Phosphorylation of the Lysine-Rich Histones throughout the Cell Cycle†

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ABSTRACT: The phosphorylation of the lysine-rich histone at various stages in the cell cycle has been studied. In rapidly dividing cell populations the lysine-rich histone is phosphorylated rapidly after synthesis and more slowly once bound to the chromosome. The half-life of hydrolysis of such interphase phosphorylation is 5 hr except during mitosis when the phosphate hydrolysis increases almost threefold. During mitosis there is extensive phosphorylation at sites different from those phosphorylated during interphase and a smaller measure of sites common to both mitotic and interphase cells. The sites of mitotic phosphorylation are most critically distinguished from those phosphorylated in interphase by the rapid hydrolysis of M-phase phosphohistone when the cells divide and enter the G_1 phase of the cell cycle.

Earlier studies with regenerating rat liver (Balhorn et al., 1971), mouse and rat tumors (Balhorn et al., 1972b), developing rat liver (Balhorn et al., 1972a), and synchronized HTC cells (Balhorn et al., 1972c) have demonstrated a positive correlation betweeen cell replication and histone phosphorylation.

We have shown that there is an increased rate of histone phosphorylation during S phase, a result which has recently been confirmed by Marks et al. (1973) and Gurley et al. (1973a). There is also an increase in the rate of ^{32}P incorporation during mitosis as has been shown by Lake (1972, 1973a,b) and by Gurley et al. (1973b). In addition to these peaks in rate of phosphorylation, rapidly dividing cells also phosphorylate histones (though at a lower overall rate) in other phases of the cell cycle.

Experiments exploiting inhibitors of DNA synthesis have led to additional insight into this matter (Marks et al., 1973; Tanphaichitr et al., 1974). Inhibition of DNA synthesis per se does not lead to an immediate decrease in the rate of lysine-rich histone phosphorylation. However, when both DNA and histone synthesis are inhibited, the rate of phosphorylation is reduced to about 35% of control (Tanphaichitr et al., 1974). We have recently shown (Tanphaichitr et al., 1974) that this represents phosphorylation of preexisting histone and that in uninhibited cells 65% of the accumulation of phosphate groups is onto newly synthesized histone. A major conclusion derived from these observations was that the rate constant for phosphorylation of newly synthesized histone is much larger than the rate constant for old histone phosphorylation. This conclusion has recently been verified directly (Jackson et al., 1975).

These observations, of course, explain why an increased rate of phosphorylation occurs in S phase, the period of histone synthesis, and why a lower level is observed in the G_1 phase when only old histone is being phosphorylated at the lower rate. In HTC cells in exponential growth (i.e., no G_0 phase, where phosphorylation is not occurring) the phosphorylation of histone is maintained in a steady-state dynamic equilibrium through the action of a phosphatase ($t_{1/2}$ = 5 hr), so that a roughly constant level of phosphorylated F_1 histone (~65%) is maintained even in G_1 phase cells.

Curiously enough, after synchronizing cells, we have observed that the bulk levels of phosphorylation were unexpectedly low after the cells entered G_1. This was the more surprising as the high levels of phosphorylation in mitosis were not in evidence, nor was there a maintenance of phosphorylated levels from the previous S phase. Additional studies showed that if the cells were not blocked in metaphase *no decrease* in the level of phosphorylated species was observed in the subsequent G_1 phase.

An analysis of F_1 phosphorylation rates and phosphatase activity in interphase and in mitosis has permitted us to resolve this apparent contradiction. We will show that phosphate groups added to the lysine-rich histone in interphase are hydrolyzed more rapidly in metaphase, and further that the phosphate groups added to histone during metaphase are hydrolyzed with an astonishing rapidity when the cells enter G_1, an observation in agreement with recent reports by Tobey and Gurley (1973). Based on these observations, and those of other groups, it appears likely that the phosphorylation of lysine-rich histone is involved in at least two (and perhaps more) disparate functions of the eucaryote chromosome.

Materials and Methods

Cell Culture. Suspension cultures of HTC cells were grown in Swimm's S78 medium as previously described (Balhorn et al., 1972c). The cells were maintained in exponential growth at cell densities between 2×10^5 and 8×10^5 cells/ml.

HTC cells were synchronized by the selective detachment of mitotic cells following an 8-hr colcemid block as described previously (Balhorn et al., 1972c). Synchronization experiments with less than 95% of the cells in mitosis following detachment were discarded. The mitotic cells were rinsed and resuspended in fresh medium at 200,000 cells/ml. Following mitosis, the cells then approached 400,000/ml.

DNA Synthesis Determination. DNA synthesis was monitored by labeling 10-ml aliquots of HTC cells at 400,000 cells/ml with 2 μCi/ml of [^3H]thymidine for 15

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min. The cells were centrifuged and the DNA was precipitated with trichloroacetic acid as previously described (Balhorn et al., 1972c).

Electrophoresis and Radioactivity Determination. The lysine-rich histones were separated on long gels (25 cm) by high-resolution gel electrophoresis (Balhorn et al., 1972d). The gels were scanned on a Gilford microdensitometer and the ^{32}P-labeled histone bands sliced out, dried, and counted on a Biospan planchet counter. Tritium-labeled bands were sliced, digested in 0.3 ml of 30% hydrogen peroxide at 60° for 3 hr, and mixed with Bray's solution (Bray, 1960). Specific activities are expressed as cpm/unit area as determined from the microdensitometric scans.

