Asynchronous Appearance of Newly Synthesized Histone H1 Subfractions in HeLa Chromatin

STEPHEN R. SIZEMORE and R. DAVID COLE
Biochemistry Department, University of California at Berkeley, Berkeley, California 94720

ABSTRACT Incorporation of radioactive alanine into chromatin-bound subfractions of H1 histone was studied in HeLa cells synchronized by the double thymidine block technique. The subfractions were resolved into three chromatographic peaks by Biorex-70. In the period 5–7 h after release from the thymidine block, peaks I and III showed twice as much incorporation as they did in the period 1–3 h after release, whereas peak II showed three times the incorporation at 5–7 h that it did at 1–3 h. Thus, the H1-histone subfraction in peak II appears in chromatin somewhat later in S phase than do the subfractions in Peaks I and III.

Although the synthesis[1] of histones is largely coordinated with DNA synthesis (2, 20), there have been reports of an additional low level of synthesis of H1 histones outside of S phase (1, 9, 23), suggesting that the synthesis of the H1 histones is controlled somewhat differently than that of the core histones. Although it might be regarded as a special case, in oocyte maturation and early development of *Xenopus laevis*, differential regulation of H1 histones relative to core histones was clearly demonstrated (6). Moreover, differential control is demonstrated by the presence of H1 histones in chromatin at half the molar amounts characteristic of the other histones.

In addition to the difference between the synthesis of H1 histones and that of the core histones, there may be differences among the H1-histone subfractions, a small number of which is found in any organism (14). Differential control of synthesis may underlie the variation in subfraction proportions that is observed among tissues (3, 12). A more direct indication of differential regulation among H1 histones is the difference in the rates with which subfractions of rat tissues incorporate lysine (10, 17).

A difference among H1-histone subfractions in the timing of their synthesis can be inferred from previous observations of Hohmann and Cole (10). The H1 histones of mouse mammary tissue did not seem to be synthesized in complete synchrony. Lactogenic hormones caused a change in the recipe of H1-histone subfractions that were synthesized during the wave of cell division that preceded the induction of milk proteins. Although synthesis of the H1-histone subfractions seemed to be coupled to DNA replication, the effects of hormones on their synthesis were not uniform throughout S phase. The suppression of synthesis of one subfraction was seen in early as well as in late S phase, whereas the enhancement of another subfraction was observed only in late S phase. One explanation of these results is that the various H1-histone subfractions were under different controls even within a single cell. However, the study of Hohmann and Cole (10) was done on cultured tissue segments in which there was a mixture of cell types, thus complicating the interpretation. Obviously, if cell types with different recipes of subfractions traversed S phase differently, the observed results would have been produced. To remove this ambiguity, we studied the rates of alanine incorporation into different H1-histone subfractions in cultured HeLa cells at different times in S phase. Even in this single cell type, the subfractions lack synchrony in their synthesis.

MATERIALS AND METHODS

HeLa cells in spinner culture were synchronized by a double thymidine block. During logarithmic growth phase, thymidine was added to the growth medium to a concentration of 2 mM. After 16 h, the cells were resuspended in fresh medium. 8 h later, thymidine was again added to 2 mM and maintained for 16 h. Synchronous cell division was then achieved by quickly washing and resuspending the cells in fresh medium. Synchrony was confirmed by cell counts, showing a doubling within about 13 h after release from the block, and by [^{3}H]thymidine incorporation (Fig. 1). To compare the H1 histones synthesized and incorporated into chromatin within the S phase, 2-h pulses of [^{3}H]- or [^{14}C]alanine were given to cells three times during their 8-h S phase. To make more rigorous comparisons, a dual label technique was used: before analysis, cells labeled with [^{14}C]alanine between hours 1 and 3 were combined with cells that had been labeled with [^{3}H]alanine from hours 3 to 5; similarly, 3- to 5-h cells labeled with [^{3}H]alanine were mixed with 5- to 7-h cells labeled with [^{14}C]alanine. Chromatin was isolated (21) from these mixtures, and H1 histone was then

[1] The appearance of newly synthesized histone in chromatin is the result of three processes, i.e., synthesis, transport into the nucleus, and deposit onto chromatin; but for simplicity we will follow long-established practice and refer to the overall phenomenon as histone synthesis. The difference between this loose use of the term "synthesis" and true synthesis is emphasized by the recent report of Groppi and Coffins (7).

