THE CYTOPLASMIC SYNTHESIS OF HISTONES IN HELA CELLS AND ITS TEMPORAL RELATIONSHIP TO DNA REPLICATION*

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There have been many attempts to establish the temporal relationships of histone and DNA synthesis. By histochemical techniques, both are synthesized concurrently in liver and muscle fibroblasts;1 Prescott,2 using elegant autoradiographic technique, has also found concurrence in the unicellular organism Euplotes eury-stomus. In amethopterin-synchronized cells, however, histone synthesis may begin prior to DNA synthesis;3 and there is evidence that their synthesis is similarly dissociated in regenerating liver.4, 5 In other studies on HeLa cells synchronized with thymidine or amethopterin,4 synthesis of “basic nuclear proteins (histones)” is detected throughout interphase, but markedly increases during DNA replication. These conflicting results probably reflect the varying techniques used for synchronization, the different cell systems examined, and the frequently inadequate means used to identify histones. Although the spatial relationships of histone and DNA synthesis have received less attention, it is generally assumed that histones are synthesized in the nucleus, since they have not been found in the cytoplasm;7 and the synthesis of a lysine-rich histone fraction by isolated thymus nuclei has recently been reported.8 However, this finding does not rule out the cytoplasmic synthesis of histones, and Bloch8 has presented suggestive evidence for such cytoplasmic synthesis in developing grasshopper spermatids. The present report describes the time and locus of histone synthesis in synchronized HeLa populations obtained by selective detachment of mitotic cells.10

Materials and Methods.—Cells: HeLa cells, subline S9, maintained in Eagle's suspension culture medium11 were used throughout. Since this cell grows equally well in monolayer and suspension culture, large populations of synchronized cells from monolayers were routinely obtained with minimal trauma, as previously described.10

Histone isolation and characterization: Logarithmically growing HeLa cells, 2 × 10⁶, were centrifuged from spinner culture and resuspended in 50 ml of lysine-tryptophan-deficient medium containing 0.5 μc/ml H¹-labeled arginine (0.5 μc/ml) was substituted for lysine. The specific activity of the isolates was the highest commercially available but varied from one batch to the next. After 20 min at 37°C, cells were washed in Earle's salt solution, swollen in hypotonic Tris buffer,12 and broken with a precalibrated Dounce-type glass homogenizer. Nuclei were then collected by centrifugation in the cold at 600 × g for 5 min. Following globulin extraction with isotonic NaCl,4 the histones and certain other proteins were solubilized from washed nuclei with 0.5 ml of 0.25 N HCl at 0°C for 30 min. Sodium dodecylsulfate (SDS) was added to the extract to a final concentration of 2%, and after 12 hr dialysis against 0.01 M phosphate buffer (pH 7) containing 0.1% SDS, samples were electrophoresed on 7.5% acrylamide gels of various lengths for 3–12 hr, depending on the desired resolution.13 Gels were mechanically fractionated13 into Packard scintillation vials containing 1 ml of H₂O, allowed to soak overnight, and then diluted with 10 ml of Bray’s fluid. Isotope distribution was measured in the Tri-Carb liquid scintillation counter. Histones were characterized on the acrylamide gel electropherograms as those HCl soluble nucleoproteins which showed active lysine uptake and no tryptophan incorporation above background levels.

