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Molecular Nature of F_1 Histone Phosphorylation in Cultured Hepatoma Cells†

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ABSTRACT: We have studied the molecular nature of lysine-rich (F_1) histone phosphorylation in hepatoma tissue culture cells. In a series of pulse-labeling experiments we have shown that (1) both newly synthesized (new) and preexisting (old) F_1 histone are phosphorylated, (2) greater than 85% of all the newly synthesized F_1 is phosphorylated in a single, unique event during each cell cycle, and finally (3) there is a time lag of some 30–60 min between the time of histone synthesis and its subsequent phosphorylation. These data tend to exclude the event of F_1 phosphorylation as a mechanism whereby specific genes are activated or repressed; nor do they support the idea that F_1 phosphorylation is a transport device. Although we have no direct evidence as to the function of lysine-rich histone phosphorylation, our results from these and previous studies make it seem highly likely that this process plays an important and integral part in chromosome replication.

It is now clear that there is a positive temporal relationship between the enzymatic phosphorylation of the lysine-rich (F_1) histone and cellular replication. This correlation has been demonstrated for a number of rapidly dividing tissues (Balhorn *et al.*, 1971, 1972a), including both tumor cells grown *in situ* and cells grown in tissue culture (Sherod *et al.*, 1970; Balhorn *et al.*, 1972b,d). It is tempting to interpret the phosphorylation of the F_1 histone as a mechanism which may

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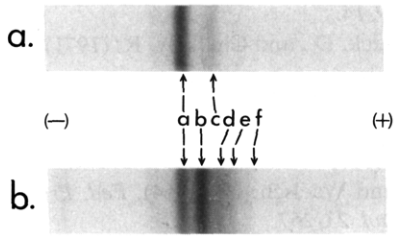

FIGURE 1: The microheterogeneity of HTC F_1 histone from exponentially growing (b) and stationary phase (a) cells. F_1 histone was isolated from appropriate cells as described in Materials and Methods. Electrophoresis was as described previously, Balhorn *et al.* (1972d) Bands "a" and "c" represent parental unphosphorylated lysine-rich histone molecules; bands b, d, e, and f are the various phosphorylated forms of the F_1 histones as demonstrated recently (Balhorn *et al.*, 1971).

either facilitate or even control the biosynthesis of DNA at the time the chromatin of eukaryotic cells undergoes replication, particularly in light of the fact that phosphorylation is restricted to the S phase of the cell cycle (Balhorn *et al.*, 1972c). However, the synthesis of histone also occurs coincidentally with DNA biosynthesis (Irwin *et al.*, 1963; Hohmann and Cole, 1969; Spalding *et al.*, 1966) and thus it is conceivable that F_1 phosphorylation is related in some way to the biosynthesis or to the transport of these basic nuclear proteins to their site of deposition upon the chromosome. In this report we present evidence which argues against the latter possibility. We will show that in exponentially growing hepatoma tissue culture (HTC) cells, F_1 phosphorylation is a unique event occurring only once to each F_1 histone molecule in each round of cell replication. Both the newly synthesized (new) and preexisting (old) lysine-rich histones are phosphorylated. Furthermore, there is a detectable delay between the time of histone biosynthesis and the ensuing phosphorylation of the lysine-rich histone.

In this study, as in others, we have used HTC cells as a model system. The F_1 histone from these cells is extensively phosphorylated (~70%) in exponential phase but the phosphorylated form of this histone is entirely absent in stationary-phase cells (Balhorn *et al.*, 1972d).

Materials and Methods

HTC cells were grown at 37° in medium S-77 buffered with 50 mM *N*-tris(hydroxymethyl)methylglycine and supplemented with 5% fetal calf and 5% bovine serum. The cultures are maintained in exponential growth at cell densities between 2×10^5 and 8×10^5 cells per ml and enter stationary phase at cell densities greater than 10^6 cells/ml.