Preparation of ^{32}P-Labeled Histone. Aliquots of synchronized cells were labeled with 7 μCi/ml of [^{32}P]phosphoric acid (Amersham-Searle neutralized with 0.01 *N* sodium hydroxide) for 1 hr in experiments in which the bulk levels of phosphorylation were examined.

Cells from which histones were isolated for tryptic digestion were collected by centrifugation at 28° and resuspended in one-tenth the original volume of medium. They were then labeled with 10 μCi/ml of [^{32}P]phosphoric acid for 1.5 hr.

Histone was isolated from the frozen cell pellets as previously described (Balhorn et al., 1972d). Lysine-rich histones were extracted from total histone by the method of Johns (1964).

Tryptic Digestion and Peptide Mapping. ^{32}P-labeled lysine-rich histones were dissolved at 0.1–0.5 mg/ml in water and titrated with 0.05 *N* ammonium hydroxide to pH 8.2. The samples were centrifuged at 12,000*g* for 15 min if turbid and the clear supernatants brought to room temperature (20°). Freshly dissolved trypsin (1 mg/ml in water) was added in two aliquots, 20 μl at zero time and a second, 20 μl after 30 min. The pH was continuously maintained at pH 8.0–8.2 by titration with 0.05 *N* ammonium hydroxide. After 3 hr of digestion, the reaction was terminated by lowering the pH to 3.0 with glacial acetic acid and the reaction mixture lyophilized. The tryptic peptides were subsequently dissolved in 1 ml of electrophoresis buffer.

Peptide (tryptic) maps were chromatographed either in 1-butanol–acetic acid–water (4:1:2) at pH 2.5 or 1-butanol–pyridine–acetic acid–water (90:60:18:72) at pH 4.5 and electrophoresed as described by Sherod et al. (1974) and the peptide spots visualized by spraying with 0.1% ninhydrin containing 9% collidine (Bennett, 1967). Radiograms were obtained by exposing RP/L X-Omat Medical X-ray film to the chromatograms in an X-ray film cassette for time periods varying between 24 hr and 1 week. To ensure reproducibility, samples to be compared were chromatographed simultaneously in the same cabinet and all electrophoretic runs were performed, one immediately following the other, after an initial "blank run" warm-up.

Results

Phosphorylation Maintenance. We have previously shown by high-resolution electrophoretic gel analysis of the lysine-rich histone of HTC cells that about 65% of the histone is phosphorylated (Balhorn et al., 1972d). This presumably represents a steady-state balance between the action of a phosphatase with a half-life ~5 hr which is essentially constant in activity throughout interphase (Balhorn et al., 1972e), the rapid phosphorylation of new histone (Jackson et al., 1974) and the slower phosphorylation of the much larger amount of the old histone (Tanphaichitr et al., 1974). If this interpretation is correct, a short pulse of [^3H]lysine should distribute itself between parental and phosphorylated histone molecules in a fashion which reflects the subsequent degree of phosphorylation of the labeled histone at times long after the moment of its synthesis. If histone were phosphorylated but once each cell cycle, then label should shift back to the parental band several hours after the S phase labeling (i.e., in G_1). On the other hand, if the histone and histone phosphate are maintained in a dynamic steady state, then the distribution of [^3H]lysine between parental and phosphorylated species should remain approximately constant. Histones were pulse labeled for 2 hr and the labeled cells then resuspended in fresh medium without label. Cell samples were taken at the indicated times and histone was isolated. Immediately after resuspension a part of the ^3H label migrates electrophoretically with the parental, unphosphorylated F_1, as shown in Figure 1, indicating that it is still in the process of being phosphorylated. However, after 1 hr a substantial number (60–70%) of these [^3H]histone molecules have been phosphorylated. These labeled histones represent an incorporation of label into a specific population of cells, namely those in S

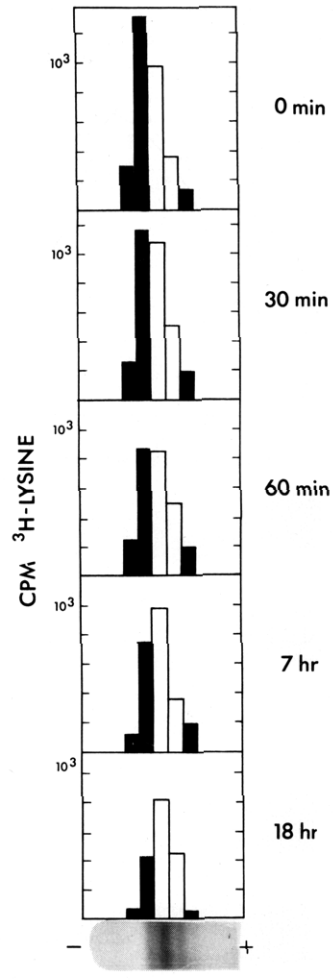

FIGURE 1: Maintenance of phosphorylation of F_1-histone throughout the cell cycle in rapidly dividing HTC cells. Exponentially growing HTC cells were pulsed with [^3H]lysine (1 μCi/ml) for 20 min. The cells were collected, washed, and resuspended in fresh, unlabeled medium and permitted to resume growth. Samples were withdrawn at the times indicated; histone was isolated in the standard manner (see Materials and Methods) and analyzed electrophoretically. After destaining, individual bands were dissected out, dissolved in 30% H_2O_2, and counted in Bray's solution. The open bars on the histogram are the cpm associated with phosphorylated forms of histone.

