cell-cycle traverse by cultured Chinese hamster cells synchronized in G_1 by isoleucine deprivation followed by resynchronization at the G_1/S boundary with hydroxyurea. Following removal of hydroxyurea, cells entered the S phase as indicated by autoradiography with a 15-min pulse-label with 2 μCi/ml ^{3}H-thymidine (open data points) and subsequently divided as indicated by cell concentration determinations (solid data points). Data from two different experiments are shown (● and ■).

TABLE I. Distribution of cells in various cell-cycle positions in cultures of Chinese hamster cells synchronized by isoleucine deprivation followed by resynchronization using hydroxyurea

Cell-Cycle Position	Synchronized Cultures		
	S-Rich	G_2-Rich	M-Rich
G_1	18	0	2
S	82	45	15
G_2	0	54	18
M	0	1	65

TABLE 2. Histone phosphorylation in Chinese hamster cells synchronized by isoleucine deprivation followed by resynchronization using hydroxyurea

Histone Fraction	Relative Rate of Histone Phosphorylation, $^{32}PO_4/^{3}H$-Lysine		
	S-Rich	G_2-Rich	M-Rich
f1	0.123	0.302	0.255
f2a2	0.177	0.289	0.317
f3	0.025	0.050	0.118
f2a1	0.060	0.097	0.065
f2b	0.064	0.104	0.101

G_2-rich cultures (Fig. 2). In addition, the major f3 fraction was observed to be phosphorylated in M-rich cultures (Fig. 2). Although the amount of f3 phosphorylation was small, it was reproducible and was not observed in G_1 cultures (5) nor in S-rich or G_2-rich cultures (Fig. 2). This new phosphorylated f3 fraction is clearly different in electrophoretic mobility from the small phosphorylated f3 subfraction which has been observed trailing the major f3 fraction in all phases of the cell cycle (ref. 5 and Fig. 2).

By taking the $^{32}P/^{3}H$ ratios of the histone fractions in Fig. 2, it was found that the relative rate of f1 phosphorylation increased by a factor of 2.5 in going from S into G_2 and that this increased rate continued on into mitosis (Table II). The relative rate of f2a2 phosphorylation increased by a factor of 1.6 in going from S into G_2, and this increased rate also continued into mitosis. Histone f3 phosphorylation increased by a factor of 4.8 in mitosis over the S-phase rate due to the appearance of the new phosphorylated f3 fraction (Table II). The small degree of phosphorylation in f2a1 and f2b was also observed to increase in the latter part of the cell cycle.

DISCUSSION

It is concluded that, once f1 phosphorylation is initiated, it continues through G_2 into mitosis and, therefore, is not solely dependent on the continuation of DNA replication. These experiments suggest that consideration be given to the idea that f1 phosphorylation is initiated as a preparation for cell division rather than because of some requirement of DNA replication for f1 phosphorylation. This interpretation is consistent with our previous work which showed that both cell division and f1 phosphorylation can be inhibited by x-irradiation without inhibiting DNA replication (4). This interpretation is also consistent with the observations of Balhorn *et al.* (17), who found a

