Histone H1 Subfractions and H1^0 Turnover at Different Rates in Nondividing Cells†

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ABSTRACT: When cell division was inhibited in mouse neuroblastoma cells by high cell density or by serum deprivation, levels of histone H1^0 increased by a factor of 3. Inhibition of cell division by butyrate increased H1^0 7-fold. In addition to the increase in H1^0, changes were seen in the proportions of H1 subfractions whenever division was inhibited. These changes were only partly due to phosphorylation. When cell division was very active, a pulse of radioactive lysine labeled the various forms of H1 nearly in proportion to their protein mass. A chase of the radioactivity from such pulse-labeled cells showed a rapid turnover of the subfraction H1c, while the other forms of H1 turned over slowly, like the core histones. The inhibition of cell division reduced lysine incorporation into the subfractions of gel band H1a,b nearly as much as it reduced core histone synthesis, but H1c and H1^0 were not so drastically affected. Relative to incorporation into core histones, incorporation into H1c and H1^0 was 4-fold and 20-fold greater, respectively, in mitotically inactive cells. When this radioactivity was chased with unlabeled lysine, H1^0 was quite stable, but H1c and one of the H1 subfractions of the H1a,b band turned over rapidly. Unequal turnover of H1 subfractions and the difference in behavior between H1^0 and the more common H1's show how the recipe of H1 components can be varied even in the absence of DNA synthesis. These results are consistent with a chromatin repair process and also with a designed modulation of chromatin functions.

The synthesis of DNA and that of the major forms of histone are coupled. This coupling has been observed in several systems and has been widely accepted as being nearly absolute (Robbins & Borun, 1967). However, there have been some reports suggesting that some histone, particularly H1, is produced in the absence of DNA synthesis (Gurley et al., 1972; Ohba et al., 1975; Appels & Ringertz, 1974; Tarnowka et al., 1978). Although the significance of the non-S-phase production of H1 is not known, this protein has been observed to have a measurable turnover rate (Gurley et al., 1972). Non-S-phase production and turnover was demonstrated clearly for a specialized histone H5, which is found only in nucleated red blood cells. In chicken erythrocytes, this histone is synthesized and turns over without significant synthesis of DNA or other histones (Appels & Wells, 1972; Sung et al., 1977). This unusual metabolic behavior is probably at least partially responsible for the observed accumulation of this histone as avian red blood cells mature.

H1^0 is an H1 variant that was found originally in tissues with little or no cell division and which occurs at reduced levels in tissues with active division (Panyim & Chalkley, 1969b). Studies of regenerating and developing tissues showed an inverse relationship between mitotic index and H1^0 levels (Varricchio, 1977; Varricchio et al., 1977; Benjamin, 1971; Garrard & Bonner, 1974; Bregnard et al., 1979). We have previously reported that the H1^0 levels in HeLa and mouse neuroblastoma cultured cell lines increased 3-4-fold when cell division was inhibited (Pehrson & Cole, 1980). This indicated that the previously reported changes in H1^0 content were not due to shifts in the distribution of cell types within a tissue but rather were due to alterations within individual cells. Such an increase seemed inconsistent with the notion that the synthesis of this histone is tightly coupled to that of DNA. If the metabolism of H1^0 is similar to that of H5 and if it continues to be made in the absence of DNA synthesis, its accumulation in mitotically inactive cells could be explained. The work about to be described was undertaken to demonstrate that H1^0 turns over in the absence of DNA synthesis. Incidentally it revealed that the more common H1 subfractions also turn over, some much more rapidly than others.

Materials and Methods

Gel Electrophoresis. NaDodSO$_4$[1]-polyacrylamide gel electrophoresis was run according to the method of Laemmli (1970); these gels had a 12.5% acrylamide separating gel. Two-dimensional gels used an acetic acid-urea-polyacrylamide gel (Panyim & Chalkley, 1969a) for the first dimension. This gel was stained very briefly just to visualize the lanes. The lightly stained gel was soaked in distilled water for 3 min, and the lanes of interest were cut out. These strips were placed in 50-mL screw top tubes and equilibrated with 30 mL of 0.0625 M Tris and 1% NaDodSO$_4$, pH 6.8, for 1 h. This was repeated for another 1-h period with fresh buffer, and the equilibrated strip was cast into the normal NaDodSO$_4$ stacking gel. Electrophoresis was carried out as usual, and gels were stained and destained as previously described (Pehrson & Cole, 1980). Stained gels were scanned at 525 nm.