DNA and histone synthesis during the cell life cycle: HeLa cells, 2 × 10⁶, were harvested in
mitosis and placed in a 1-liter suspension culture. DNA synthesis was monitored at 20-min intervals by adding C\(^{14}\)-thymidine to 1-ml aliquots and measuring its incorporation into TCA-precipitable material.\(^{11}\) Starting 3 hr after mitosis, histone and DNA synthesis were assayed concomitantly. For histones, 50-ml culture aliquots (10\(^6\) cells) were centrifuged and resuspended in lysine-deficient spinner medium containing 0.5 \(\mu\)g/ml of C\(^{14}\)-lysine and 2% fetal calf serum. After 20 min the cells were washed twice in Earle's salt solution; the histones were then isolated and processed for gel electrophoresis as above. In some experiments lysis was effected by suspending the cells for 15 min in a 0.5% solution of the detergent Nonidet P40 (Shell Chem. Co.) made up in isotonic buffer (0.01 \(M\) Tris\-HCl pH 7.2, 0.0015 \(M\) MgCl\(_2\), 0.14 \(M\) NaCl). Although the nuclei remaining after this latter treatment retained a halo of cytoplasm,\(^{14}\) results were similar to those obtained in cells broken mechanically. The technique gave quantitatively reproducible results and eliminated the necessity of a separate step for the extraction of globulin.\(^5\)

_Locus of histone synthesis:_ A. Pulse-chase kinetics: The pulse-chase technique was used to determine whether the histones were actually synthesized in the nucleus. Synchronized cells, \(2 \times 10^8\), in either \(G_1\) or mid-\(S\) stages of the life cycle were suspended in 20 ml of amino acid-free medium and pulsed at 37\(^\circ\)C for 2 min in a 0.01% solution of \(\alpha\)-aminoisobutyric acid (\(\alpha\)-AIB) and H\(^+\)-lysine (2 \(\mu\)g/ml). Incorporation was stopped by adding 200 ml of medium at 37\(^\circ\)C containing 10 mM each of unlabeled lysine and tryptophan, effectively diluting the specific activities by a factor of about 1000. Half the sample was immediately poured over an equal volume of crushed frozen Earle's salt solution. In the other half, radioactivity was "chased" for 5 min at 37\(^\circ\)C before cooling to 0\(^\circ\)C as above (i.e., synthesis was allowed to continue in the absence of new incorporation of isotope). The cells were centrifuged at 0\(^\circ\)C, washed twice in Earle's salt solution, and fractionated into nucleus and cytoplasm. Then 20% TCA-precipitable material from samples of each fraction was dissolved in 0.5 ml of 1 N NaOH, neutralized with an equal volume of 1 N HCl, and reprecipitated. This precipitate was then collected on Millipore filters which were dissolved in Bray's fluid and assayed in the Tri-Carb liquid scintillation counter. In some experiments part of the doubly labeled HCl-soluble fraction was electrophoresed on acrylamide gels in order to follow the kinetics of individual histone fractions.

B. Tryptophan-lysine double labeling of nascent protein on cytoplasmic polysomes: Histones do not contain tryptophan;\(^6\) if they are synthesized on cytoplasmic polysomes, this should be reflected by differences in lysine:tryptophan ratios in nascent polypeptides on different classes of polysomes. To study this point, 10\(^6\) synchronized cells in either \(S\) or \(G_1\) were suspended in 5 ml of Earle's balanced salt solution (pH 7.2) containing 10 \(\mu\)g/ml of C\(^{14}\)-tryptophan and 15 \(\mu\)g/ml of H\(^+\)-lysine. After 2 min the pulse was stopped by adding 50 ml of ice-cold Earle's salt solution. The cells were washed three times at 4\(^\circ\)C and disrupted with 0.5% Nonidet P 40 in isotonic Tris buffer. Following centrifugation to separate nuclei and cytoplasm, deoxycholate was added to the cytoplasmic supernatant to a final concentration of 0.5% and the suspension layered on a 15–30% sucrose density gradient. The techniques of centrifugation, polysome collection, and measurement of isotope incorporation have been described elsewhere,\(^{11}\) except that in the present experiments the double label was measured by liquid scintillation counting.

In some experiments cells in \(S\) were treated for 2–10 hr with 20 \(\mu\)g/ml cytosine arabinoside (Sigma) to inhibit DNA synthesis\(^{12}\) before labeling nascent peptides, in an attempt to determine whether this inhibition affected protein synthesis on specific polysome classes.

