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Further Studies on Histone Phosphorylation in the Presence of Inhibitors of DNA Synthesis†

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ABSTRACT: Further studies on the effect of inhibitors of DNA and protein synthesis upon continued histone phosphorylation are presented. The capacity to phosphorylate the lysine-rich (F_1) histone in hydroxyurea-treated cells appears to be maintained while the rate of phosphorylation is decreased. In the presence of cycloheximide histone phosphorylation is decreased while histone phosphatase is activated, probably as a result of the inhibition of protein synthesis. The cycloheximide-induced activation of phosphatase occurs both in G_1- and S-phase cells. The rate constant for the phosphorylation of preexisting histone appears to be unchanged by the addition of cycloheximide. In addition, upon removal of cycloheximide newly synthesized histone is extensively and rapidly phosphorylated while the "old" preexisting histone is restored to the normal bulk phosphorylation levels at a much slower rate.

The phosphorylation of certain specific histone fractions associated with the process of cell multiplication is now well established (Balhorn *et al.*, 1971, 1972a,b,d; Sherod *et al.*, (1970). Phosphorylation of the lysine-rich histone appears to be particularly active during the S-phase of the cell cycle (Balhorn *et al.*, 1972c; Gurley *et al.*, 1973a; Marks *et al.*, 1973), though a measure of phosphorylation does continue after this phase of the cell cycle is complete (R. Balhorn *et al.*, manuscript in preparation; Gurley *et al.*, 1973a,b; Lake, 1973a,b; Oliver *et al.*, 1972).

The possibility has been raised that histone phosphorylation and DNA synthesis might in some way be interdependent. At this time we know of no data concerning the dependence of DNA synthesis upon histone phosphorylation since specific means for inhibiting histone phosphorylation are not available. On the other hand DNA synthesis can be rapidly inhibited. The attendant effect upon histone phosphorylation depends upon the inhibitor used. Hydroxyurea has little effect on the rate of histone phosphorylation for 2 hr, but during a subsequent 5-hr period the rate of phosphate incorporation falls to about 40% of control values (Balhorn *et al.*, 1973). Cycloheximide, which rapidly inhibits DNA and histone synthesis in HTC cells, has a more immediate effect upon the rate of histone phosphorylation which falls to about 40% of control within 90 min after adding the drug (Balhorn *et al.*, 1973).

We have further characterized the nature of histone phosphorylation in the presence of these two inhibitors of DNA synthesis. In hydroxyurea the nature of histone phosphorylation appears in all its properties to be indistinguishable from that occurring in uninhibited cells. In the presence of cycloheximide, however, the most significant contribution to the decrease in phosphorylation rate appears to be due to the loss of a continuing supply of fresh histone. Upon removal of cycloheximide, DNA synthesis is rapidly resumed and it was of interest to ask if rephosphorylation of histone preceded this event, or not.

Materials and Methods

Cell Culture. HTC cells, derived from Morris minimal deviation hepatoma 7288c, were grown in suspension culture in a modified Swims S-77 medium (Grand Island Biological Co.) and were maintained under conditions of exponential growth at a cell density of between 2×10^5 and 8×10^5 cells/ml as previously described (Balhorn *et al.*, 1972c).

Isolation and Analysis of Histone. Histones were isolated from HTC cells as described previously (Balhorn *et al.*, 1972d). Precipitation of the histone sulfate was accomplished by dialyzing against ethanol. The histone samples were dissolved in 0.9 N acetic acid containing 20% sucrose and 0.5 M β-mercaptoethanol. Electrophoresis was performed on either short or long polyacrylamide gels using modifications of earlier techniques (Balhorn *et al.*, 1972d).

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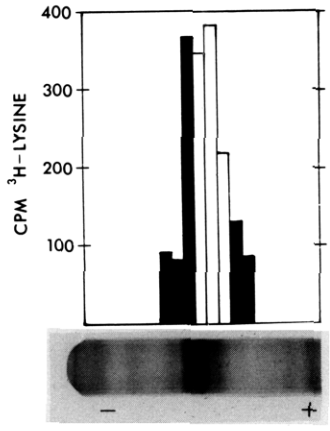

FIGURE 1: Phosphorylation of lysine-rich histones in the presence of hydroxyurea (5 mM). Electrophoresis was performed for 80 hr at 4° in 25-cm gels and radioactivity assayed as described in the text. The open bars indicate [^{3}H]lysine incorporated into phosphorylated species of the F_1histone. The filled bars show the presence of [^{3}H]lysine in the parental (unphosphorylated) form and also give a measure of gel background radioactivity.