Labeling of F_1 Histone. In each experiment 0.5 mCi of [^{3}H]-lysine (Schwarz–Mann, N. Y.) with a specific activity of 45 Ci/mmole was added to each flask of HTC cells which were growing at a cell density of 500,000 cells/ml (exponential phase) in a volume of 1200 ml. The cells were incubated at 37° in the presence of label for various times. The labeling period was terminated by centrifuging the cells, washing once and then resuspending the cells in fresh, nonradioactive culture medium. Incubation was then continued in the absence of radiolabel for various time periods depending upon the particular experiment.

Histone Preparation. Approximately 4–5 mg of whole histone can be extracted from chromatin prepared from nuclei isolated and purified from 3×10^8 HTC cells as previously described (Panyim *et al.*, 1971). After precipitation in five volumes of ethanol the histone is stored as a dry powder until ready for use.

Assay for Histone Phosphorylation. Whole histone was dissolved in solution containing 0.9 N acetic acid, 0.5 M β-mercaptoethanol, and 15% sucrose and analyzed by electrophoresis in 20-cm polyacrylamide gels at 4–5° for 80–100 hr following the procedure of Balhorn *et al.* (1972d). This method resolves five distinct electrophoretic bands in the F_1 histone isolated from those HTC cells which are growing exponentially. After electrophoresis the gels were fixed and stained with Amido-Schwarz for 8 hr before destaining electrophoretically. Microdensitometer traces of the histone bands were obtained using a Gilford 2000 gel scanner. The amount of protein in the various F_1 bands was estimated by measuring the average area from the densitometer curves using a Du Pont curve analyzer.

Radioactivity Determinations. To determine the amount of [^{3}H]lysine incorporated into each of the F_1 subfractions, histone samples were electrophoresed as described above, after electrophoretic destaining specific bands were cut out of the gels (*i.e.*, those bands corresponding to the parent and phosphorylated F_1 histone), digested in 30% hydrogen peroxide at 60°, and dispersed in Bray's solution containing 3% Cab-O-Sil. The samples were counted in a scintillation counter (Nuclear-Chicago Unilex III) with an efficiency of 20–25%.

Results

Comparison of F_1 Histone from Exponential and Stationary Cells. Figure 1 confirms that the F_1 histone extracted from exponentially growing HTC cells displays band microheterogeneity (two major and three minor bands) which is greatly reduced as the cells are grown into stationary phase where now only a major and a minor band are seen. That the observed microheterogeneity is produced by the phosphorylation of at least one parent F_1 molecule has been shown previously by treating HTC F_1 samples with *Escherichia coli* alkaline phosphatase, thus eliminating the band heterogeneity (Balhorn *et al.*, 1971) which distinguishes exponentially growing HTC cell F_1 histone from the histone of stationary phase cells. For convenience we have assigned letters a–f to each of the F_1 components beginning at the cathodal end. Bands a and c correspond to the major and minor parent F_1 species and bands b, d, e, and f represent the phosphorylated components. The mobility of the minor parent band (c) is intermediate between the phosphorylated bands "b" and "d" and in all probability is also phosphorylated in cells which are growing exponentially. It is clear from the gel shown in Figure 1 that the method of electrophoretic analysis we have employed is easily capable of resolving the phosphorylated F_1 components from the parent, unphosphorylated F_1 molecules. By microdensitometry and curve analysis we can accurately quantify the amount of protein in each of these electrophoretic bands and, in the case of radiolabeled F_1, we can cut specific electrophoretic bands from the gel and thereby determine the radioactivity associated with the phosphorylated and parent forms of the F_1 protein.

Phosphorylation of Newly Synthesized (New) F_1 Histone. HTC cells growing exponentially at a density of 500,000 cells/ml were labeled with 0.5 mCi of [^{3}H]lysine. Following a 6-hr pulse, the cells were collected by centrifugation and resuspended in fresh, nonradioactive medium. A cell sample (5×10^8 cells) was taken immediately following the pulse. Twenty-four hours later, after an additional cell cycle, the