Alkaline Phosphatase Treatment of H1 and H1^0. H1 and H1^0 were selectively extracted from whole neuroblastoma cells with ice-cold 0.74 M PCA (Johns & Butler, 1962). The extracted histone was dissolved in 50 mM Tris, pH 8.2, and *Escherichia coli* alkaline phosphatase (Worthington, BAPF) was added to a final concentration of 1 mg/mL. The mixture was incubated at 37 °C for 16 h, and the reaction was terminated by the addition of 3 × NaDodSO$_4$ sample buffer (30% glycerol, 9% NaDodSO$_4$, and 0.188 M Tris, pH 6.8; to dissolve this mixture, it must be warmed to 37 °C). The proteins were electrophoresed in NaDodSO$_4$-polyacrylamide gels.

Labeling of Histones. Mouse neuroblastoma cells were grown as described (Pehrson & Cole, 1980), on 100 × 20 mm tissue culture dishes. Rapidly dividing cells were labeled when

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[1] Abbreviations: NaDodSO$_4$, sodium dodecyl sulfate; PBS, phosphate-buffered saline; DME medium, Dulbecco's modified Eagle's medium; PCA, perchloric acid; PMSF, phenylmethanesulfonyl fluoride; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Quantitative Changes of H1's and H1^0 When Cell Division Was Inhibited [a]

growth state	H1/core [b]	H1^0/core [b]	H1 + H1^0 core [b]	H1a,b/ H1c
rapidly dividing, $n = 6$	0.19 ± 0.01	0.017 ± 0.001	0.21 ± 0.01	2.8 ± 0.3
density inhibited, $n = 4$	0.20 ± 0.02	0.054 ± 0.006	0.25 ± 0.03	4.4 ± 0.3
serum deprived, $n = 5$	0.19 ± 0.01	0.050 ± 0.002	0.24 ± 0.01	3.9 ± 0.4
sodium butyrate, $n = 4$	0.18 ± 0.02	0.12 ± 0.01	0.30 ± 0.02	3.6 ± 0.3

[a] Histones were quantified by scanning $NaDodSO_4$–polyacrylamide gels of 0.4 N H_2SO_4 extracts from isolated nuclei. The data are presented as the average ± the average deviation, and n is the number of extracts analyzed. Density-inhibited cells were grown on the same dish for 10–12 days, serum-deprived cells were analyzed 4–5 days after serum withdrawal, and butyrate-treated cells were cultured in 5 mM sodium butyrate for 4 days. [b] Core = core histones: H2A, H2B, H3, and H4.

the plates were approximately one-third confluent; serum-deprived cells were labeled 4–5 days after serum withdrawal. The viability of these cells was measured (by exclusion of the dye Trypan Blue), and more than 90% of the cells in the monolayer was viable even after 8 days of serum deprivation. The medium was removed from the plate, and the cells were washed once with PBS. Two milliliters of lysine-free DME medium that contained 5 μCi of L-[^{14}C]lysine (Schwarz/Mann) per mL (this brought the lysine concentration to 16 μM) was put on the washed cells, with care taken to avoid detachment of cells. The labeling medium for rapidly dividing cells was supplemented with dialyzed fetal calf serum to 10%. The cells were incubated in the L-[^{14}C]lysine-containing medium for 3 h; culture dishes were gently shaken periodically to prevent dry spots. At the end of the 3-h incubation period, the radioactive medium was removed, and the cells were harvested or, after being washed once with PBS, they were cultured with lysine-containing DME medium for 2 or 4 days before harvest. When harvested, rapidly dividing cells were diluted approximately 15-fold with unlabeled cells to lower the specific activity of their histone to a level similar to that of serum-deprived cells. Nuclei were isolated from these cells by use of the procedure of Lawson & Cole (1979), except that 250 μM PMSF was included in all buffers. The histones were extracted from the nuclei with 0.4 N H_2SO_4, precipitated with 20% trichloroacetic acid, and electrophoresed on $NaDodSO_4$ gels. Stained gels were soaked for 1 h in the aqueous autoradiographic image enhancer Autofluor (National Diagnostics) and then dried. The dried gel was autoradiographed, and the exposed X-ray film was scanned with a densitometer.