After destaining the gels were sliced and either counted directly (^{32}P) in a gas flow counter or were dissolved in 0.3 ml of hydrogen peroxide (30%) at 60° for 12 hr and counted in a scintillation counter using Bray's solution (Bray, 1960). Specific activities of histone fractions were determined as counts per minute per unit of histone area measured from gel microdensitometric scans.

Turnover of F_1 Histone Phosphate in the Presence of Cycloheximide. Two liters of HTC cells were labeled with 10 mCi of [^{32}P]phosphate for 3 hr. The [^{32}P]phosphate was then removed by centrifuging the cells at 200*g* and resuspending them in fresh medium without [^{32}P]phosphate. Cycloheximide (10 μg/ml) was added at the appropriate time as indicated in the text. Cells (250 ml) were collected, pelleted, and frozen at various times after adding the inhibitor. Histone was isolated and the specific activity of ^{32}P-labeled F_1 phosphate was determined as described above. Control experiments indicated that incorporation of ^{32}P continued for 20 min after the completion of the pulse, whereafter the observable chase begins. That >90% of the ^{32}P label can be removed after 3 hr in cycloheximide indicates that little reutilization is occurring at this time.

DNA Synthesis after Removal of Cycloheximide. HTC cells were treated with cycloheximide at a concentration of 10 μg/ml for 3 hr. The cells were then centrifuged at 600*g* for 5 min at 25° and resuspended in fresh medium without the drug. At various times after the resuspension, 10-ml aliquots were pulsed with 1 μCi/ml of [^{3}H]thymidine for 15 min. Control experiments were performed without cycloheximide pretreatment. At the end of the pulse, cells were centrifuged at 600*g* for 2 min at 4° and stored frozen. The frozen cell pellets were resuspended in distilled water (1 ml), sonicated, and treated with 1 ml of 40% trichloroacetic acid. The suspension was sonicated again for 15 sec. Macromolecules were sedimented at 12,000*g*. The pellets were washed twice with trichloroacetic acid and dissolved in 1.8 ml of 0.3 N potassium hydroxide. The solution was incubated at 37° for 18 hr. Sulfuric acid was added to a final concentration of 0.4 N to precipitate DNA. The DNA was sedimented at 12,000*g*, resuspended in 0.4 N sulfuric acid, and then hydrolyzed at 90° for 20 min. The protein residue was sedimented and removed. Aliquots (100 μl) of the supernatants (which contains deoxyribonucleotides) were assayed by the diphenylamine reaction and counted in 10 ml of Bray's solution.

F_1 Histone Phosphorylation after Removal of Cycloheximide. Cycloheximide-pretreated HTC cells were prepared as described above. At specific times 250-ml aliquots were removed and pulsed with 2 mCi of [^{32}P]phosphate (Amersham–Searle) for 30 min. At the end of the pulse, the cells were centrifuged at 600*g* for 2 min and stored frozen. Histone was isolated from the frozen cell pellets and analyzed as described above.

Phosphorylation of Preexisting Histone after Cycloheximide Removal. One liter of HTC cells was labeled with 2 mCi of [^{3}H]lysine for 30 min. At the end of the pulse, the cells were pelleted by centrifugation and resuspended in fresh medium without radioactivity. Cycloheximide was either added immediately or 9 hr after resuspension. Three hours after treatment with the drug, cycloheximide was removed as described before. An aliquot of cells (250 ml) was collected at 0 and 45 min after cycloheximide removal. After electrophoresis of the isolated histones on long (25 cm) gels the phosphorylated and parental F_1 bands were dissected out, digested with hydrogen peroxide, and counted.

Phosphorylation after Cycloheximide Removal. Cycloheximide-pretreated cells (500 ml) were prepared as above. Ten minutes after removing cycloheximide, the cells were labeled with 1 mCi of [^{3}H]lysine for 10 min. A control experiment was performed with untreated cells. An aliquot of cells (250 ml) was collected, pelleted, and frozen. The remaining cells were resuspended in fresh medium and collected 20 min later. Histone was isolated and the ^{3}H label on F_1 was assayed as above.