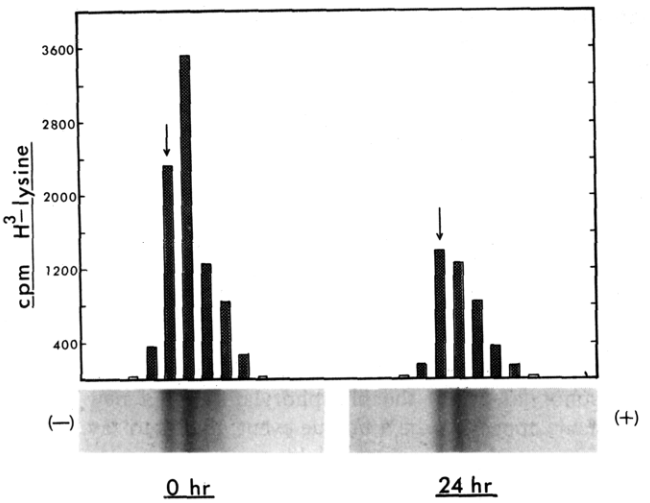

FIGURE 2: Incorporation of [^{3}H]lysine into phosphorylated and parent lysine-rich histones. HTC cells were incubated with [^{3}H]-lysine as described in the text. F_1 histone was isolated from cells collected at 0 and 24 hr after termination of the pulse. After electrophoresis, the gels were photographed, sliced, and dispersed in 30% H_2O_2 and prepared for radioactive counting as described in the Methods section. The major unphosphorylated parental histone is denoted by an arrow; both samples have identical electrophoretic mobility as measured by applying a control F_1 histone to all gels 1 hr after the beginning of electrophoresis for alignment purposes.

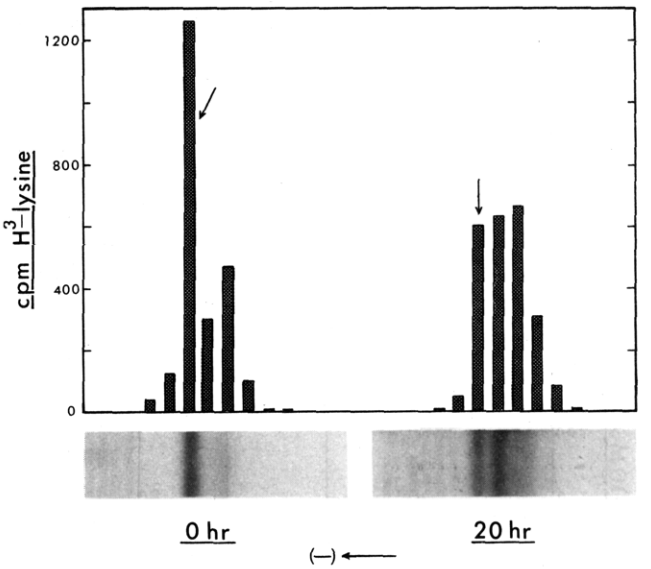

FIGURE 3: Demonstration of the phosphorylation of preexisting ("old"), lysine-rich histone. HTC cells were grown to stationary phase in the presence of [^{3}H]lysine. F_1 histone was isolated from a portion of the cells and analyzed in the standard manner (0 hr). The remaining cells were subcultured into nonradioactive media and grown for 20 hr so that all labeled histone must have been synthesized before subculture. Isolation and analysis of the F_1 histone were as before.

remaining cells were harvested (sample 2). Histones were prepared as described previously and subjected to high-resolution electrophoretic analysis. Since we can adequately resolve both parent and phosphorylated histone species, we can assay for the amount of [^{3}H]lysine in the various bands, and in this way assess the extent of phosphorylation of those histone molecules which had been synthesized during the 6-hr pulse period. In Figure 2, the distribution of label (hatched bars) is shown along with the band patterns of the gels from this experiment. The F_1 histone of sample one (0 hr, Figure 2) shows the presence of label in both the parent (band a) and phosphorylated species (bands b–f), clearly demonstrating that newly synthesized F_1 histone is phosphorylated.

Analysis of the gel pattern and radioactivity distribution in the lysine-rich histone 24 hr after the radiolabel was removed (Figure 2, 24 hr) indicates that [^{3}H]lysine is again found in the phosphorylated region. In view of the turnover rate of phosphate groups on the F_1 histone described in the previous paper ($t_{1/2} \approx 5$ hr), we interpret this latter observation as a first indication that phosphorylation of preexisting histone had occurred.