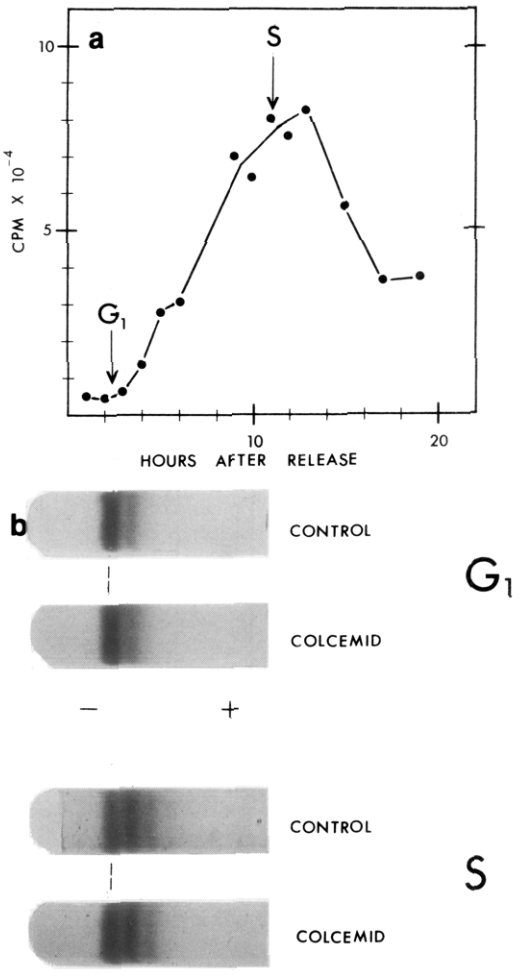

FIGURE 2: Synchronized cells. HTC cells were synchronized as described under Materials and Methods. (a) The efficiency of synchrony was measured by pulsing [^{3}H]thymidine (1 μCi/ml in 10 ml of medium containing 4×10^5 cells/ml) for 15 min. (b) Samples for G_1 and S phase histone (control) were collected at the points indicated by the arrows in 2a; histone was isolated and analyzed electrophoretically. The effect of colcemid was studied by adding the drug 1 hr prior to collection at the above time points.

phase. At later intervals, when the cells containing the [^{3}H]histone are no longer in S phase, the level of phosphorylated [^{3}H]histone remains essentially unchanged. This continues for at least 18 hr (approximately 4 half-lives for phosphate removal), by which time the labeled cells are reentering S phase, indicating that the lysine-rich histones are repeatedly phosphorylated throughout the cell cycle.

Clearly then in these very rapidly dividing cells the bulk level of phosphorylation is maintained even in the G_1 phase of the cell cycle. At this stage of the cell cycle no histone is being made, and we can describe the steady-state system in simple kinetic terms for the reaction

$$\text{histone-OH} \underset{k_2'}{\overset{k_1'}{\rightleftharpoons}} \text{histone-O-P} \qquad (1)$$

where k_1' and k_2' are the pseudo-first-order rate constants (assuming levels of ATP and H_2O are constant throughout the G_1 phase of the cycle).

If this assumption is valid then in the steady state

$$k_1'(\text{histone-OH}) = k_2'(\text{histone-P})$$

or

$$k_1' = k_2' \,(\text{histone-P})/(\text{histone-OH})$$

Table I: Specific Activity of ^{32}P-Labeled Lysine-Rich Histones in the Cell Cycle.

Cell Cycle	cpm/Unit Area F_1
G_1 phase	161
S phase	459

The ratio of parental to phosphorylated species is known, k_2' can be deduced directly from the half-life for phosphate turnover, and thus k_1' can be computed to be equal to 0.14 hr^{-1}. It is perhaps instructive to note that if k_1' were to be held unchanged and k_2' were to be increased (greater phosphatase activity), then the ratio of parental histone to the phosphorylated form would increase by a calculable amount if the new value for k_2' were known.

Bulk Phosphorylation Levels in G_1 and S. If histones are indeed phosphorylated throughout the cell cycle, so that bulk levels are maintained as these results demand, we would expect the lysine-rich histones isolated from G_1 cells to be phosphorylated to the same degree as that indicated by the distribution of [^{3}H]lysine in histones at the same stage of the cell cycle. In order to test this proposal we have isolated histones from cells synchronized into various stages of the cell cycle. HTC cells were synchronized by selective detachment of colcemid-blocked mitotic cells. The cells were washed free of colcemid. They subsequently divided and progressed through the cell cycle as shown in Figure 2a. The gel electrophoretic patterns of F_1 histone from G_1 and S phase HTC cells are shown in Figure 2b. While the S phase histones are highly phosphorylated, as is evidenced by the increased microheterogeneity, the level of phosphorylated species found in G_1 cells is quite low. Approximately 70% of the lysine-rich histone is in the parental, unphosphorylated form in contrast to the prediction based on the [^{3}H]lysine experiment above, which argued that in G_1 cells only 30% of this histone fraction should be in the parental form. The incorporation of [^{32}P]phosphate into F_1 (Table I) occurs at only 35% of the rate observed in S phase, even though there are clearly more free serine residues in G_1 histone. We interpret this latter observation as a reflection of the lower rate constant for phosphorylation of "old" histone relative to the higher rate constant for newly synthesized histone. However, there is a critical difference between the synchrony experiment which shows low bulk levels of phosphorylated F_1 histone in G_1 phase of the cell cycle, and the [^{3}H]lysine distribution studies indicating a high level of bulk phosphorylation during the G_1 phase. This difference lies in the time period spent in metaphase. In normal cells this amounts to a few minutes, whereas during the production of the synchronized cells, they are maintained for up to 8 hr in metaphase. We surmized that critical changes might be occurring in metaphase on the histone species which were phosphorylated in the previous interphase. It seemed likely that these changes might be related to one or more of three factors. These were (1) the known extensive phosphorylation in metaphase (Lake, 1973a–c), (2) the possible loss of interphase kinase during extended metaphase, or (3) a change in phosphatase activity during metaphase. We will argue below that the last point is critical.