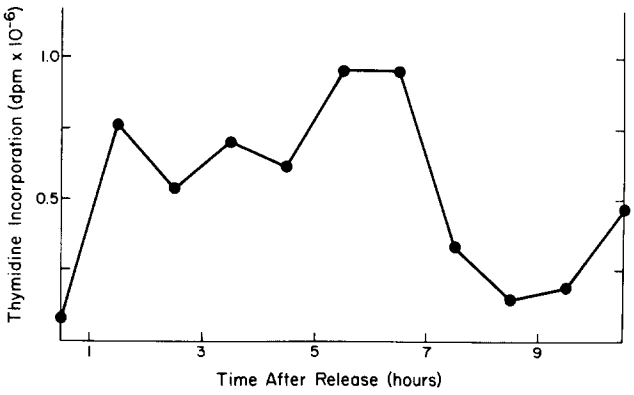

FIGURE 1 Thymidine incorporation after release of double thymidine block. HeLa cells at 3.5 × 10^5 cells/ml (21) were synchronized by a double thymidine block. Immediately after release, the cells enter an S phase of 6–8 h. At hourly intervals after release, 5-ml aliquots were incubated for 15 min with [^{3}H]thymidine added to 5 μCi/ml. The incubation was stopped by addition of 5 ml of TCA (0°C). After 15 min, the precipitate was collected on a Millipore filter. Filters were solubilized with 1 ml of NCS and counted, using internal standards.

extracted by use of 5% trichloroacetic acid (5). H1-histone subfractions are uniquely resolved on Biorex-70 ion exchange columns (10, 13); the radioactivity in the fractions was measured using double label counting techniques.

RESULTS AND DISCUSSION

Ion exchange chromatograms for HeLa H1 histone showed three major peaks of protein which we identified as H1-histone subfractions and tested for purity by amino acid analysis and gel electrophoresis (18, 22). The chromatogram in Fig. 2*A* allows a comparison between the [^{14}C]alanine incorporated into chromatin-bound H1-histone subfractions in early S phase (1–3 h) and the [^{3}H]alanine incorporated in mid S phase (3–5 h). Similarly, in Fig. 2*B*, incorporation in late S phase (5–7 h) may be compared to that in the middle period (3–5 h). It must be kept in mind that each panel represents a single chromatogram, even though separate curves are plotted for each isotope. Because this dual label technique eliminates discrepancies that might otherwise arise from variations in isolation, and analytical resolution, comparisons within either panel may be made with considerable rigor. Although comparisons between panels are somewhat less rigorous, the results fit together smoothly anyway. Moreover, because the incorporation for the middle period in both panels represents a single batch of cells and a single incubation with [^{3}H]alanine, the early and late period incorporations can be compared rigorously by their normalization to that of the middle period. Thus, the incorporation of alanine in the late period relative to that in the early one can be expressed by the ratio ^{14}C/^{3}H (Panel *B*) ÷ ^{14}C/^{3}H (Panel *A*). Comparing points in peak I, this ratio is found to be 0.48/0.23; for peak II the ratio is 0.52/0.16; for peak III it is 0.42/0.22. In other words, the H1-histone subfractions in peaks I and III doubled their incorporation from early to late S phase, whereas the H1 histone in peak II trebled it.

An alternative way of using the data in Fig. 2 is to integrate the areas under each of the peaks for each isotope separately. For each part of the S phase, the Table I shows the fraction of the total isotope that occurs in each peak. These figures reveal that the contribution of peak II to the total alanine incorporation almost doubles from early S phase (12%) to late (21%). In contrast, the contributions of peaks I and III are hardly

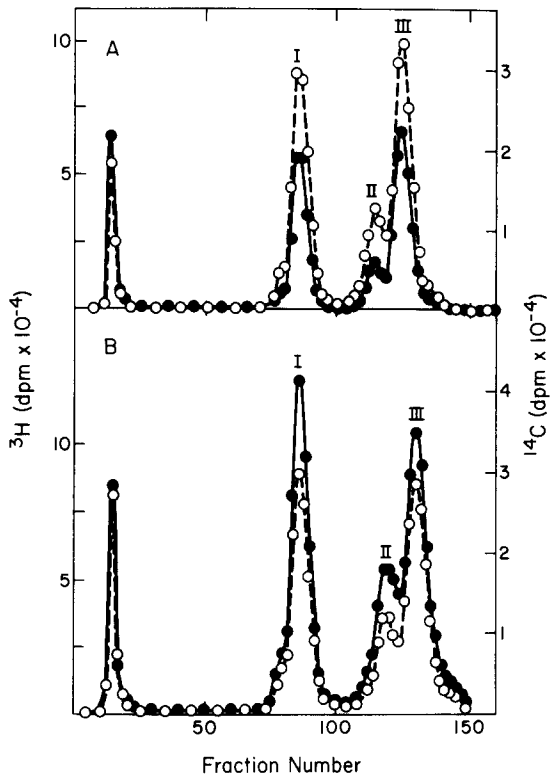

FIGURE 2 Biorex-70 chromatograms of H1 histones. The cell culture described in Fig. 1 was used to measure incorporation into H1 histones. Half of the cell culture was labeled from 3 to 5 h with ^{3}H-Ala (0.5 μCi/ml). The other half was labeled with ^{14}C-Ala (0.25 μCi/ml): one-quarter from 1 to 3 h and one-quarter from 5 to 7 h. Cells labeled 1–3 h were combined with half the 3- to 5-h cells (panel *A*). Cells labeled 5–7 h were combined with the rest of the 3- to 5-h cells (panel *B*). H1 histone was extracted from prepared chromatin and chromatographed on Biorex-70 (0.8 × 20 cm). Open circles, ^{3}H; and filled circles, ^{14}C.