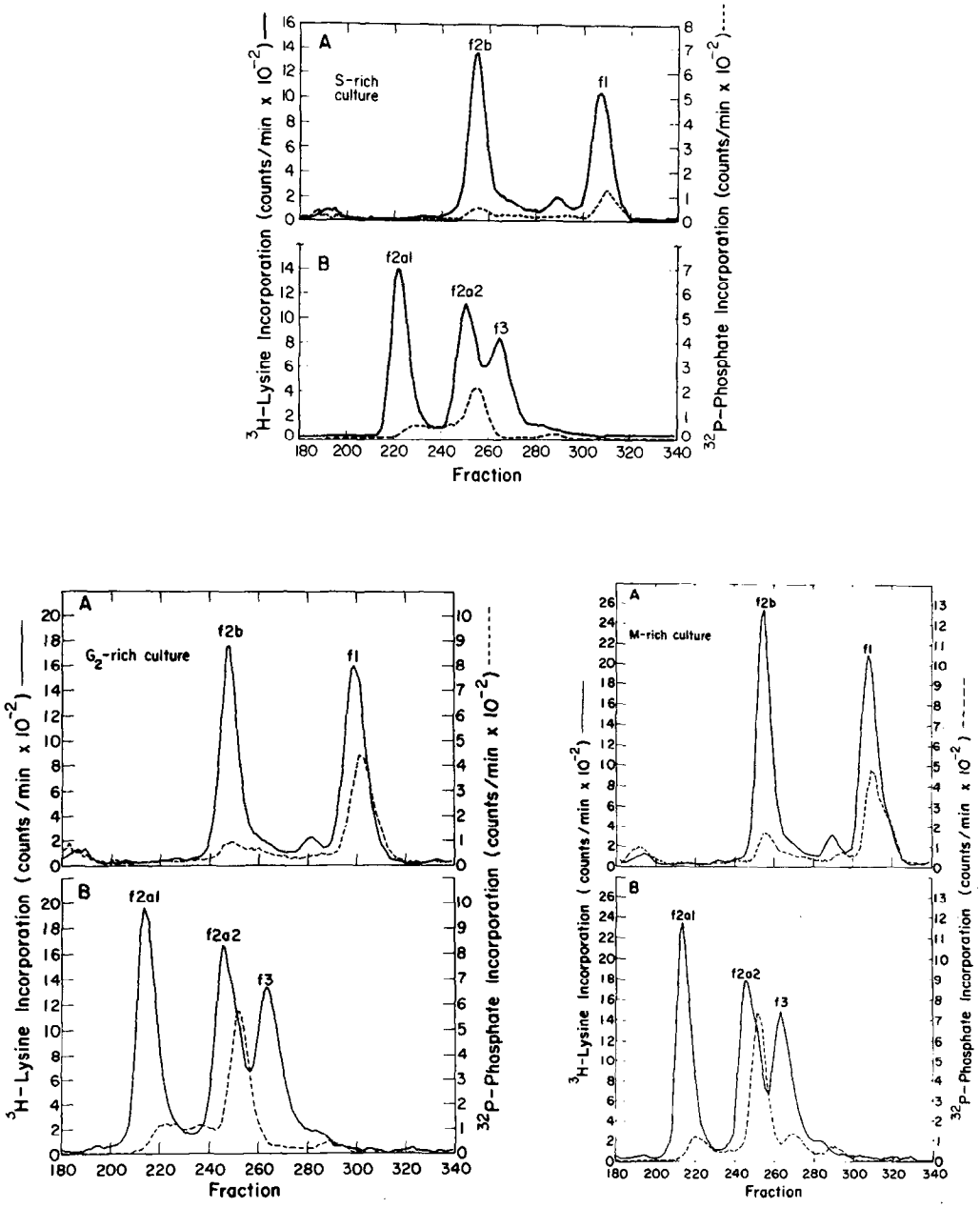

Fig. 2. Preparative electrophoresis of histones prepared from cultures enriched in S-phase cells, G_2-phase cells, and mitotic cells. Cultures were harvested at the times shown by arrows in Fig. 1. The lysine-rich histones (A) and arginine-rich histones (B) were subjected to electrophoresis on separate polyacrylamide gel columns to facilitate separation of f2b from f2a2 and f3, as described previously (4,5). 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 1-hr pulse incorporation (– – – –). The total amount of histone extracted from 2.5 x 10^8 cells was subjected to electrophoresis.

linear correlation between tumor growth rate and extent of f1 phosphorylation, and the work of Lake *et al.* (18), who found dephosphorylation of f1 when dividing cells go from mitosis into G_1.

In our studies on early interphase (5) and in this report on late interphase, we have shown that f2a2 phosphorylation occurs in all phases of the cell cycle. These results cast doubt on whether this process is involved in cell-cycle-specific events the way f1 phosphorylation appears to be.

The shoulder on the ^{32}P peak of histone f1 in M-rich cultures indicated that those cultures contained more than one phosphorylated species of f1 at that particular time. It is possible that this additional f1 phosphorylation began in the G_2 phase but resolution of the preparative electrophoresis was not adequate to detect it. The small f3 phosphorylation observed exclusively in M-rich cultures is curious but must await further developments for interpretation.

The relationship of histone phosphorylation to the cell cycle in Chinese hamster cells was found to be similar to that observed in synchronized HTC cells by Balhorn *et al.* (3) only to the extent that f1 phosphorylation was absent in early G_1 (5). Those authors (3) could not detect any f1 or f2a2 phosphorylation in Colcemid-arrested HTC cells nor any f2a2 phosphorylation in G_1 HTC cells, as we did in Chinese hamster cells. However, our observations on f1 phosphorylation in mitosis do agree with those of Lake *et al.* (18), who found a large amount of phosphorylated f1 in mitosis, followed by dephosphorylation in G_1. At the end of the S phase we found an increase in both f1 and f2a2 phosphorylation. In contrast, Balhorn *et al.* (3) reported a decline in f1 and f2a2 phosphorylation at this time. We cannot explain the differences observed between HTC and Chinese hamster cells at the present time. It is possible that differences observed in cell-cycle-dependent histone phosphorylation in different cell lines may reflect cell-specific differences in the balance of histone kinase and histone phosphatase (19,20) at various times in the cell cycle.

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