Identification of a Distinction between Cytoplasmic Histone Synthesis and Subsequent Histone Deposition within the Nucleus†

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ABSTRACT: Two different approaches have been used to show that in rapidly dividing tumor cells the size of the free histone pool is exceedingly small. It is nonetheless finite and amounts to approximately 0.2% of the total chromosomal histone content. The site of this free histone pool is extranuclear, and evidence suggests that it is not in the perinuclear space between the inner and outer nuclear membranes.

Hepatoma tissue culture (HTC) cells, a tissue culture line established from Morris minimal deviation hepatoma 7288c (Thompson *et al.*, 1966), phosphorylate lysine-rich histone in proportion to the rate of cell growth (Balhorn *et al.*, 1972b). Further studies showed that HTC cells, synchronized by colcemid block, greatly increase the rate of phosphorylation of histones F_1 and F_{2a2} during the S phase of the cell cycle (Balhorn *et al.*, 1972a). Both newly synthesized and preexisting F_1 histones are phosphorylated.

During the course of the experiments which led to the latter conclusion we noted that newly synthesized lysine-rich histone was phosphorylated after a lag period of approximately 30 min. We were curious as to the nature of this lag period and wondered whether the histone was in an extractable pool during this time or if it was associated with the chromosome in an apparently normal fashion. From a study of the synthesis and deposition of histones in the rapidly growing HTC line, we will show that histone is associated with the chromatin during most of this lag period.

There is increasingly strong evidence that histones are synthesized in the cytoplasm (Borun *et al.*, 1967) and subsequently deposited upon the chromosomal material. In order to learn more about these processes it would be advantageous to dissociate them so that they can be studied independently. Such a system has been developed and its description forms a part of this report.

Materials and Methods

Cell Culture and Isotopic Labeling. Hepatoma tissue culture (HTC) cells were grown in suspension culture at cell densities between 4 and 6 $\times$ 10^5 cells/ml in a modified Swim's S77 culture medium containing 50 mM tricine [*N*-tris(hydroxymethyl)methylglycine] buffer and fetal calf and calf sera at final concentrations of 5% each (Granner *et al.*, 1968). HTC cell histone was labeled during 20-min incubations with 0.5 mCi of [^{3}H]lysine/l. of cells. The labeled cells were removed by centrifugation at 160*g* for 5 min at room temperature, washed once with unlabeled medium, and then resuspended in fresh S77 medium warmed to 37° for the chase period.

Novikoff cells were grown either *in vivo* within the peritoneal cavity of the rat or *in vitro* in culture flasks containing the modified Swim's S77 culture medium. For the *in vivo* experiments, 0.5 mCi of [^{3}H]lysine was injected into the cell-laden peritoneal cavity of a rat. At the conclusion of the pulse, the radiolabeled cells were removed, washed once with the standard culture medium, and reinjected into the peritoneal cavity of a second, uninoculated rat for the chase period. Aliquots of cells were removed from the latter animal at various times, centrifuged as described above, and quickly frozen. In the *in vitro* experiments, Novikoff cells were removed from the animal, centrifuged once, and resuspended in 100 ml of warm culture medium. The cells were incubated with 0.5 μCi of [^{3}H]lysine for 20 min at 37°. After the pulse, the cells were centrifuged, washed once, and resuspended in nonradioactive culture medium for the chase period. At appropriate times, cells were removed from the incubation medium, centrifuged, and washed once and then stored frozen at −17°.

DNA was labeled by incubating 2-ml aliquots of either HTC or Novikoff cells, at a concentration of 5 $\times$ 10^5 cells/ml, with 3.3 μCi of [^{3}H]thymidine for 10 min at 37°. The *in vivo* incorporation of [^{3}H]thymidine was determined by injecting the radioisotope (2 μCi/ml of cells) into the peritoneal cavity of the rat for a 15-min incubation at which time an aliquot was removed and the cells frozen. Estimation of the specific radioactivity of the incorporation of [^{3}H]thymidine into DNA was determined by using a modification of the Schmidt–Thannhauser (1945) procedure.