Results

Quantitative Changes in H1's and H1^0 When Cell Division Was Inhibited. An increase in the amount of H1^0 in mouse neuroblastoma cells when cell division was inhibited by serum deprivation was like the increase in H1^0 observed when the mitotic rate was reduced by high cell density (Table I). Serum deprivation of neuroblastoma results in two things, cessation of cell division and differentiation (neurite formation). This indicates that the increase of H1^0 seen during serum deprivation was correlated with inhibition of cell division but not with the differentiation process which results from withdrawal of the serum. In addition to the increase in H1^0, there were

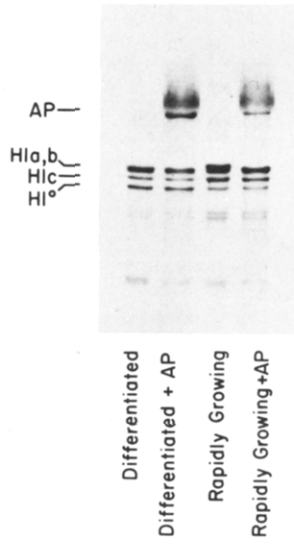

FIGURE 1: Identification of phosphorylated H1. H1 extracted with PCA was treated with alkaline phosphatase (AP) and electrophoresed on $NaDodSO_4$–polyacrylamide gels. The first two lanes contain H1 from serum-deprived neuroblastoma cells before and after alkaline phosphatase treatment. The next two lanes contain H1 from rapidly dividing neuroblastoma cells before and after alkaline phosphatase treatment.

changes in the H1 subfraction pattern when cellular division was inhibited by high cell density or serum deprivation. In $NaDodSO_4$–polyacrylamide gel electrophoresis, rapidly dividing cells showed three bands of H1 in addition to H1^0; these bands were termed H1a, H1b, and H1c. Although density-inhibited and serum-deprived cells gave essentially identical patterns, that pattern was different from the one given by rapidly dividing cells. The pattern for growth-inhibited cells did not have a distinct band corresponding to H1a, but instead it had a larger, somewhat diffuse band in the H1b region. Moreover the amount of the H1c was somewhat reduced in comparison to the remaining H1 (H1a, H1b). These changes in H1 subfractions are reported in Table I along with the increase in H1^0 content.

An especially dramatic increase in H1^0 levels was obtained by treatment of neuroblastoma cells with sodium butyrate, which blocks the cells in the G1 phase of the cell cycle (D'Anna et al., 1980b). This treatment induced levels of H1^0 that nearly matched the H1 level (see Table I).

Identification of Phosphorylated H1. Since H1 and H1^0 are phosphorylated in a cell cycle dependent fashion (D'Anna et al., 1980a) and phosphorylation can effect the mobility of some H1 subfractions on $NaDodSO_4$–polyacrylamide gels (Billings et al., 1979; Fischer & Laemmli, 1980), it seemed likely that some of the electrophoretic differences between rapidly dividing and mitotically inhibited cells were due to phosphorylation. H1 histones were selectively extracted by PCA from rapidly dividing and serum-deprived neuroblastoma cells and were then treated with bacterial alkaline phosphatase (see Figure 1), which can remove the phosphate groups from H1 (Balhorn et al., 1971). This treatment eliminated the distinct H1a band seen in rapidly dividing cells. The H1a band merged into the H1b band, giving an H1b band similar in intensity and shape to that seen in serum-deprived cells. This indicates that H1a is a phosphorylated form of H1, which, when dephosphorylated, migrates electrophoretically in a position at or very near that of H1b. Serum-deprived cells have no H1a since these cells are mitotically inactive, and when serum-deprived cells were treated with alkaline phosphatase, there was no measurable change in the H1 pattern. Quan-