Results.—Histone characterization: Figure 1 shows the electropherogram count pattern from the HCl-soluble fraction of cells labeled with lysine, tryptophan, and arginine. The two most prominent groups of rapidly migrating proteins, arbitrarily designated \(A\) and \(C\), are seen to incorporate more lysine than the others, with a disproportionately small uptake of tryptophan. The group designated \(A\) is also characterized by its high arginine incorporation. Although specific activities were not obtained, the rapid migration, HCl solubility, nuclear location, the active lysine incorporation, differential arginine uptake, and the absence of tryptophan incorporation strongly suggest that the \(A\) and \(C\) groups are histones, and that the \(A\) group includes the arginine-rich histones. These interpretations have been supported by findings to be reported elsewhere\(^{18}\) correlating the F1 (very lysine-rich) histone
of Johns and Butler with the C group, and the F2a, F2b, and F3 fractions with the A group. 19

**Histone synthesis during the cell life cycle:** Lysine incorporation into individual protein peaks in the HCl-soluble fraction increased uniformly as G1 progressed, following the pattern expected on the basis of increasing cell mass, but the ratios between peaks in the electropherograms remained unchanged. The dashed plot in Figure 2 is from G1 cells processed six hours after mitosis; electropherograms at various times in G1 gave qualitatively similar results. Up to this time there was no detectable DNA synthesis above background levels (Fig. 2, insert). Twenty minutes later, however, DNA synthesis had started, as shown by a twofold rise in C14-thymidine incorporation. Concomitantly (dotted plot, Fig. 2), there was a marked increase in lysine incorporation into the two histone peaks. The minor synthesis of these two proteins in G1 cells (dashed plot, Fig. 2) probably reflects the contamination of the original "mitotic" population with about 5 per cent cells in interphase at the time of synchronization, and protein turnover may also contribute to that baseline incorporation. Therefore, it is unlikely that there is appreciable net synthesis of these proteins prior to the onset of DNA synthesis. (We have been unable to confirm the synthesis of unextractable histone during G1 which subsequently becomes extractable during S as reported by Spalding et al. 6) In any event, the steep rise in rate of their synthesis coincides with the start of DNA replication. It is of interest that the pattern of lysine incorporation into all the other proteins of the electropherogram is unchanged in the transition from G1 to S (cf. Fig. 2).
Seven hours after mitosis and one hour after the beginning of S, C$^{14}$-thymidine incorporation had risen to seven times basal level, and incorporation into the A peaks in particular became considerably more prominent (solid plot, Fig. 2). This pattern of count distribution was retained throughout the period during which synchrony was maintained,\textsuperscript{10} i.e., up to late S.

It is therefore clear that histones, represented by the A and C groups in the gel electropherograms here described, are synthesized simultaneously with DNA.

\textit{Locus of histone synthesis}: Pulse-chase experiments indicate that in the intact HeLa cell most of the histones are made in the cytoplasm. When synchronized cells at the peak of histone synthesis are pulse-labeled for two minutes with C$^{14}$-tryptophan and H$^{3}$-lysine, incorporation of the isotopes may be effectively stopped by addition of excess unlabeled tryptophan and lysine; there is no increase in counts on subsequent incubation. There is, however, a striking redistribution of the counts between the various intracellular compartments, and that redistribution is different for tryptophan and lysine. The lysine counts in the nucleus and in its HCl-soluble fraction rise sharply, while counts in the cytoplasm and in the HCl-insoluble fraction of the nucleus fall. Clearly, the lysine-labeled protein lost from the cytoplasm must pass into the nuclear HCl-soluble fraction, since the total cellular count remains constant. The decrease in the C$^{14}$-lysine counts of the HCl-insoluble proteins of the nucleus could represent either their conversion to HCl-soluble material or their exit from the nucleus. The kinetics of the tryptophan label during the chase (Table 1) shows that the latter is the correct interpretation. The decrease in