Histone Isolation and Electrophoresis. Whole histone was isolated from HTC and Novikoff tumor cells by the method of Panyim *et al.* (1971). The yield from 4 $\times$ 10^8 HTC cells was approximately 6 mg and a comparable yield was obtained from the same number of Novikoff cells, Electrophoretic separation of each of the histone fractions was accomplished in 9-cm gels (15% acrylamide) containing 2.5 M urea and 0.9 N acetic acid at 130 V for 3.5 hr as previously described (Balhorn *et al.*, 1971). [^{3}H]Lysine incorporation into each of the histone fractions was determined by slicing each band from the gel, digesting with 30% hydrogen peroxide, and counting in Bray's solution (Bray, 1960).

Binding of [^{3}H]Histone to Nuclei. Approximately 1 g of mouse liver was gently disrupted (Virtis) in a grinding medium

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which contained 0.01 M $MgCl_2$, 0.01 M Tris-HCl (pH 8.2), 0.05 M sodium bisulfite, and 0.25 M sucrose. The suspension was centrifuged at 500*g* for 10 min and resuspended in 20 ml of the grinding media. After centrifugation the partially purified nuclei were resuspended in washing medium (same constituents as the grinding medium with the addition of 1% Triton X-100) containing 300 μg of [^{3}H]lysine-labeled HTC histone at a specific activity of 70,000 cpm/mg. The nuclear suspension was gently agitated for several minutes and after centrifugation, as above, 97% of the counts were found in the pellet fraction. Similar results were obtained when the [^{3}H]lysine-labeled histone was added prior to homogenization.

DNA Extraction of [^{3}H]Histone from Nuclei. The efficient removal of exogenously added [^{3}H]histone from nuclei (described above) was performed by extracting the nuclear suspension (either mouse liver, HTC, or Novikoff) with sonicated DNA (Worthington) at a concentration of 0.5 mg/ml in a modified grinding medium (in which the $MgCl_2$ concentration is 0.001 M rather than 0.01 M). Two incubations with the DNA-containing grinding medium were sufficient to remove 98% of the exogenously added [^{3}H]histone. To exclude the possibility that DNA might leach histone through the nuclear membrane, the experiment was also done using nuclei from cells previously labeled with [^{3}H]lysine, *i.e.*, endogenously labeled histone. No extraction of this label was seen.

Results

Experimental Rationale. The strategy employed to discriminate between histone synthesis and its subsequent deposition onto the chromosome was simple and direct. Cells were labeled for 20 min with [^{3}H]lysine and the specific activity of chromosomal histone was measured immediately upon conclusion of the pulse and at a later, defined time. If a portion of the histone synthesized during the 20-min pulse period was not deposited by the conclusion of the pulse we expected to observe an increase in the specific activity of deposited histone as a function of time during the chase. On the other hand, if no significant increase in specific activity was observed during the chase we would conclude that the bulk of the histone synthesized in the 20-min pulse period was deposited by the conclusion of the pulse. Our original definition of deposition simply meant [^{3}H]histone that was extractable from purified chromatin; however, as is discussed below, newly deposited histones appear to interact with DNA in a manner analogous to that found for the histone already bound to DNA in the chromosomal material.

Histone Synthesis and Deposition in HTC Cells. HTC cells were pulsed for 20 min in the presence of [^{3}H]lysine, after which they were centrifuged and washed once in fresh medium, as described above. A portion of the cells was collected for the zero time point, and the remainder was resuspended in fresh medium containing unlabeled lysine and permitted to grow for an additional 2 or 5 hr before collection. Histones were isolated from the cells using standard methods (Panyim *et al.*, 1971). Unless otherwise mentioned, specific activities were determined for histones fractionated electrophoretically as described under Materials and Methods.

Specific activities were obtained for all histone fractions at the conclusion of the pulse (time zero) and after a 2- and 5-hr chase period. The data are presented in Figure 1. There is no significant change in specific activity of any histone fraction during the course of the experiment other than that due to isotope dilution as a result of continued histone synthesis