Phosphorylation of Preexisting (Old) Histone. To further establish that phosphorylation of old histone occurs, HTC cells were grown for three generations in the presence of [^{3}H]-lysine and then allowed to grow to stationary phase before removal of the radioisotope. The first sample of cells was taken at this point (sample, 0 hr). In this way all cells have completed the period of histone synthetic activity, and thus upon induction of new rounds of cell division the histones which contain label will be totally preexisting, old histone (*i.e.*, the histone deposited on the chromosome in previous rounds of replication). The cells were then subcultured into nonradioactive medium at a cell density of 500,000 cells/ml to reinitiate cell division. After 20 hr a second sample was taken. Histones were prepared from the two samples as described previously and electrophoresed in long gels. The results from this experiment are shown in Figure 3. The F_1 histone from cells at 0 hr show the typical electrophoretic pattern of F_1 from stationary cells in that it contains only major (a) and minor (c) parent (unphosphorylated) bands, both of which are heavily labeled (crosshatched bars in Figure 3). Following subculture, the cells rapidly initiate cellular replication which is accompanied by the usual, extensive phosphorylation of the F_1 histone, as is shown by the slow-moving electrophoretic bands in the gel (20 hr) in Figure 3. The presence of label in those electrophoretic bands corresponding to the phosphorylated F_1 clearly shows that preexisting (old) histone is phosphorylated, since only this contains [^{3}H]lysine.

F_1 Phosphorylation is a Single, Unique Event. Having established that both newly synthesized and preexisting histone are capable of being phosphorylated, we then sought to determine whether the phosphorylation of an F_1 molecule is repetitive in nature (*i.e.*, does the F_1 molecule become phosphorylated and dephosphorylated several times in the course of a single cell cycle) or whether it is simply a single, unique event for each F_1 molecule, within each S period. In addition, we wanted to determine if there is a time lapse between the synthesis of F_1 histone and phosphorylation. These experiments were performed by a 20-min pulse labeling of exponentially growing HTC cells.

In this way we labeled a small, discrete population of F_1 histone which was synthesized during this period of incubation. The cells were separated from the culture medium after the pulse by centrifugation and the pellets resuspended in fresh, nonradioactive medium at a cell concentration of approximately 500,000/ml. The cells continued to grow for one complete cell cycle. Aliquots were taken for histone phosphorylation analysis at various time periods following the pulse: zero time, 40 min, 2 hr, 5 hr, 12 hr, and 24 hr. Histones were prepared from each of these cell samples and electrophoresed as previously described.

The results from this experiment are shown in Figure 4.