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<th>Table 1</th>
<th>Pulse-Chase Kinetics of C$^{14}$-Tryptophan and H$^{3}$-Lysine Labeled Protein in Cells during S</th>
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<td>Cell fraction</td>
<td>C$^{14}$-Tryptophan</td>
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<td></td>
<td>Cpm</td>
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<td></td>
<td>0-2-min</td>
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<tr>
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Synchronized cells, 2 × 10$^6$, were pulsed and chased with C$^{14}$-tryptophan and H$^{3}$-lysine as described in the text. Radioactivity was assayed on indicated cell fractions after the 2-min pulse (0–2) and at the end of the 10-min chase (2–12). The discrepancies between total counts and the summation of the subtotals indicate the degree of error inherent in the system. "Per cent change" is indicated to emphasize that in certain cases a relatively large change in absolute counts during the chase represents a small and probably negligible percentage of the total counts in that fraction while the converse holds true in other cases.

tryptophan counts in the HCl-insoluble nuclear protein (2875) exceeds the rise in the HCl-soluble fraction (760), and reflects a net loss of labeled tryptophan from the nucleus. This efflux of tryptophan implies a concomitant loss of labeled lysine which approaches the loss observed from the HCl-insoluble nuclear pellet. (The loss of labeled lysine may be estimated as follows: The HCl-insoluble nuclear proteins contained 3580 tryptophan counts at the end of the chase and 7300 lysine counts, or approximately two lysine counts for every tryptophan. If one assumes that the exiting and residual HCl-insoluble proteins have the same average tryptophan/lysine ratio, the 3010 tryptophan counts lost from the nucleus would be accompanied by about 6000 lysine counts. Considering the approximation, this is reasonably close to the observed 4500 lysine counts lost from the HCl-insoluble fraction,
thus disallowing the possibility that counts from this fraction pass into the HCl-
soluble fraction.) It follows that little if any labeled lysine is transferred from
HCl-insoluble to HCl-soluble proteins and that there is an active exchange across
the nuclear membrane in both directions: HCl-soluble proteins pass from cyto-
plasm to nucleus, while HCl-insoluble proteins pass from nucleus to cytoplasm.

The nuclear HCl-soluble fraction contains histone and nonhistone material.
Since both increase in radioactivity after quenching of incorporation by addition
of "cold" lysine (Fig. 3), they are both presumably synthesized in the cytoplasm,
and only subsequently find their way to the nucleus. It is also to be noted in
Figure 3 that at the end of a two-minute pulse, the incorporation of labeled lysine
into the A group of histones is less prominent than that into the C group; yet by
the end of the ten-minute "chase" the A group contains substantially more counts.
Since there is no additional incorporation during the "chase," and since there is
no significant turnover in ten minutes,20 more C14-lysine must be incorporated into
the A-group precursors during the pulse. The reversal of count ratios observed
during the "chase" suggests a difference in the transport rate of the two histone
groups from the cytoplasmic sites of synthesis to the chromosomes.

Evidence for polysome-associated histone synthesis: Figure 4A shows the 260-mu
absorbance of a sucrose gradient run on cytoplasm from synchronized cells during
the peak of DNA synthesis. Plotted on the same graph are the normalized distribu-
tions of C14-tryptophan and H3-lysine incorporation into nascent protein. While

Fig. 3.—Radioactive electropherograms of HCl-soluble nuclear fraction from cells pulsed
in C14-tryptophan for 2 min and "chased" in medium containing a large excess of unlabeled lysine
for 10 min. Pulse, ----; chase, ---.