Phosphorylation in Mitosis. The phosphorylation of lysine-rich histones by mitotic cells has been previously reported in HeLa (Marks et al., 1973) and CHO cells (Lake, 1973a–c; Gurley et al., 1973a,b). HTC cells blocked in metaphase with colcemid show an extensive phosphorylation of

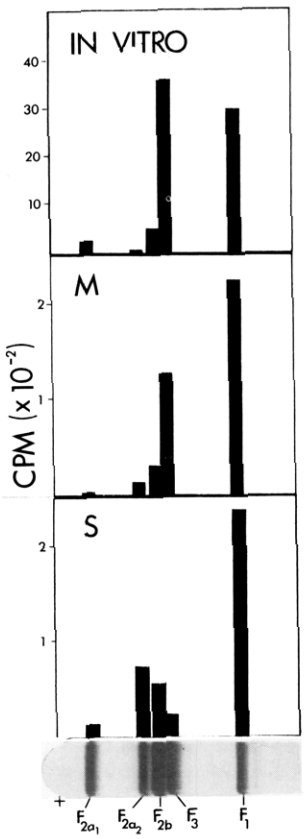

FIGURE 3: Labeling of various histone fractions in different phases of the cell cycle. HTC cells in either metaphase (M) or S phase were labeled with [^{32}P]phosphate (10 μCi/ml for 1.5 hr). Histones were subsequently isolated and analyzed electrophoretically. Radioactivity was detected as described in Figure 1. The radioactivity scale shows the cpm present after subtracting background counts. Background counts never exceeded 15% of the amount shown in individual bands. Histone was labeled in vitro by the action of cytoplasmic protein kinase on intact nucleohistone using [γ-^{32}P]ATP.

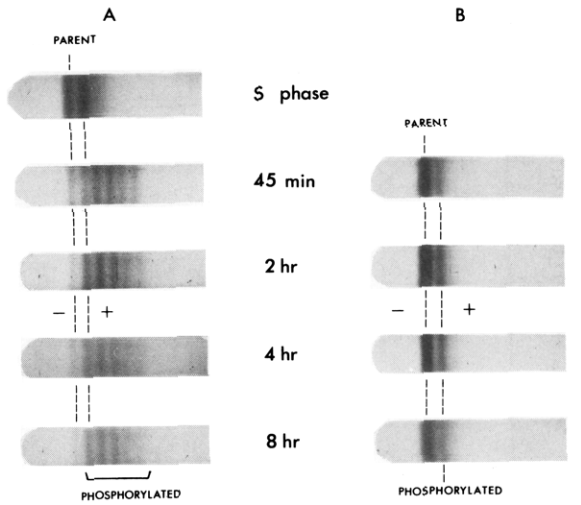

FIGURE 4: The extent and rate of phosphorylation of the F_1-histone in metaphase. (A) Cells were blocked in metaphase for the times indicated. Samples of essentially pure mitotic cells were collected and the F_1 histone isolated and analyzed by high-resolution electrophoresis. (B) HTC cells were blocked for various time periods in metaphase and subsequently washed and released for 2.5 hr into G_1. The cells were collected; histone was isolated and analyzed as discussed in the text.

histone fractions F_3 and F_1 as seen in Figure 3. In contrast when S phase cells are labeled with [^{32}P]phosphate the predominant histone fractions incorporating radioactivity in long term pulses are F_1 and F_{2a2}. It is of interest to note that nucleohistone phosphorylated in vitro by cytoplasmic kinases also specifically labels only F_1 and F_3 (Figure 3). Not only does F_3 incorporate a considerable amount of ^{32}P, but the observed heterogeneity is more extensive than that due to the normal acetate-induced microheterogeneity. However, the phosphate-induced microheterogeneity in the F_1 histone is truly remarkable and is clearly demonstrated in the long gel analysis shown in Figure 4. At least 90% of the lysine-rich histones from 8 hr colcemid-blocked cells are phosphorylated, and to even higher levels than observed in S phase. The rapidity with which F_1 is phosphorylated after blocking M cells is indicated in Figure 4A. Extensive phosphorylation has occurred within 45 min and we note that 2 hr after treatment with colcemid the lysine-rich histones are as highly phosphorylated as after an 8-hr treatment, indicating that not only is the characteristic M phase phosphorylation pattern obtained quite rapidly, but also it speedily arrives at steady-state levels.