Results

Previous experiments had shown that the rate of phosphate incorporation into the F_1 histone in cells blocked with hydroxyurea gradually falls until it reaches a constant value which is about 40% of control (Balhorn *et al.*, 1973). Preliminary studies had suggested that, as the rate of histone synthesis gradually decreased upon exposure to hydroxyurea, the availability of new sites for phosphorylation was reduced and under these conditions becomes primarily dependent upon production of available serine residues by the on-going phosphatase action. In this case the reduced rate of ^{32}P incorporation in the presence of hydroxyurea might simply reflect an unaltered phosphokinase activity and a reduced supply of sites. This has been tested in two ways. The first approach was to assay the capacity of hydroxyurea-blocked cells to phosphorylate newly synthesized histone, a material which necessarily has a plentiful supply of fresh sites. HTC cells were blocked with 5 mM hydroxyurea for 6 hr. The blockade was continued for an additional 2 hr in the presence of [^{3}H]lysine. Although the ability of the cells to manufacture histone at this stage is reduced, a significant degree of histone synthesis does indeed occur in cells which are not making DNA, as has been documented previously (Balhorn *et al.*, 1973). The cells were collected and histone isolated. Electrophoretic analysis showed the typical lysine-rich histone heterogeneity due to the presence of phosphorylated histone. Individual bands were dissected out and analyzed for [^{3}H]lysine to assay for a shift of parental histone into a phosphorylated form of the molecule. The data of Figure 1 show that the newly synthesized histone is extensively phosphorylated. Approximately 75% of the radiolabel is found in the phosphorylated bands (even triphosphorylat-

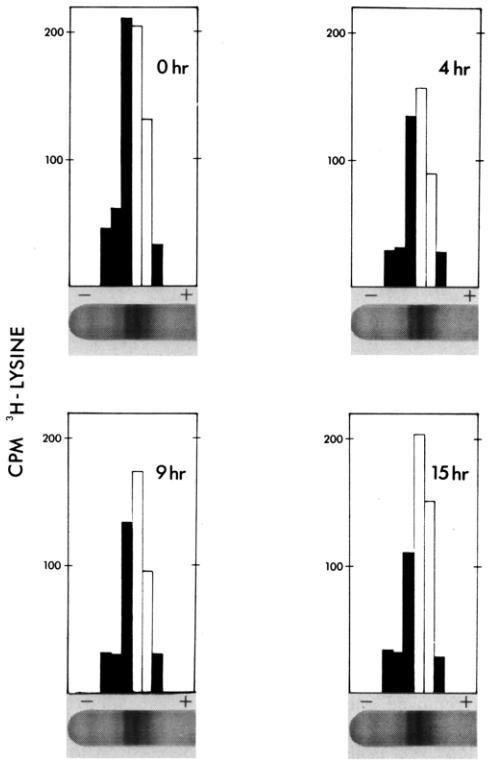

FIGURE 2: Rephosphorylation of lysine-rich histone in hydroxyurea-treated cells. Exponentially growing cells were pulse labeled with [^{3}H]lysine (1 μCi/ml) for 2 hr. The label was removed, the cells were resuspended in 1600 ml of fresh medium and samples were collected at the times indicated. Hydroxyurea (5 mM) was present throughout. Electrophoresis and radioactivity determinations were performed as described in the text. The open and filled bars have the same meaning as in Figure 1.

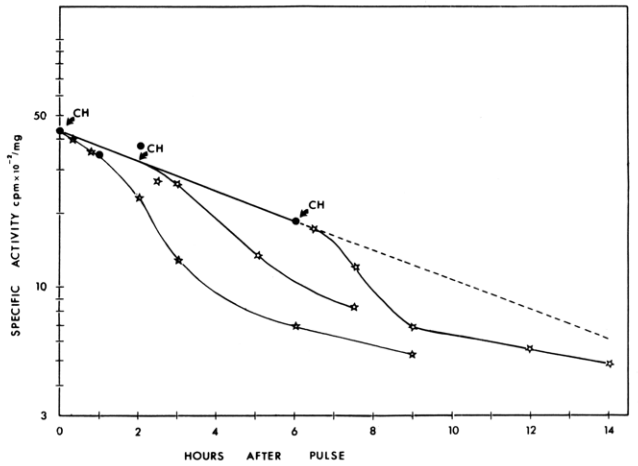

FIGURE 3: Cycloheximide-induced activation of histone phosphate turnover occurs in several phases of the cell cycle. Exponentially growing HTC cells were pulse labeled with 10 mCi of [^{32}P]orthophosphate for 2 hr. Radioactivity was then removed and cycloheximide (10 μg/ml) was added either immediately, 2 hr, or 6 hr later (arrows). Samples of cells (250 ml) were collected at various times after the addition of cycloheximide. Histone was isolated and the incorporation of [^{32}P]phosphate into the F_1 fraction was determined. The turnover rate of F_1 phosphate under normal conditions (untreated exponentially growing HTC cells) (●), the extrapolated turnover rate of F_1 phosphate under normal conditions (- - -), and the turnover rate of F_1 phosphate of the cycloheximide-treated exponentially growing HTC cells are shown (★).

ed F_1 histone can be detected), indicating the substantial capacity of hydroxyurea-treated cells to phosphorylate available substrate.