Fig. 4.—(A) Polysome OD 260 profile and isotope incorporation patterns into nascent proteins
from synchronized HeLa cells labeled during S with C14-tryptophan and H3-lysine. C14-trypto-
phan, O—O—O—O; H3-lysine, •—•—•—•; OD 260, ———. Ordinate has been normalized for
convenient comparison of the individual curves. (B) Polysome OD profile and isotope
incorporation patterns into nascent proteins from synchronized HeLa cells labeled during G1 with
C14-tryptophan and H3-lysine. C14-tryptophan, O—O—O; H3-lysine, •—•—•—•; OD 260,
———. Ordinate has been normalized for convenient comparison of individual curves.
the two count profiles are quite similar in the heavy polysome region, there is a pronounced disparity in the region of the small polysomes. The curve for lysine is skewed to the right, and, compared to tryptophan, a significantly greater percentage of the total lysine counts are located on these small polysomes. In contrast, when synchronized cells are pulsed during G₁, the curves for polysome-associated lysine and tryptophan incorporation closely overlap throughout (Fig. 4B). The difference between S and G₁ cell polysomes is also evident in the optical density profiles where, owing to an increase in small polysomes, the S profile is skewed to the right compared to G₁; we consider these small polysomes as the probable sites of histone synthesis. This hypothesis is strengthened by the effects of DNA inhibitors on these polysomes. Figure 5A compares optical density tracings from cells pulsed during S with and without exposure to the DNA inhibitor cytosine arabinoside, which also markedly depresses histone synthesis.¹⁸ The arrest of DNA synthesis leads to the disappearance of some of the small polysomes in that region characteristic of cells in S, and results in a polysome profile closely resembling that normally obtained in early G₁. On the other hand, when the same experiment is performed with G₁ cells (Fig. 5B), cytosine arabinoside has no effect, indicating that this inhibition affects only histone-specific polysomes. As was to be expected, the differences between the patterns of tryptophan and lysine incorporation found in S cells is not seen in cytosine arabinoside–treated cells (Fig. 6A vs. B). Similar results were obtained when FUdR was substituted for cytosine arabinoside. With either inhibitor the polysome shift occurs during the first 90 minutes, and the profile then remains unchanged for at least ten hours.

Discussion.—The present experiments demonstrate a dynamic and sensitive relationship between histone and DNA synthesis. Initiation of DNA synthesis is accompanied by the activation of cytoplasmic histone-producing polysomes, while the arrest of DNA synthesis causes not only a rapid decline of histone synthesis, but the disruption of those specific polysomes.

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![Graph of polysome OD 260 profiles](image)

**Fig. 5.**—(A) Polysome OD 260 profiles from synchronized cells in S. Treated for 2 hr with cytosine arabinoside (20 μg/ml), ——; untreated control, ———. (B) Polysome OD 260 profiles from synchronized cells in early G₁. Treated for 2 hr with cytosine arabinoside (20 μg/ml), ——; control, ———.
Although certain aspects of these results differ from some previously reported, they are not necessarily inconsistent. Reid and Cole\(^8\) have described synthesis of histones in isolated thymus nuclei, but the thymocyte used by these workers is unusual in that the nucleus is reported to contain ribosomes\(^21\) and is able to carry out independent protein synthesis in vitro. Neither is true of HeLa cells. Furthermore, while our results demonstrate the cytoplasmic locus of histone synthesis, they do not rule out nuclear histone synthesis which on a small scale could escape detection by our methods. The fluctuating histone:DNA ratio found by Umana et al.\(^8\) in regenerating rat liver at varying times after partial hepatectomy may, as with thymocytes, reflect a profound difference between these cells and the heteroploid cells used in the present experiments. Finally, differences in the results obtained with cells synchronized by selective detachment and those obtained with drug-induced synchronous populations\(^4, 6\) emphasize the importance of using untreated cells in such experiments.

**Summary.**—In synchronized HeLa cells there is a precise correlation between the initiation of histone and DNA synthesis. Pulse-chase labeling of cells in G\(_1\) and S and examination of cytoplasmic polysomes doubly labeled with tryptophan and lysine have shown that histones are made in cytoplasm on small polysomes. The DNA inhibitors cytosine arabinoside and 5 fluorodeoxyuridine cause the selective disruption of these when they are added during S, but have no effect on small polysomes of the same size when added during G\(_1\).

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