Table II: Turnover of H1's and H1^0 in Rapidly Dividing and Serum-Deprived Neuroblastoma Cells [a]

growth state	label	rel radioactivity [b] H1a,b	H1c	H1^0
rapidly dividing	pulse	20 ± 1	9 ± 1	0.8 ± 0.2
	chase [c]	18 ± 4	5 ± 0.2	0.8 ± 0.1
serum deprived	pulse	34 ± 3	40 ± 10	18 ± 7
	chase [d]	13 ± 1	13 ± 1	19 ± 1

[a] Neuroblastoma cells were labeled with L-[^{14}C]lysine; the radioactive histones were electrophoresed in NaDodSO$_4$–polyacrylamide gels, and the amount of label in each of the various forms of H1 and core histones was quantified by scanning autoradiographs of these gels with a densitometer. Each value given in the table is the average of four experiments ± the average deviation. [b] Expressed as percent of core histone radioactivity. [c] Two-day chase. [d] Four-day chase.

titation of the amount of H1c present in rapidly dividing cells, relative to the H1a and H1b, showed no change within experimental error, after treatment with alkaline phosphatase (data not shown).

Turnover of H1 and H1^0 in Rapidly Dividing and Serum-Deprived Cells. Metabolic studies were done to better understand how the pattern of H1 subfractions and H1^0 is regulated when cell division is inhibited. Cells were labeled with L-[^{14}C]lysine and analyzed either immediately or after 2 or 4 days of culture in medium containing unlabeled lysine. Nuclei were isolated from these cells, and histones were extracted with 0.4 N H_2SO_4. The H_2SO_4 extracts were run on NaDodSO$_4$–polyacrylamide gels, and the dried gels were autoradiographed. The proteins from such extracts were compared to PCA-extracted H1 histone as shown in Figure 2, left-hand panel; the right-hand panel shows the autoradiographic image obtained from the same electrophoretic gel. A quantitative analysis of several such experiments was performed by scanning the autoradiograms; the data are shown in Table II. . The core histones were used as internal standards since their synthesis is tightly coupled to DNA synthesis (Robbins & Borun, 1967). The rate of incorporation of radioactivity into these proteins ought to be essentially proportional to the rate of DNA synthesis. However, since there may be a small amount of histone synthesis in the absence of DNA synthesis, due to the slow turnover of these proteins, the absolute rate of histone synthesis may be somewhat higher than that of DNA synthesis, particularly in mitotically inactive cells. The slow turnover of the core histones (Gurley et al., 1972) makes it possible to use them as references for measuring the degradation of other proteins. Although this approach does not allow the calculation of absolute rates of turnover, it does give an approximation of relative rates.

The distribution of label among H1 components (Figure 2) when rapidly dividing cells were pulsed with radioactive lysine was similar to the distribution of protein mass as measured by staining, although there was proportionately slightly more label in H1c and somewhat less in H1^0. When these labeled, rapidly dividing cells were grown in unlabeled medium for 2 days, the pattern was significantly different (Figure 2). The most significant change that occurred was the loss of radioactivity from H1c. When the core histones were used as a reference, there was a 45% loss of label from H1c during the 2-day chase period (see Table II). This shows that H1c turned over fairly rapidly in these cells. The distribution of label in PCA extracts from pulse-labeled, rapidly dividing cells gave a pattern (Figure 2) of H1 similar to that seen for H_2SO_4 extracts, suggesting that the pattern of labeling was due to H1 rather than to some contaminant.