In a second experiment, normally growing HTC cells were labeled with [^{3}H]lysine for 2 hr. The cells were collected, washed and resuspended in fresh medium containing 5 mM hydroxyurea, but no radiolabel. At the times indicated in Figure 2 samples were withdrawn and histone isolated. The results shown in Figure 2 indicate the degree of phosphorylation found at the end of the pulse and before the addition of hydroxyurea. The extent of phosphorylation is about 60% and as such is somewhat less than that seen for freshly synthesized histone in the presence of hydroxyurea. During the subsequent 15 hr of the chase period the [^{3}H]lysine in the phosphorylated bands does not return to the parental bands, but rather the extent of phosphorylation is maintained at an essentially constant level. This can be interpreted in two ways. Either (a) there is no removal of phosphate from phosphorylated histone when hydroxyurea is present or (b) there is an active phosphate removal, but the histone is subsequently rephosphorylated so that constant bulk levels of phosphorylated species are maintained. That the second alternative is correct was shown by an analysis of phosphate turnover in these cells in the presence of hydroxyurea. Cells were labeled with [^{32}P]phosphate for 3 hr either before adding the hydroxyurea or immediately after its addition. After the 3-hr pulse the ^{32}P was removed and in both cases the chase–turnover part of the experiment was conducted in the presence of hydroxyurea. The turnover (data not shown) was the same in both cases and showed a $t_{1/2} \simeq 6$ hr.

Cycloheximide-treated HTC cells lose the ability to direct DNA and histone synthesis within 10 min after the addition of the drug (Balhorn *et al.*, 1973). Incorporation of ^{32}P into phosphorylated histones decreases to approximately 35% of control values during the ensuing 60 min. This reduced rate is then maintained essentially unchanged for 7 additional hr of cycloheximide treatment. Previous studies had indicated a general activation of the histone phosphate phosphatase over a 3-hr period following cycloheximide addition (Balhorn *et al.*, 1973).

We wondered if the histone phosphate phosphatase responded in the same way to cycloheximide addition in phases of the cell cycle other than the S-phase. This was studied by pulse labeling the cells for 2 hr in the presence of [^{32}P]phosphate. The radiolabel was removed and the cells were permitted to grow for an additional 2–6 hr. By the sixth hour most of the cells in the S-phase during the pulse period would now have moved on to other phases of the cell cycle and a substantial proportion of such cells would have entered the G_1-phase. Cycloheximide was added at either 2 or 6 hr after removing ^{32}P and the effect of the drug on histone phosphate turnover was assayed. The results shown in Figure 3 indicate that in both cases a substantial activation of the phosphatase occurs about 30–60 min after addition of cycloheximide but the activation is independent of elapsed time since the initial incorporation of radioactive phosphate. Thus we conclude that it is a property common to all interphase cells. The data of Figure 3 also show that a constant small fraction (about 10%) of the ^{32}P associated with histone is largely resistant to the action of the activated phosphatase, though this is difficult to assess precisely as the amount of ^{32}P still associated to histone after extended exposure to cycloheximide is very small.

Since cycloheximide treatment leads to an activation of the phosphatase, the net effect is to lower the amount of phosphorylated F_1 species to low levels (see Figure 4a,b).

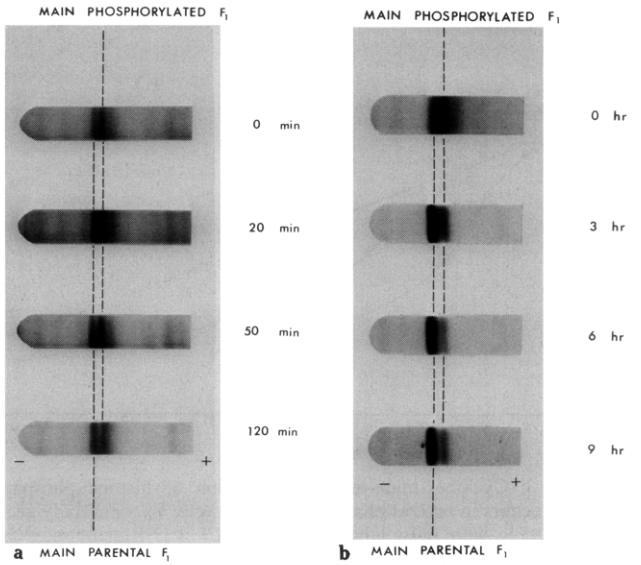

FIGURE 4: The effect of protein synthesis inhibitors on the extent of F_1 histone phosphorylation as a function of time. Exponentially growing HTC cells were treated with either (a) cycloheximide (10 μg/ml) for 0–120 min; (b) cycloheximide (10 μg/ml) for 0–9 hr. Aliquots (250 ml) were collected at various times after the addition of the drug. Histones were isolated and electrophoresed on high-resolution polyacrylamide gels. The change in microheterogeneity of the F_1 histone species is shown as a function of time in the presence of the drugs.