TABLE I
^{14}C and ^{3}H in Fig. 2

Chromatogram	In S phase: Time period	Fraction of total radioactivity*: Peak I	Peak II	Peak III
A	Early	0.41	0.12	0.47
	Middle	0.36	0.17	0.47
B	Middle	0.40	0.17	0.43
	Late	0.40	0.21	0.40

* The peaks from Fig. 2*A* were integrated to calculate the fraction of the total ^{14}C (for early S phase) and ^{3}H (for middle S phase) that occurred in each. Similar integrations were done for Fig. 2*B*, where the ^{3}H profile again represented middle S phase and ^{14}C represented late S phase.

changed. peak III seems to show a decrease, which, although modest when viewed as a percentage, is observed consistently in repeated experiments. In any case, whereas all the subfractions increase their synthesis in response to DNA replication, and it is clear that the increases in the H1-histone subfractions are not at all parallel, the major increase for peak II occurs later than those for peaks I and III.

It is not likely that the difference between peak II incorporation and that of the other peaks is an artifact due to phosphorylation, lack of cell synchrony, or chromatographic crosscontamination. Gurley et al. (8) showed that the extensive phosphorylation associated with metaphase causes H1-histone

subfractions to elute from Biorex 70 columns well ahead of peak I. Such components are not detected in Fig. 2. The phosphorylation associated with S phase is much more modest and causes only a slight acceleration in elution; all three of the major H1 histone peaks in Fig. 2 must have this degree of phosphorylation because they are all newly synthesized. The difference between peak II incorporation and that of the other peaks is not an artifact due to lack of cell synchrony or chromatographic crosscontamination because both of these technical imperfections would tend to reduce differences between time periods and chromatographic peaks. Therefore, the magnitude of the difference we report, between peak II incorporation and incorporation into peaks I and III, is a minimal representation of the real difference.

What we have here called synthesis is actually the appearance of new H1 histone in chromatin, and it thus depends on transcription, RNA processing, translation, and transport of the histone to the chromatin. Differential control of H1 histones appearing in chromatin might involve any of these steps. The regulation of histone gene transcription could be differential even in sea urchins where there are blocks of genes containing one gene for each of the five classes of histone (H1, H2A, H2B, H3, H4), and where those blocks are reiterated tandemly (11). The possibility for differential transcription is even more pronounced, however, in the recent finding that the gene order is different from one gene block to the next in *X. laevis* (26) and in the report that *Drosophila* histone gene blocks are bipolar, with some histones coded on one DNA strand while the remaining histones are derived from the other (15). However, differential histone synthesis might just as easily be pictured at the posttranscriptional level, because Melli et al. (16) showed that histone mRNA was synthesized throughout the entire HeLa cell cycle, even though substantial levels of the message are maintained in the cytoplasm only during S phase. In apparent conflict with the findings of Melli et al. (16), Groppi and Coffino (7) report that equal amounts of histones are synthesized throughout the cell cycle, but that transport of histones into the nucleus is dependent on S phase. If this were true, what is termed asynchronous synthesis of H1-histone subfractions in our studies could represent differential control at the level of transport. Our concern in this work was not, however, the cause behind the asynchrony of subfraction appearance, but the existence of it.

The observation that H1-histone subfractions are not synthesized synchronously might represent a mechanism for the designed manipulation of subfraction recipes, or it might be merely incidental. The designed selection of particular recipes would imply functional distinctions among the different types of H1 histone. Such functional differences are made plausible by the observation that individual subfractions, which differ significantly in amino acid sequence (14, 19), differ in their ability to condense supercoiled and relaxed DNA (24, 25). H1 histone is generally thought to find its role in the condensation of the nucleosomal strand into a higher order of folding; if so, the H1-histone subfractions may introduce variations in the geometric parameters of the folded structure. Conceivably, specific patterns of folding are involved in processes such as the programming of the cell cycle, and the commitment to the differentiation state. It is intriguing to note, in this context, that there is an asynchronous replication of euchromatin and heterochromatin (4), just as there is the asynchronous synthesis of H1-histone subfractions.

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