Turnover of Metaphase F_1-Phosphate. If interphase histone is labeled with ^{32}P and turnover analyzed during a chase period longer than the cell cycle (Balhorn et al., 1972e) then only a single rate constant is detected for phosphate hydrolysis ($t_{1/2}$ = 5 hr), indicating no detectable interval of rapid turnover of such phosphorylated material at any stage in the cycle. However, since the metaphase phosphorylation microheterogeneity is very complex and that described above for early G_1 phase is rather simple it would appear that, in order to obtain the G_1 bulk levels of phosphorylated lysine-rich histone within 3 hr after release from the colcemid block, the metaphase phosphorylated species must selectively turn over extremely rapidly. Thus we might expect that interphase and metaphase phosphorylation are sufficiently distinct so that after passage through mitosis, only metaphase phosphorylated histone is selectively dephosphorylated. This proposal can be tested directly. Cells growing as a monolayer were pulse labeled with [^{32}P]phosphate. Colcemid was added to the medium and all cells entering mitosis in the ensuing 2 hr were selectively detached and rejected. Thus the bulk of the radiolabel in the residual monolayer would be from S phase cells. During the next few hours these cells entered and were blocked in metaphase by the continued presence of colcemid. These cells were detached and resuspended in colcemid-containing medium. By taking samples of cells at appropriate times the turnover of S phase histone phosphate in metaphase was determined. Finally, some of the cells were washed free of colcemid and resuspended in fresh medium so that continued turnover after release from the metaphase block could be determined. In a parallel experiment cells were blocked in metaphase and collected. The suspension of metaphase cells (still in colcemid) was labeled with [^{32}P]phosphate in a brief pulse. The added label was removed and the turnover of histone phosphate studied both in metaphase and also upon removal of the block as the cells entered the G_1 phase of the cell cycle.

The results of such turnover experiments are presented in Figure 5. S phase-labeled histone phosphate which has a half-life of 5 hr in normal cells (Balhorn et al., 1972e) shows a half-life of approximately 1.8 hr during the time in which it is artificially blocked in metaphase, but it resumes its slower rate of turnover upon release from the block as it enters G_1 phase. In contrast, metaphase-labeled phosphohistones turn over with $t_{1/2} \approx 2$ hr during metaphase, but

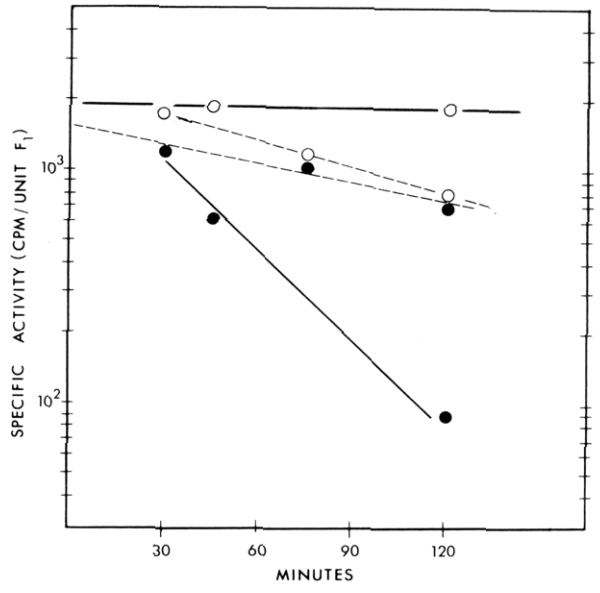

FIGURE 5: Turnover of interphase ^{32}P-labeled and metaphase ^{32}P-labeled F_1 histone when present in mitotic and G_1 cells. Interphase and mitotic HTC cells were labeled with [^{32}P]phosphate as described in the text. The turnover of the ^{32}P-labeled histone was studied in cells blocked in metaphase and also in cells released from mitotic block. (●—●) turnover of metaphase-labeled phosphohistone in G_1 immediately following release from mitotic block; (● - - - ●) turnover of metaphase-labeled phosphohistone in metaphase; (○ - - - ○) turnover of interphase labeled phosphohistone in metaphase; (○ — ○) turnover of interphase labeled histone after release from mitotic block.

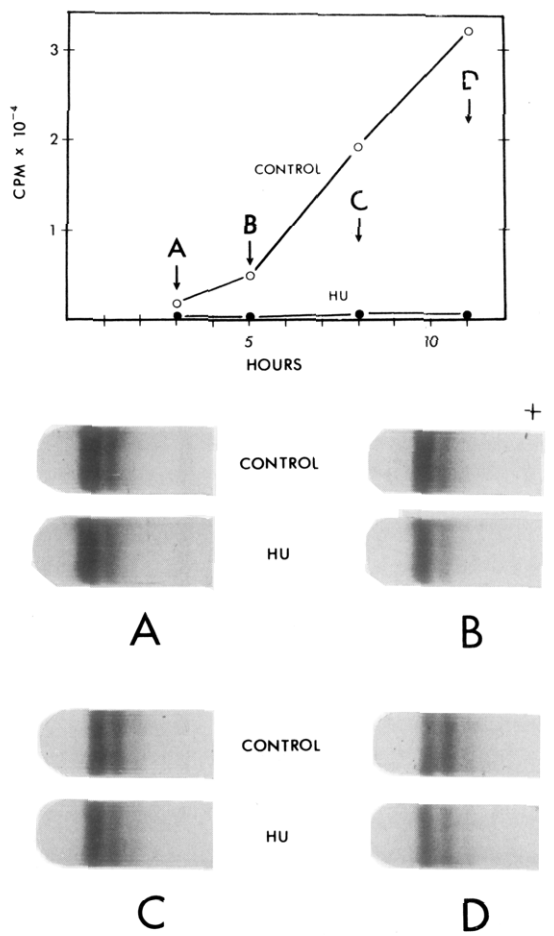

FIGURE 6: Effect of hydroxyurea on histone phosphorylation during transit through the G_1 phase of the cell cycle. HTC cells were synchronized and released into G_1. Two hours after release hydroxyurea (5 mM) was added to one-half of the cells. Aliquots were then removed at the times indicated in the upper panel, which also shows [^3H]thymidine incorporates into normal and hydroxyurea blocked cells. The heterogeneity in normal and hydroxyurea-treated cells at the various time points was analyzed in the usual manner.

the rate of turnover is increased to a half-life of about 25 min immediately upon entering G_1 phase, thus accounting for the absence of typical metaphase histone microheterogeneity following mitosis. We recognize that these turnover graphs are based on *relatively few points in a single experiment*. This, of course, derives from the technical problems of dealing with rather small numbers of cells in the monolayer aspect of the synchrony, and the need for moderately large numbers of cells for the chromatin and histone isolations. However, the turnover rates are reproducible among (three) repeated experiments, though we make no claims other than the observation that the phosphatase activities are grossly different, recognizing that the error on any given half-life could easily be of the order of 20%.