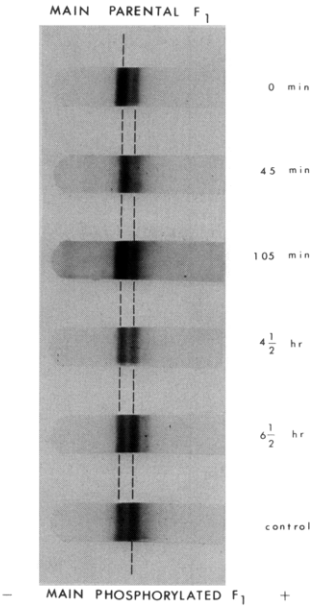

FIGURE 6: Recovery of extensive phosphorylation of F_1 histone following removal of cycloheximide. Exponentially growing HTC cells were treated with cycloheximide (10 μg/ml) for 3 hr. Cycloheximide was then removed and the cells were resuspended in fresh medium. Samples of cells (250 ml) were taken at various times after resuspension. Histone was isolated and electrophoresed on the high-resolution polyacrylamide gel. The microheterogeneity of the F_1 species is shown as a function of time after release from the drug.

During the time span between the third and the ninth hour of treatment the ratio of phosphorylated species to parental form is essentially constant, indicating a balance has been established between the rate of phosphorylation on the one hand and the phosphatase on the other. This would appear to indicate either that the kinase is not turning over to any great extent or that the rates of turnover of both kinase and phosphatase are similar. This appears to be a response induced by inhibitors of protein synthesis and is not specifically a property of cycloheximide itself. This was shown by an identical response upon treatment with puromycin.

We have attempted to exploit the ability of cycloheximide to lower gross phosphorylation levels. HTC cells previously blocked in cycloheximide recover the capacity to make DNA relatively quickly after removal of the drug and we have therefore asked the question of whether histone phosphorylation recovers at a comparable or slower rate. Thus, for instance, if histone phosphorylation were not increasing toward normal at a time in which DNA synthesis had fully recovered one would be justified in concluding that DNA synthesis was not dependent upon the act of histone phosphorylation. HTC cells were treated with cycloheximide for 3 hr until the bulk levels of F_1 histone phosphate were reduced to the lowest levels attainable. The cycloheximide-containing media was removed by centrifugation and cells were resuspended in fresh media and subsequently assayed for DNA synthesis and histone phosphorylation by standard techniques. The results shown in Figures 5 and 6 may be summarized as follows. (1) DNA synthesis is resumed within 30 min of removal of cycloheximide though it rarely attains more than 70% of control cultures which had not been treated with cycloheximide. (2) Phosphate incorporation is likewise elevated rapidly, though it too does not return to the values seen for the control cultures. (3) Bulk levels of phosphorylated F_1 histone return to that characteristic of an exponentially growing culture over a 4–6-hr period (Figure 6), a significantly slower time period than that required for a resumption of DNA synthesis.

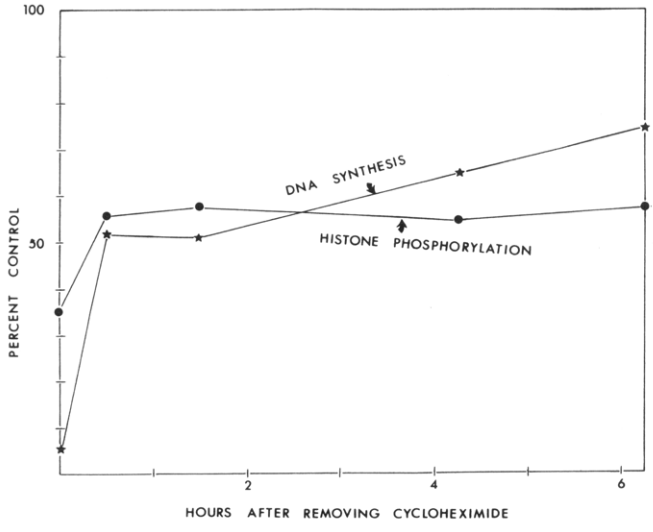

FIGURE 5: Recovery of DNA synthesis and histone phosphorylation following removal of cycloheximide. Exponentially growing HTC cells were treated with cycloheximide (10 μg/ml) for 3 hr. Cycloheximide was then removed and the cells were resuspended in the fresh medium. F_1 histone phosphorylation (●) and DNA synthesis (★) were assayed at different times after release from the drug as described under Materials and Methods.