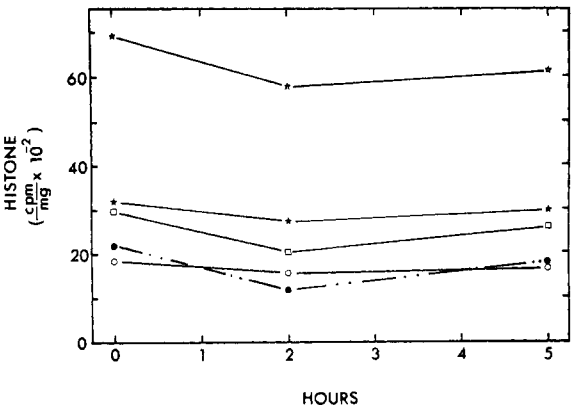

FIGURE 1: Deposition of histone in short pulse period. HTC cells were pulsed with [^{3}H]lysine for 20 min and subsequently chased for 5 hr in the absence of label as described under methods. Histones were isolated and analyzed by gel electrophoresis. Specific activities of individual fractions were computed as described in the text. The zero time point represents histones isolated from the cells immediately upon initiation of the pulse period. The individual histone fractions are: (☆), F_1 histone; (★), F_{2b} histone; (⊡), F_3, histone; (●) F_{2a2} histone; (⊙), F_{2a1} histone.

(~15%). We conclude that either (1) histones are synthesized within the nucleus (or the perinuclear space) and are extractable from within this organelle in the form of an artifactual complex with chromatin even if deposition has not occurred *in vivo* or (2) that the elapsed time between cytoplasmic synthesis and nuclear deposition is very short. We estimate that a difference of 20% in histone specific activity could be reliably detected with our methods; however, the differences noted are much less in the three experiments we have performed. Thus, it seems likely that greater than 80% of the histone synthesized during the 20-min pulse was also deposited during this period. We therefore estimate that the average time spent by a histone molecule in transit (site of synthesis to a site of deposition) is less than 4 min in HTC cells.

The data of Figure 2 confirm our previous observation that phosphorylation of the lysine-rich histone occurs after deposition onto the chromosome (Oliver *et al.*, 1972). This is shown by the observation that immediately following the 20-min pulse (and the ensuing deposition of most of this histone) much of the [^{3}H]lysine labeled histone is present in the nonphosphorylated parental molecules. However, 3.5 hr after the initial chase period a much higher level of the ^{3}H label is now associated with the slower moving phosphorylated histone bands. Evidently some phosphorylation of lysine-rich histones occurs at a time greater than 20 min and less than 3.5 hr after deposition upon the chromosome. Previous studies have indicated that phosphorylation most likely occurs within 30–40 min after deposition (Oliver *et al.*, 1972).

Histones bind rapidly to chromosomal material at physiological ionic strengths and the existence of a "free" pool within the mileau of the nucleus seemed unlikely without involving an as yet undescribed nuclear compartmentalization. However, the possibility existed that a histone pool might reside in the perinuclear space and that in the cellular disruption during extraction, such histone might bind to the nuclear envelope and ultimately become artifactually bound to chromatin. Control experiments were performed in which ^{3}H-labeled histone was added to purified, intact nuclei. As shown in Table I, efficient binding of [^{3}H]histone to nuclei was observed. This bound histone, however, is readily dissociated by adding DNA to the suspension of nuclei (Table I),

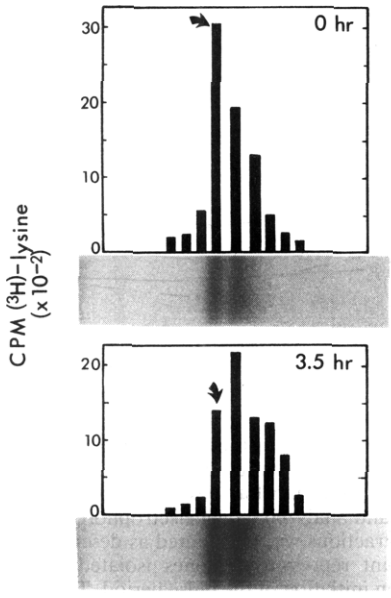

FIGURE 2: Phosphorylation of F_1 histone as a function of time after deposition. HTC cells were pulsed with [^{3}H]lysine for 20 min and chased for 3.5 hr in the absence of label. Histones were isolated as described above and the lysine-rich histone analyzed by high-resolution gel electrophoresis to give the heterogeneity photographed above. Bands were cut out using a razor blade and dispersed and counted as described under Materials and Methods. The arrow indicates parental species of F_1 histone. The total number of cpm applied to each gel was not quite the same and no attempts were made to normalize these numbers in the figure presentation.