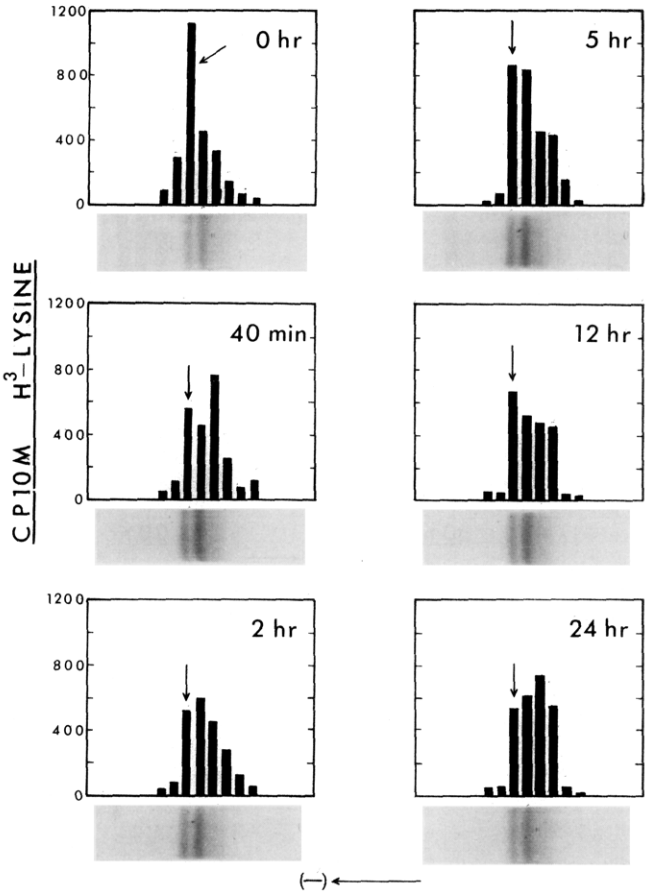

FIGURE 4: Incorporation of [^{3}H]lysine into various forms of F_1 histone during one cell cycle. Exponentially growing HTC cells were pulsed with [^{3}H]lysine for thirty minutes as described in Materials and Methods. After washing and resuspending the cells in fresh media cell samples were collected at the indicated time. Histone was isolated and analyzed by standard methods. The major parent histone, band "a," is indicated by an arrow.

Typical gel patterns and the radioactivity associated with each separate band (solid bars) are shown in this figure. The radioactivity for the phosphorylated and unphosphorylated electrophoretic species was determined by cutting the bands from the gel and counting [^{3}H]lysine as previously described. Data for each of the samples (0–24 hr) were obtained in this fashion.

Immediately following the pulse (*i.e.*, at zero time) most of the radiolabel is associated with the parent lysine-rich histone (indicated by an arrow in Figure 4). After a forty minute chase, a dramatic shift in the electrophoretic position of the ^{3}H-labeled histone can be seen, so that 75% of the label is now associated with those F_1 molecules which are phosphorylated. During the subsequent 12 hr we observe that the radioisotope in the lysine-rich histone shifts back toward the parent, unphosphorylated form, though as expected from the half-life of dephosphorylation discussed in the previous paper (Balhorn *et al.*, 1972e) the return to the parent form is not complete. However, if the experiment is continued into the next round of replication, 24 hr after the initial pulse, then once more the labeled histone reappears in the phosphorylated position which we interpret as phosphorylation of old histone as discussed above. One can see from these results that phosphorylation of newly synthesized histone occurs as a unique event in the course of the cell cycle (which is followed by phosphate removal with a $t_{1/2} \approx 5$ hr), and that if one extrapolates the time course of the phosphorylation back to zero time, it appears that greater than 85% of all newly synthesized F_1 histone is phosphorylated. Perhaps the most surprising result is the observation that there appears to be a short lag period between the time of F_1 histone synthesis and its subsequent phosphorylation. The implications of this observation are discussed below.

Discussion

Several points emerge from the observations described above. These are (1) both newly synthesized histone and preexisting F_1 histone are phosphorylated; (2) the newly synthesized F_1 histone is probably completely (or nearly so) phosphorylated, *i.e.*, every newly synthesized F_1 molecule becomes phosphorylated; (3) the phosphorylation of a new histone molecule appears to be a unique event, that is to say, a given F_1 histone molecule is not available again for phosphorylation until the next round of the cell cycle; (4) there is a time lag of about 30–60 min between the time of histone synthesis and its subsequent phosphorylation; and (5) there is no detectible phosphorylation of histones in stationary phase cells. Taken collectively these points provide overwhelming support for the notion that F_1 phosphorylation is an obligatory event in chromosome replication. Certainly at this time we find no convincing evidence to support the notion that histone phosphorylation is a device whereby specific genes may be activated or repressed.

Although both new and old F_1 histone are phosphorylated in an event temporally correlated with DNA synthesis (Balhorn *et al.*, 1972a), we have no data indicating whether the two species of F_1 histone are phosphorylated coincidentally. Experimentally it is difficult to be precise on the timing of the phosphorylation of old histone simply because of the way it is labeled (in cells grown into stationary phase) which precludes exploiting very brief pulse-chase studies. At this time we do not see any method with which we could resolve this problem. It seems quite likely that both new and old F_1 histone might be effectively phosphorylated coincidentally, as this might simplify the phosphorylation process. Certainly, we can feel confident that the old histone is complexed with DNA when phosphorylation occurs and therefore reason that the new histone is first complexed with DNA and then phosphorylated.