Bulk levels of phosphorylated histone isolated from randomly growing cells reflect the contribution of F_1 histone from cells in all phases of the cell cycle. The possibility existed that S-phase cells (making DNA) might phosphorylate F_1 histone more extensively than cells in G_1 or *vice versa*. This possibility can be tested directly by labeling histone with [^{3}H]lysine for a short period immediately prior to adding cycloheximide. After the incubation with cycloheximide and its subsequent removal we assayed for the rate at which ^{3}H-labeled parental F_1 histone was shifted into the

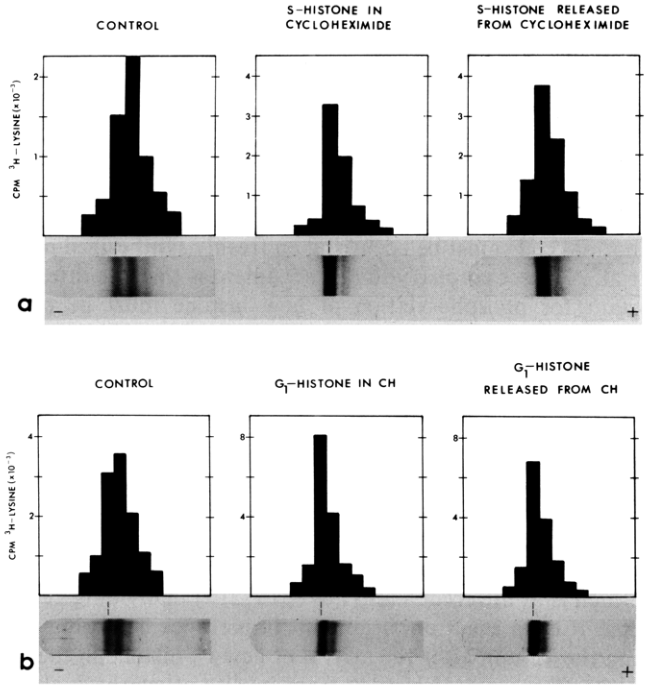

FIGURE 7: The phosphorylation upon removal of cycloheximide of (a) F_1 histone in S-phase cells and (b) preexisting F_1 histone in G_1-phase cells. (a) Exponentially growing HTC cells were labeled for 30 min with 1 mCi of [^{3}H]lysine. Radioactivity was then removed and cycloheximide (10 μg/ml) was added immediately. An aliquot of cells (250 ml) was taken at the end of the third hour of cycloheximide treatment. Cycloheximide was then removed from the medium and 45 min later another 250-ml aliquot of cells was taken. Histone was isolated and the level of phosphorylation of F_1 fraction was assayed as described under Materials and Methods. A control experiment was performed similarly without treating the cells with cycloheximide. (b) The level of phosphorylation of preexisting histone in G_1-phase cells was determined in a similar experiment except that after the labeling period the cells were permitted to grow for 9 hr, at which time the bulk of the labeled histone would be in G_1-phase cells, and then were treated with cycloheximide.