TABLE I: Binding of [^{3}H]Histone to Purified Nuclei and Its Subsequent Removal by the Addition of DNA.

Sample	Amount of Radioact Bound [^{3}H]Histone (cpm)	% Bound
[^{3}H]Histone added to nuclei	20,000	(100)
[^{3}H]Histone bound to nuclei following two washes[a]	12,000	60
[^{3}H]Histone extracted from nuclei after washing twice in the presence of DNA[a]	860	4.3

[a] As described under Materials and Methods.

though histones are not removed from within the nucleus, since the latter retained a normal histone complement following this treatment as judged by electrophoretic analysis. Strictly analogous results are obtained if labeled histones are added to HTC cells immediately prior to homogenization. The bulk of the label becomes associated with the nuclei and can be efficiently removed by the addition of DNA. Thus, if a perinuclear histone pool exists we could expect that upon nuclear isolation the histones would bind to the outside of the inner nuclear envelope and that they should subsequently be extractable with DNA as described above. As a result, the specific activity of zero time chromosome histone should be reduced accordingly. When this approach was applied to nuclei isolated from HTC cells from a pulse-chase experiment identical with that presented previously (Table II), no decrease in specific activity of histone was observed in material isolated either immediately after the pulse (time zero) or after a 3-hr chase. This does not exclude the possibility that a perinuclear pool may exist, but we conclude that it is either undetectably small, or is in some way resistant to extraction with exogenously added DNA.

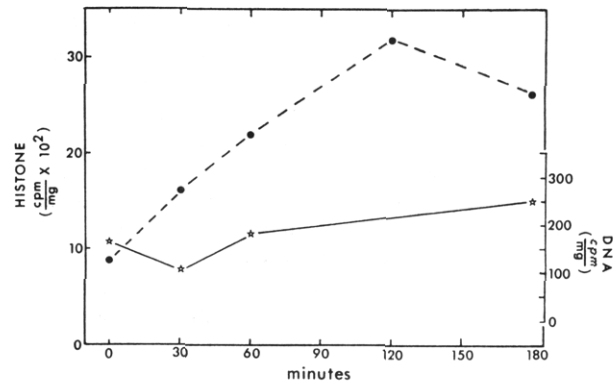

FIGURE 3: Deposition of F_1 histone in Novikoff cells *in vitro*. Cells collected from the peritoneal cavity of a single rat were washed in S-77 medium and then resuspended in fresh, warm medium (37°). The cells were pulsed with [^{3}H]lysine for 20 min and again resuspended in fresh unlabeled medium. The specific activity of F_1 histone (●) was recorded during the chase period. DNA synthetic capacity (☆) was measured after the same time periods in parallel cultures as described under Methods.

TABLE II: A Comparison of the Specific Activities of the Five Major Histone Fractions Extracted from Novikoff Nuclei Prepared in the Presence (+) or Absence (−) of DNA.[a]

Histone Fraction	0 hr		3 hr	
	DNA (−)	DNA (+)	DNA (−)	DNA (+)
F_1	6900	6340	5900	5320
F_3	1840	1850	1600	1630
F_{2b}	3100	3100	2800	2110
F_{2a2}	2950	3160	2400	2450
F_{2a1}	2200	1340	1350	1110

[a] Specific activity measured in cpm/mg of histone.

Histone Synthesis and Deposition in Novikoff Tumor Cells. The ability to uncouple histone synthesis and deposition was discovered by serendipity in Novikoff tumor cells. In order to obtain increased yields of labeled histone we had removed Novikoff tumor cells from the peritoneal cavity of the rat, suspended them in tissue culture medium, and pulsed them with [^{3}H]lysine for 20 min. The specific activity of histone isolated from the nuclei of these cells was unexpectedly low, compared to *in vivo* cultures, yet cytoplasmic protein synthesis had continued at a high rate, as judged by Cl_3CCOOH precipitable radioactivity. The experiment was repeated, except that after a 20-min pulse with [^{3}H]lysine the cells were resuspended in fresh, unlabeled medium (containing nonradioactive lysine) and samples were collected at subsequent times for histone isolation. The specific activity of nuclear histones at various times after the pulse (Figure 3) increases several-fold with time until a plateau is reached approximately 180 min after the conclusion of the pulse. We interpret these data as indicating that histone synthesized during the 20-min pulse period is only slowly deposited during the subsequent 180-

min chase period, the dissociation of synthesis and deposition presumably occurring as a result of the sudden shift to *in vitro* growth.