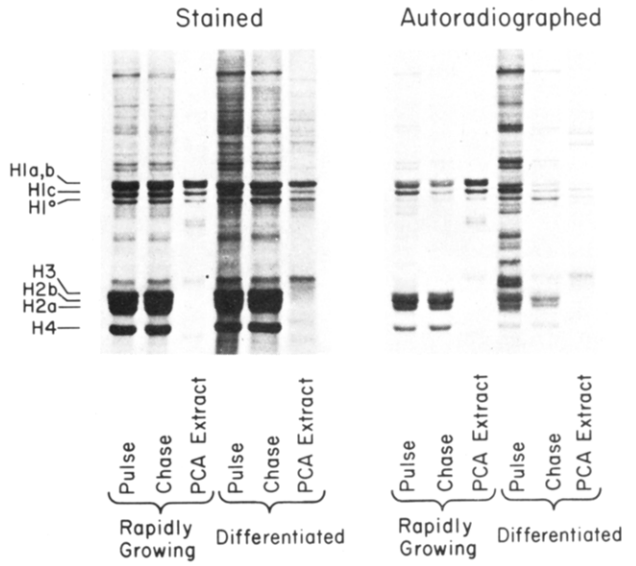

FIGURE 2: NaDodSO$_4$–polyacrylamide gel electrophoresis and autoradiography of radiolabeled histones from mouse neuroblastoma cells. The left-hand panel is a stained NaDodSO$_4$ gel, and the right-hand panel is an autoradiograph of that gel. From left to right the lanes contain histones from pulse-labeled rapidly dividing cells, 2-day chased rapidly dividing cells, PCA extract of pulse-labeled rapidly dividing cells, pulse-labeled serum-deprived cells, 4-day chased serum-deprived cells, and PCA extract of pulse-labeled serum-deprived cells.

The pattern of lysine incorporation into the H1's was significantly different when the mitotically inhibited, serum-deprived, neuroblastoma cells were pulsed. Here, in contrast to the situation in rapidly dividing cells, the incorporation of lysine into H1c was as great as that into H1a,b. The relative labeling of H1^0 was also greatly enhanced by inhibition of mitosis. While there was radioactivity in the H1a,b region of the gel, it no longer dominated the H1 part of the autoradiogram as it did when rapidly dividing cells were pulsed. When normalized to that of the core histones, lysine incorporation into H1c increased approximately 4-fold and that into H1^0 increased by more than 20-fold, when cell division was inhibited. These relative increases do not represent an absolute increase in the synthesis of these proteins since the synthesis of the core histones was markedly inhibited in these mitotically inactive cells. Although the slight incorporation of isotope into core histones could have been due to a few dividing cells rather than to the turnover of these proteins in the nondividing ones, the large increases observed in the ratio of the synthesis of H1c and H1^0, relative to the core, is a clear indication that the latter were produced in the absence of DNA synthesis. When pulse-labeled, serum-deprived cells were chased with unlabeled medium for 4 days, there were significant losses of label from both the H1c and the H1b regions of the gel. When compared to the core histones, the amounts of label in the H1b and H1c were reduced to about a third of that seen after the pulse phase. H1a,b, which was stable in rapidly dividing cells, does turnover in mitotically inactive cells. PCA extracts of pulsed, serum-deprived cells (Figure 2) gave patterns of H1 labeling similar to those seen for the complete mixture of histones, extracted by H_2SO_4 as expected, and demonstrated that this unusual pattern of H1 labeling seen in nondividing cells is due to H1 rather than to some contaminant. Pulse-labeled H1 was extracted from rapidly dividing and serum-deprived cells and treated with bacterial alkaline phosphatase. The change in the pattern of labeling of H1 from rapidly dividing cells paralleled the changes in staining seen in Figure 1. There were no changes in the labeling of H1 from serum-deprived cells

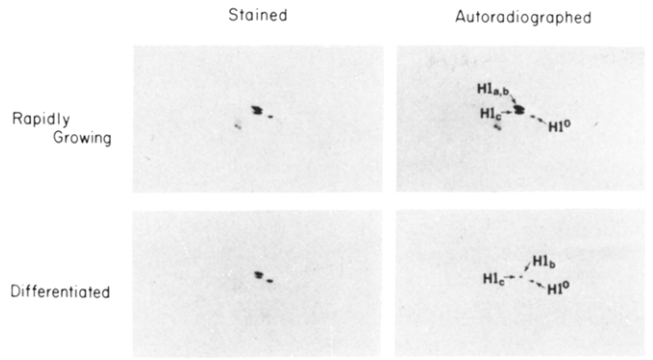

FIGURE 3: Two-dimensional gel analysis of pulse-labeled H1 and H1^0. Pulse-labeled H1 and H1^0 were extracted from neuroblastoma cells with PCA and analyzed by two-dimensional electrophoresis. The upper left-hand panel is a stained gel of H1 and H1^0 from rapidly dividing cells; the upper right-hand panel is an autoradiograph of the same gel. The lower left-hand panel is a stained gel showing H1 and H1^0 from serum-deprived cells; the lower right-hand panel is an autoradiograph of this gel. The first dimension was an acetic acid–urea–polyacrylamide gel run from left to right, and the second dimension was an $NaDodSO_4$ gel run from top to bottom.

upon treatment with alkaline phosphatase. This strongly indicates that the differences in labeling seen between rapidly dividing and serum-deprived cells were not due to differences in phosphorylation.