These results, therefore, not only explain the rapid decrease in bulk levels of phosphorylated lysine-rich histones from M to G_1, but also demand that the two types of phosphorylation be different. They do not, however, exclude the possibility that during the colcemid block both metaphase and interphase types of phosphorylation occur, and studies described below are relevant to this point. In addition, these observations offer a convincing explanation for the unexpectedly low levels of phosphorylated histone observed in G_1 cells obtained by the normal synchronization procedures. This is simply explained in as much as the phosphohistone had been exposed to a more active phosphatase during the artificially extended stay in metaphase. In unblocked cells the transit time through metaphase is a matter of minutes and thus no significant loss of interphase phosphohistone is observed as was concluded from the [^3H]lysine pulse-chase experiment and from ^{32}P-turnover studies (Balhorn *et al.*, 1972e).

In order to complete our analysis of the behavior in metaphase of histone phosphorylated during the previous interphase we have posed the following questions: (1) is phosphorylation at the interphase sites suspended during metaphase or (2) is a new steady-state balance developed between a continuing interphase site phosphorylation and a more active phosphatase? It is possible to distinguish between these two possibilities by measuring the amount of phosphorylated histone remaining (as viewed in G_1) after varying time periods in metaphase. Thus by exposing the cells to colcemid for up to 8 hr in metaphase, we can subject the interphase sites to as much as 4 half-lives of phosphate turnover and this would be easily detected in terms of changes in bulk levels of phosphohistone after release. The data shown in Figure 4b indicate that a new steady state had indeed been established for the interphase type of phosphorylation during metaphase. In the new steady state, as expected, because of the more active phosphatase, the bulk level of phosphohistone is much reduced so that only 30% of the lysine-rich histone is phosphorylated. Since the $t_{1/2}$ for the phosphatase activity is known ($t_{1/2}$ = 1.8 hr, see above) and the steady-state level of histone and phosphohistone are measured in this experiment we can calculate the rate constant for the interphase histone kinase activity in metaphase. The value for the interphase rate constant in metaphase is 0.16 hr^{-1} compared to 0.26 hr^{-1} found for histone fully associated with DNA in interphase cells. Thus we incline to the notion that the interphase kinase is moderately