Additional documentation was obtained by quantitative isolation of the lysine-rich F_1 histone by the method of Johns (1964). Its purity was established by gel electrophoresis and the specific radioactivity of the various experimental samples was measured. Again, a large increase in specific radioactivity with time was noted. That we are not observing continued synthesis of histone from a residual [^{3}H]lysine pool is considered unlikely since cytoplasmic proteins do not show a significant shift in specific activity during these time periods.

DNA synthesis was also monitored during the course of this experiment and these results are also shown in Figure 3. Removal of the cells from the peritoneal cavity with resuspension in artificial medium generated a substantial reduction in the rate of DNA synthesis (150 cpm/mg of DNA as compared to *in vivo* rates of 3800 cpm/mg of DNA), which none the less increased somewhat during the course of the experiment. This suggested a possible explanation for the uncoupling of histone synthesis and subsequent deposition noted above. We assume that histone synthesis continued actively during the pulse (as did synthesis of other proteins) but that DNA synthesis was drastically reduced by the change in the environment. Further, we envision that in *in situ* Novikoff cells the size of the histone pool and the rate of DNA synthesis are in reasonable balance, but that this balance is disturbed when DNA synthesis is decreased without a concomitant reduction in histone synthesis.

We can test this notion directly since we recently have observed that Novikoff ascites tumor cells continue DNA synthesis unabated if they are maintained in the intraperitoneal cavity of the rat during a pulse-chase experiment. Accordingly, Novikoff cells were pulsed *in vivo* for 20 min with [^{3}H]-lysine after which the cells were removed from the rat, washed in fresh intraperitoneal fluid, resuspended in such fluid, and reinjected into a host rat for the chase period. Cells were harvested at selected times and the histones were isolated and specific radioactivities determined. The results shown in Table III indicate that only a small (10%) increase in specific activity is observed during the 2-hr chase. This suggests that during the process of active DNA synthesis, synthesis and deposition of histones are not significantly uncoupled and that the pool size in Novikoff cells is not greater than 2 min, a finding consistent with the data obtained from the HTC cells.

TABLE III: The *In Vivo* Synthesis and Deposition of Novikoff Histone.

Time	Sp Act. (cpm/mg)
0 hr	10,263
30 min	10,857
120 min	11,583

Discussion

The results presented in this paper argue that the size of the free histone pool in actively growing cells is small and equivalent to less than 2 min of histone synthesis time. Figuring an S phase duration of 8 hr, this amounts to approximately 0.4% of the histone content of a given replicating cell. In Novikoff cells in which DNA synthesis has been reduced to ~2% of normal by a change in the environment we find that histone synthesis and deposition can be uncoupled. The histone pool in this case can support chromosomal histone deposition (at the reduced DNA synthetic rate) for 3 hr. This again gives us a measure of the pool size since it can accommodate 3 hr at 2% of the normal rate, relative to 24 hr at 100% of the rate. Thus, the pool represents $3 \times {}^2/_{100} \times {}^1/_{24} \times 100$ of the total histone, *i.e.*, approximately 0.25% of the total histone, a value in good agreement with that calculated more indirectly above.

That an uncoupling between histone synthesis and deposition can occur (*i.e.*, that newly synthesized histones cannot be isolated from chromatin) indicates the small histone pool is extranuclear and is not able to become nuclear bound to a significant extent during tissue disruption, thus providing further strong support to the proposal that histone synthesis itself is extranuclear.

A significant part of the rationale for these experiments derives from the observation that histone phosphorylation occurs within approximately 30 min after histone synthesis (Oliver *et al.*, 1972). It is thus apparent that the phosphorylation event must take place on the histone while it is a part of the nucleoprotein complex and that such phosphorylation is therefore not a histone transport device. Unless histones are deposited on the dividing chromosome at least 30 min ahead of the replication fork, it is likely that the phosphorylation of newly synthesized histones is a postreplicative event. It is clear that a better understanding of the function of histone phosphorylation must await the results of current studies on the relationship of the site of histone deposition relative to the DNA replication point.

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