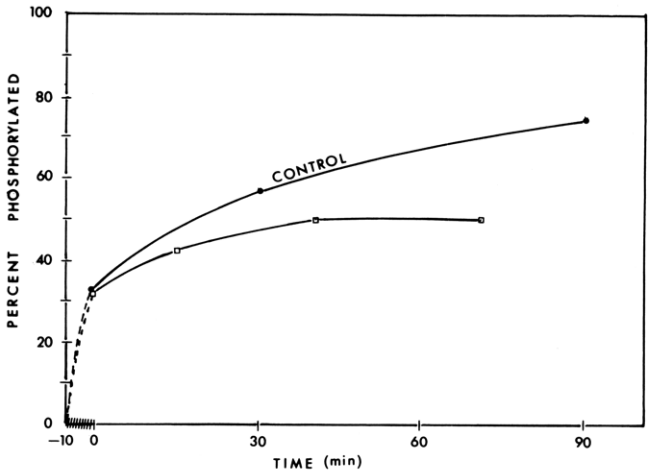

FIGURE 8: Phosphorylation of new histone synthesized immediately following release from cycloheximide blockade. Exponentially growing HTC cells were treated with cycloheximide (10 μg/ml) for 3 hr. Cycloheximide was then removed and 10 min afterward the cells were pulsed with 4 mCi of [^{3}H]lysine for 10 min so that newly synthesized histone is labeled. The radioactivity was removed and an aliquot of 250 ml of cells was taken immediately. The rest of the cells were allowed to grow for the times indicated. Histone was isolated and assayed for the level of phosphorylation as described under Materials and Methods. A control experiment was performed similarly except that the cells were not exposed to cycloheximide. The data are presented in terms of the extent (per cent) of phosphorylation of newly synthesized F_1 histone in control (-●-●-) and cycloheximide pretreated cells (-□-□-□-). The data are interpolated (- - -) through a value of zero phosphorylation at the beginning of the pulse period ($t = -10$ min), thereby assuming that the histone molecule incorporates no serine phosphate during its synthesis.

phosphorylated bands. The results are shown in Figure 7a. Clearly, the preexisting histone synthesized immediately prior to the addition of cycloheximide is not phosphorylated with great rapidity after removal of the drug. The fraction of the F_1 histone in the phosphorylated form increased from 40% in the presence of cycloheximide to only 42% during the 45 min following its removal, an insignificant increase. Likewise, histone was labeled with [^{3}H]lysine in the S-phase and chased for 9 hr so that the bulk of labeled histone was primarily in the G_1 region. Histones in S-phase cells can be specifically labeled with [^{3}H]lysine as one may assume that histone synthesis is restricted to the S-phase. Cycloheximide was added for 3 hr and then removed. In Figure 7b, we see that the phosphorylation recovery of G_1-phase histone is quite slow, with little additional phosphorylation occurring during the time period in which DNA synthesis has so effectively recovered. However, it has been shown above (Figure 5) that the rate of phosphorylation increases during the 45 min after release from the cycloheximide block. A major candidate for rapid phosphorylation must therefore be the histone newly synthesized after release of the block, as histones in G_1 cells and old histones in S-phase cells have been excluded. That this appears to be the correct interpretation was shown by adding [^{3}H]lysine immediately after removing cycloheximide.

The data shown in Figure 8 indicate that about 50% of the incoming, newly synthesized histone was phosphorylated in the 45-min period after release of the cycloheximide block. This amounts to some 80–90% of the efficiency with which new histone was phosphorylated in control cultures which had been treated in an identical manner throughout all the operations, except that the cycloheximide treatment was omitted.

Phosphorylation is a postsynthetic modification and thus phosphorylation of 50% of the incoming F_1 histone within a 45-min period represents a substantial and rapid addition of phosphate groups to these molecules in contrast to the slow conversion of old F_1 histone into the phosphorylated forms during this time period as shown in Figures 6 and 7.

Discussion

The observations reported in this paper indicate that an interruption in DNA synthesis *per se* apparently has no direct effect on the capacity of a given cell to phosphorylate the lysine-rich histone, though the rate of phosphorylation may change depending upon several variables encountered in the present work including levels of newly synthesized histone with phosphorylatable sites, levels of the appropriate kinase, and the activity of the histone phosphate phosphatase.

In hydroxyurea the primary variable is a gradual decrease in the amount of new histone which is synthesized, and this is reflected in a gradual fall in the rate of ^{32}P incorporation as a function of time that the cells spend in hydroxyurea. The histone which is synthesized in the presence of hydroxyurea is, however, rapidly and extensively phosphorylated. Presumably, the final value for the rate of ^{32}P incorporation reflects a balance between continued rephosphorylation of histone and histone phosphate removal.

In cycloheximide all three parameters discussed above are changing in a complex way, particularly since histone synthesis is inhibited within 10 min of the addition of the drug. An interpretation of the rate of ^{32}P incorporation into cycloheximide-treated cells is complicated by the unforeseen activation of the histone phosphate phosphatase. In a response quite different to that seen upon the addition of hydroxyurea, cycloheximide increases the turnover rate of F_1 phosphate about threefold shortly after the drug addition. This leads to a gross change in the amount of parental and phosphorylated forms of F_1 histone from a normal value characteristic of rapidly growing cells (60% phosphorylated) to that resembling F_1 in slowly growing cells (30% phosphorylated).