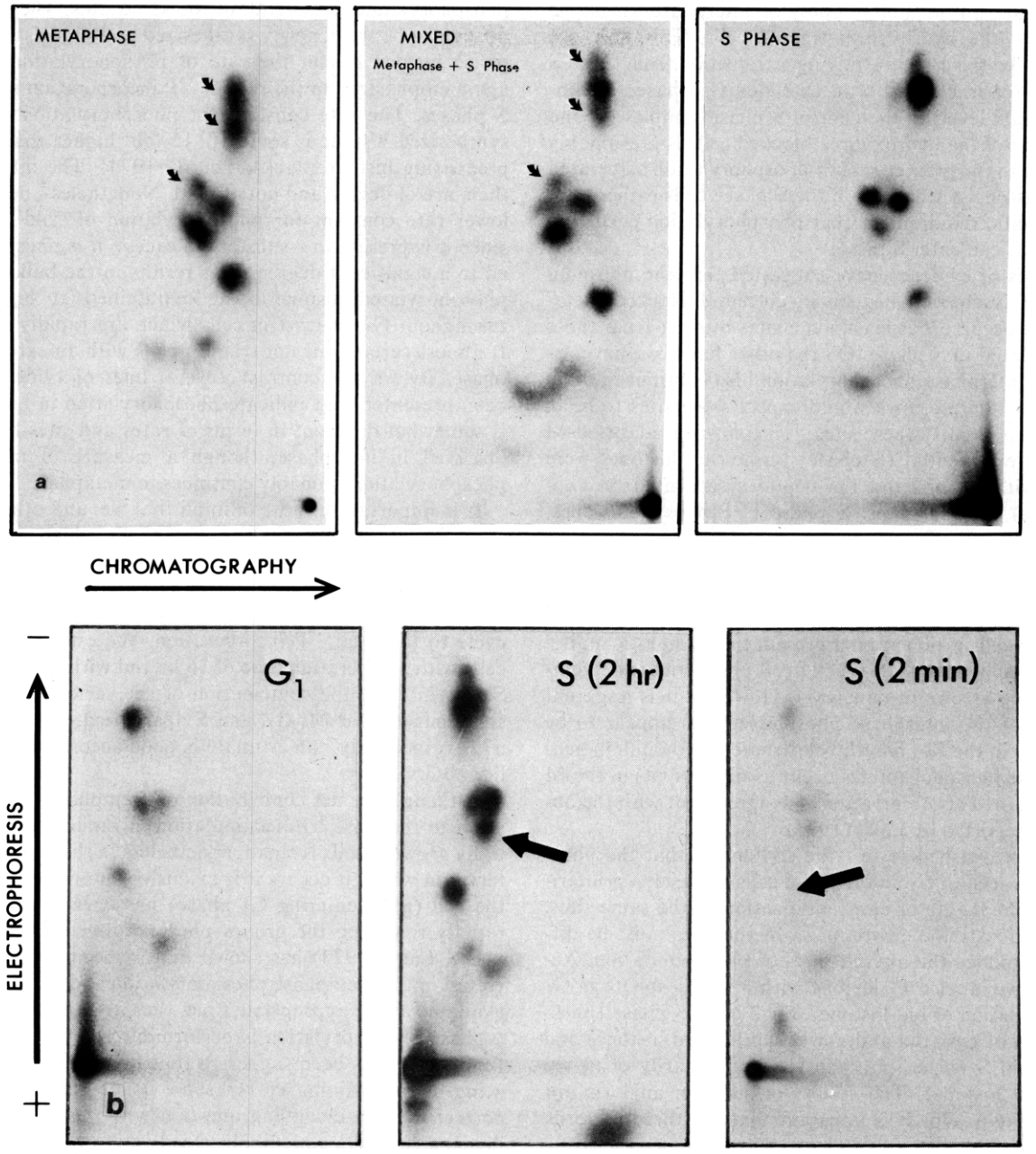

FIGURE 7: Autoradiograms of tryptic ^{32}P-labeled phosphopeptides. (a) M and S phase cells. The lysine-rich histone (F_1) was labeled either in metaphase or S phase cells, isolated, and purified from other histone fractions. Tryptic digestion and fingerprint mapping was performed as described under Materials and Methods. The chromatographic system consisted of 1-butanol–pyridine–acetic acid–water (90:60:18:72). (b) S and G_1 phase cells. The arrow in panel S (2 hr) indicates the position of a spot which is absent from the G_1 panel. The arrow in panel S (2 min) indicates the position of a spot in S (2 hr) which is absent in S (2 min).

active in metaphase and that a new steady-state balance of parental and interphase phosphohistone is set up in the blocked cells at the same time as extensive phosphorylation of the M-phase type is ongoing.

The synchrony studies reported above indicate that after a colcemid block low levels of phosphorylated lysine-rich histone are obtained in G_1 phase, but that the level of phosphorylation increases to dramatically higher levels in S phase. We have extended this approach to ask two questions. (1) Does colcemid itself modify phosphorylation when added to synchronized S phase cells? (2) Since the shift to the phosphorylated forms coincides roughly with a shift from G_1 to S phase, does the increase in phosphorylation occur if the transition is inhibited by treatment with hydroxyurea?

The results of such studies are shown in Figures 6 and 7. Clearly colcemid has no effect on either G_1 or S phase phosphorylation and the massive heterogeneity seen in mitosis is not a reflection of colcemid treatment per se, but is a function of the stage of the cell cycle itself (Figure 2b). In addition, we see that a slow increase in phosphorylation is observed in synchronized G_1 cells which were treated with hydroxyurea to prohibit their entry into S phase (Figure 6). This would appear to be consistent with our current thinking that the levels of phosphorylation in the various phases of the cycle reflect a balance between kinase and phosphatase activity. The level of phosphorylation, artificially lowered by the extended stay in metaphase, slowly recovers when the G_1 cells resume their normal ($t_{1/2}$ = 5 hr) phosphatase activity. Though of course, such an interpretation

demands that an adequate supply of interphase kinase be available in the hydroxyurea-treated cells. This may not necessarily be the case in studying a transition from G_0 to S phase cells or in cells with an extended G_1 phase. The increase in bulk levels in the control S phase samples (C and D) over that of the hydroxyurea blocked cells is presumably in response to the greater rate of phosphorylation associated with the histone synthesized in the S phase cells rather than to any specific requirement that phosphorylation occur before the cells can enter S phase.

Two lines of evidence have suggested that the phosphorylation of lysine-rich histone in colcemid blocked metaphase cells occurs in at least some sites distinct from those phosphorylated in S phase. On the other hand, we have argued that interphase phosphorylation also continues in metaphase and therefore we would expect some sites to be in common. To identify these sites, [^{32}P]phosphate-labeled-M phase and exponential (S phase) lysine-rich histones were digested with trypsin and the peptides separated on two-dimensional peptide maps. S phase [^{32}P]phosphohistones, as shown in Figure 7a, are characterized by the presence of four major spots and several minor ^{32}P-labeled peptides. M phase phosphohistones contain 5 major peptides. In this system we can identify the presence of at least three peptides in M phase cells which are not present to any significant degree in the exponential (S phase) cell phosphopeptide complement (see arrows in panel M). However, it is apparent that most of the interphase phosphopeptides appear to be represented in the M phase-labeled material, though in general S phase phosphopeptides occur as minor spots in the M phase pattern. This observation is in agreement with the observations reported by Lake (1973).

Finally, we wished to test the contention that the phosphorylation seen in G_1 and S phase cells represents primarily a differential rate of enzymatic action at the same sites, though obviously the environment of the sites must be different to produce the altered rates of phosphorylation. Accordingly, we labeled F_1 histone with [^{32}P]phosphate in G_1 (phosphorylation of old histone), for 2 hr in S phase (phosphorylation of both old and newly synthesized histone) and for 2 min in S phase (phosphorylation primarily of newly synthesized histone). The results of such an analysis are shown in Figure 7b. It is apparent that the three patterns are quite strikingly similar. The S-phase histone labeled for 2 hr contains eight spots in common with the G_1 phase material together with one phosphopeptide which is present to a much larger degree in the S phase material (arrow in panel S (2 hr)). The short pulse S phase pattern is very similar to that of the longer pulse S phase and the G_1 pattern, except that the short pulse map lacks one major peptide common to the other two samples (arrows). The material at the origin containing radiolabel does not stain with ninhydrin and, based on our previous studies, is mostly small oligonucleotides.