A similar line of reasoning also leads to the conclusion that old histone may be as extensively phosphorylated as the new histone in which we find that at least 85% of all the F_1 molecules are phosphorylated. Again our inability to effectively pulse label old histones precludes confirming this notion directly. However, it is necessary to assume this point in order to account for the amounts of parent (30%) and phosphorylated F_1 (70%) found in exponentially growing HTC cells, considering the observed rates of phosphate turnover described in this and the accompanying paper.

The half-life of phosphate turnover can be measured in two ways. It can be measured directly by observing the removal of ^{32}P from the histone molecules as described previously, or we can study the shift of ^{3}H-labeled F_1 histone from the phosphorylated to parental form. The half-life is estimated as 4–5 hr by both methods. This leads us to conclude that once a ^{3}H-labeled histone has been dephosphorylated, it remains in the parental form until the next round of chromosome replication, when it is once more phosphorylated. Thus, for new histone at least, phosphorylation is a unique event occurring only once each cell cycle.

We note that a lag of about 30 min occurs between synthesis of histone and its subsequent phosphorylation. This most likely excludes the function of phosphorylation as a trans-

port device, and argues that the new histone is associated with the chromosome *before* it is phosphorylated. It will obviously be of great importance to try to resolve the question of whether there is a delay in phosphorylation after the histone is deposited on the chromosome, or whether the lag period represents a mean time during which the histone is passing through a pool of newly synthesized material and that the phosphorylation event occurs fairly promptly after deposition upon the newly synthesized DNA. Such an analysis is currently under way. However, at this time the available evidence points toward a phosphorylation event involving essentially all histone F_1 molecules, possibly in the region of the DNA replication point. We do not know what the function of such an activity might be, but it seems unavoidable that it must play an important and integral part in chromosome replication, and as such would represent the first clearly identifiable role for a histone molecule.

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Effects of Agents That Influence Hydrogen Bonding on the Structure of Rat Liver Ribosomes†

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ABSTRACT: Rat liver ribosomes were exposed to a number of agents that affect hydrogen bonding. Ethanol, which strengthens hydrogen bonding in water, aggregated the ribosomes at 5°, and prevented dissociation to subunits at 35°. Ethylene glycol had similar but weaker effects. Urea, which weakens hydrogen bonding, first dissociated the ribosomes to active 59S and 40S subunits, with molecular weights of 3.0×10^6 and 1.5×10^6. At higher urea concentrations the subunits were converted to slowly sedimenting forms. The 39S large subunit had lost its 5S RNA, but its large RNA was still 28 S, and its molecular weight showed little change. The 27S small subunit still contained 18S RNA, but had lost 35% of its protein; its molecular weight was only 1.14×10^6. Formamide, another agent that weakens hydrogen bonding, also dissociated ribosomes. The first large subunits, 55 S, were active, but additional formamide converted most of them to 41S particles. The 5S RNA and its associated protein were detached but the residual particles showed no further loss of protein, and still contained 28S RNA. The small subunit, 27 S, still contained 18S RNA, but had lost 23% of its protein, and had a molecular weight of 1.27×10^6. The effects of both urea and formamide increased with time, and were enhanced by other factors that tend to dissociate ribosomes, such as increased temperature, decreased magnesium binding, or high pH. The effect of every agent could be attributed to its action on hydrogen bonds. Since liver ribosomes are highly hydrated, the sensitive hydrogen bonds may include those of ribosome-associated water as well as those of RNA and protein.

Since animal ribosomes can dissociate to subunits on warming, and reassociate on cooling, with no change in their total magnesium content (Petermann and Pavlovec, 1967), bonds other than salt linkages must also play an important part in subunit association. One cohesive force appears to be hydrogen bonding between the RNA bases, since ribosomal subunits do not reassociate when their amino groups are masked by formaldehyde treatment (Moore, 1966; Petermann and Pavlovec, 1969). The present study concerns the effects on ribosomal structure of agents that affect hydrogen bonding. The behavior of the ribosomal subunits was followed by analytical ultracentrifugation, and slowly sedimenting forms were isolated and characterized. Ethanol, which enhances hydrogen bonding in water (Franks, 1965), prevented dissociation on warming; ethylene glycol had a similar effect. Two agents that weaken hydrogen bonding, urea (Frank, 1965)

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