The decrease in the bulk chromosomal content of phosphorylated F_1 species upon treatment with cycloheximide is seen both in G_1- and in S-phase cells. The phosphatase activation appears to be a result of protein synthesis inhibition and puromycin gives rise to a similar response. The amount of phosphorylated species in the presence of the activated phosphatase presumably represents a steady-state balance of the kinase and the phosphatase. Simple calculations show that the rate constant for the phosphorylation of "old" histone is essentially unchanged by the addition of cycloheximide.[1]

The results obtained with cycloheximide treatment of HTC cells offered us a potent tool for probing the role of histone phosphorylation. This derives from two facts: (1) the levels of phosphorylated histone are quite low after 3 hr of treatment with cycloheximide; (2) DNA synthesis recovers very rapidly upon removal of the inhibitor. Thus, we could pose the following questions: (1) must the whole histone F_1 complement be massively rephosphorylated to permit reinitiation of DNA synthesis, (2) need only those F_1 histones in S-phase cells be rephosphorylated, (3) need only newly synthesized histone be extensively phosphorylated, and finally (4) could DNA synthesis be reactivated without a significant degree of any type of histone phosphorylation? The answers to these questions indicate that newly synthesized histone is rapidly phosphorylated upon resumption of DNA synthesis while the "old" histone is restored to the normal bulk levels of phosphorylated histone at a much slower rate, long after DNA synthesis has recovered, presumably as the phosphatase returns to its normal lower level of activity. There is no evidence that the phosphorylation of newly synthesized histone is a prerequisite for DNA synthesis, merely that the two events appear to occur concurrently.

The implications arising from this study are twofold. If the rate constant for phosphorylation of old histone is unaffected by inhibition of DNA synthesis and if the rate of phosphorylation in the presence of cycloheximide is 35–40% of normal, then the other 60–65% of phosphorylation in normal cells must be occurring on freshly synthesized material. A direct corollary to this argument is that the rate constant for phosphorylation of new histone must be much higher than that for the much larger amount of old histone. Such a high rate of phosphorylation of new histone is entirely consistent with the observation that new histone is rapidly phosphorylated when DNA synthesis resumes after removal of cycloheximide (Figure 8), whereas the degree of rephosphorylation of older histone is much less in the same time period (Figure 6).

Our current assessment of the role of interphase histone phosphorylation (as opposed to that occurring in mitosis which is at different sites (Lake, 1973b)) in dividing cells is that it is an event of paramount importance occurring during the 45 min after the arrival of new F_1 histone in the nucleus (transport of histone from its site of synthesis to the nucleus is very rapid). Perhaps it is critical to the final organization of this molecule onto the DNA itself. It seems feasible that the smaller rate of phosphorylation of old histone may be perhaps no more than a low efficiency recognition of the original phosphorylatable serine residues, now perhaps somewhat more buried in the chromosome structure. Thus, the "bulk" phosphorylation levels we see may have no physiological significance at all, merely reflecting an unavoidable low level of continued enzyme activity. Obviously, a critical test of this notion will depend on the identity of the serine residues which are phosphorylated on old and new histone molecules. These observations will be reported shortly.

[1] Application of steady-state kinetics to the constant bulk levels of phosphorylated F_1 and parental species is based upon the assumption that levels of ATP within HTC cells are constant during the process of phosphorylation of histone. In the steady state, the rate of phosphorylation = the rate of phosphate hydrolysis. $k_P(40) = k_H(60)$, where k_P is the rate constant for phosphorylation including the term for the constant level of ATP, (40) is the concentration of parental unphosphorylated histone in arbitrary units (because the concentration in the cell is unknown in absolute units; however, it is known that the ratio phosphorylated F_1/parental F_1 = 60/40 in normally growing exponential cells). (60) is the concentration of phosphorylated species and k_H is the rate constant for phosphate hydrolysis. k_H is known from phosphate turnover studies and is equal to 0.13 hr^{-1}. We can therefore calculate that $k_P = 0.18$ hr^{-1}. Again, assuming a steady state is reached during the latter stage of cycloheximide treatment (see above) with phosphorylated F_1/parental F_1 = 30/70, then $k_P'(70) = k_H(30)$ where k_P' is the rate constant for phosphorylation in cycloheximide and $k_H = 0.45$ hr^{-1} for the activated phosphatase with $t_{1/2} = 1.5$ hr. Thus, $k_P' = 0.19$ unit in good agreement with the rate constant seen in exponentially growing cells. In rapidly dividing cells, such as HTC cells, the kinase activity persists throughout all stages of the cell cycle and thus the amount of phosphorylated histone is maintained at 60% even in the G_1-phase (manuscript in preparation). Obviously, such a continued kinase activity in essentially all cells is an absolute prerequisite for the above calculations to possess validity.

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