Discussion

Based on the observations described in this paper, we can draw several conclusions about the phosphorylation of the lysine-rich histones of HTC cells in the G_1, S, and M phases of the cell cycle. The sites of phosphorylation in G_1 and in S phase are almost identical, though certain sites may be preferred to some degree in one phase over another. Since a substantial amount of the phosphorylation in S phase is on newly synthesized histone, we can conclude that newly synthesized histone and preexisting histone (e.g., in G_1) are phosphorylated, in general, at the same sites. The principal difference between newly synthesized and preexisting interphase histone lies in the rate of phosphorylation, as was again emphasized in the rate of ^{32}P incorporation in G_1 and S phases. The rate constant for phosphorylation of newly synthesized histone is some 10–15-fold higher than that of preexisting histone (Jackson et al., 1974). The differences, then, are of degree and not of kind. Nonetheless, despite the lower rate constant for phosphorylation of "old" histone, since it is present in a substantial excess, it is phosphorylated to a significant degree. This results in the bulk levels of phosphorylated histone being maintained at high levels throughout G_1, at least in cells which are rapidly dividing. It almost certainly is not true for cells with an extended G_1 phase. By way of contrast, several lines of evidence have been presented that indicate phosphorylation in metaphase is somewhat different in terms of rates and sites from that observed in interphase, though a measure of interphase phosphorylation probably continues in metaphase.

It is important to bear in mind that we, and others, have studied phosphorylation in a metaphase which was artificially extended many fold over its normal time period. Normally it is a brief episode in the cell cycle and it is possible to assess the net contribution of various phases of the cell cycle to the total ^{32}P incorporation. We estimate in HTC cells with a generation time of 16 hr and with G_1 = 6 hr and S = 8 hr, that the contribution of the various phases in a short pulse is for M, G_1, and S approximately 15, 18, and 67%, respectively,[1] an estimate in good accord with our earlier observations.

Although the net contribution of metaphase phosphorylation to the total ^{32}P accumulation in randomly, exponentially growing cells is small, nonetheless in the short time interval in which it occurs it is extensive and rapid. Likewise, the cell (after entering G_1 phase) has devised a means of rapidly removing the groups phosphorylated during metaphase. Lake (1973) has shown that a specific kinase is involved in the metaphase phosphorylation and that it is made available at the appropriate time, thus arguing that the metaphase phosphorylation is performing a key chromosomal function. It has been suggested that it is involved in generating or maintaining chromosome condensation. If this is correct it is an intriguing question why the system should demand so active a metabolic steady state with a rapid rate of phosphorylation balanced by an active phosphatase. However, it should be pointed out that HTC cells possess a highly active cytoplasmic protein kinase which is almost constitutive in its activity in the sense that it possesses little of the cAMP binding control subunit (Granner, 1972). Exposure of the chromosomes to the cytoplasm during metaphase might well lead to a measure of untoward phosphorylation which is rapidly removed after the cells have completed mitosis. This remains a moot point, but clearly caution is required before we can develop an extensive model of the function of histone phosphorylation in metaphase chromosomes (Bradbury et al., 1974; Louie and Dixon, 1973).

More detailed information is available concerning the in-

[1] This calculation is based upon rate constants calculated from steady-state phosphorylation levels in M and G_1. The rate constant for phosphorylation in S phase is calculated from the rate of shift of [^{3}H]lysine from parental to phosphorylated forms after a 1-min pulse. The rate constants are 4.0, 0.26, and 2.5 hr^{-1} for M, G_1, and new histone phosphorylation. Such a treatment assumes ATP is constant throughout and that the reaction is therefore pseudo first order, as discussed in a previous report (Tanphaichitr et al., 1974).

terphase (primarily S phase) phosphorylation, though it would be premature indeed to assign it to a precise or specific function. It appears that the critical aspect about this form of F_1 phosphorylation is that newly synthesized histone be phosphorylated promptly (it is 65% phosphorylated in 45 min), but that the bulk levels of gross phosphorylated species may serve no vital function. This latter point is deduced from the following observations: (1) cells released from G_0 can traverse much of G_1 successfully without a need to increase the bulk levels of phosphohistone (Gurley et al., 1973a); (2) rapidly cycling cells depleted of much of the phosphorylated histone by an extended stay in colcemid (this paper) appear to be able to traverse G_1 and enter S and subsequently to divide without injury; (3) cells, partially depleted of phosphohistone in S phase (by cycloheximide block), can resume DNA synthesis without increasing bulk levels of phosphohistone, though new histones are still vigorously phosphorylated (Tanphaichitr et al., 1974).

Thus interphase phosphorylation appears to play a key role in S phase, shortly after the histone is synthesized. This could involve organization of the histone into its final conformation on the chromosome or host of possibilities more directly concerned with chromosome replication itself. The answers to these problems are far from clear; however, some of the inconsistencies in the field observed during the last two years can now be explained in terms of varying rates of metabolism of modifications occurring in different phases of the cell cycle. Recent observations in this laboratory indicate that, over shorter time periods of study than those described herein, we can observe a bewildering array of acetylation and phosphorylation changes with vastly differing metabolic rates in all histone fractions, and many of these observations are made on cells not involved in DNA replication. Thus, it appears that the area of histone modification must experience a further period of increasing complexity before simplifying generalities are perceived.

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