Two-Dimensional Gel Analysis of Pulse-Labeled H1 and H1^0. For confirmation of the purity of the radioactive bands identified as H1's, H1 was selectively extracted after pulse labeling both rapidly dividing and serum-deprived neuroblastoma cells, and the extracts were submitted to two-dimensional gel electrophoresis. The upper panels of Figure 3 show that the H1's obtained from rapidly dividing cells yield a pattern of staining that is similar to the distribution of radioactivity. Two-dimensional electrophoresis of H1's from serum-deprived neuroblastoma cells (Figure 3, lower panels) confirms the conclusions based on one-dimensional electrophoresis; H1c is strongly labeled. Further selectivity is revealed in the small amount of label in the H1b region, which was confined to the right-hand portion of the usual H1b spot. It is likely that there is more than one H1 subfraction present in this spot and that the unsymmetrical labeling of this spot was probably due to the labeling of only one of these subfractions. The preferential labeling of only one subfraction in the H1b band in nondividing cells could account for the difference in turnover rates of H1b observed between serum-deprived and rapidly dividing cells. The unequal distribution of radioactivity in the H1b spot was probably not due to phosphorylation since the labeling was confined to the front of the spot and phosphorylation has been shown to slow the migration of H1 in acetic acid–urea gels (Balhorn et al., 1971). Moreover these cells are mitotically inactive and would not be expected to have much phosphorylated H1. The H1^0 spot showed very active labeling in the serum-deprived cells, confirming the results obtained on one-dimensional gels of H_2SO_4 nuclear extracts. The two-dimensional analysis makes it very unlikely that the differences seen in lysine incorporation into the different bands of H1, when rapidly dividing cells were compared to serum-deprived cells, were due to the presence of contaminants in this region of the gel.

Discussion

There are generally several molecular species of H1 histone in any organism (Kinkade & Cole, 1966a). These H1 subfractions differ from one another in amino acid sequence (Kinkade & Cole, 1966b; Rall & Cole, 1971). Histones H1^0 and H5, which are considered to be variants of H1, are highly homologous to each other (Smith et al., 1980; Pehrson & Cole, 1981) but quite distinct from H1. What the functional relationship is between the more common H1 subfractions on the one hand, and the variants H1^0 and H5 on the other, remains to be seen, but the diversity of subfractions and variants might be used in producing chromatins whose structures differ within cells as well as between cells.

The present data show that H1^0 production and an alteration of H1 subfraction recipe occur in the absence of DNA synthesis. Despite many reports of the tight coupling of histone synthesis with that of DNA synthesis, some observations have been made previously (Gurley et al., 1972; Ohba et al., 1975; Appels & Ringertz, 1974; Tarnowka et al., 1978; Appels & Wells, 1972; Sung et al., 1977) of histone synthesis oustide the S phase. Our work shows that H1 turns over in nondividing as well as in dividing cells.

For changes in the H1 complement of chromatin to occur in the absence of DNA synthesis, turnover of the H1's must occur, and the subfractions must do so unequally. The present study demonstrates that unequal turnover of H1 subfractions is a fact. Although this conclusion was implied by earlier reports (Ohba et al., 1975; Hohmann & Cole, 1969), those studies were done on whole tissues and so must be interpreted with caution. In the present study, heterogeneity of cell type was minimized by use of a cell line. Thus our studies show that the different forms of H1 are metabolized quite differently from each other even within individual cells. The major H1 subfraction in the H1a,b band behaves metabolically somewhat like the core histones. Its synthesis relative to the core histones changed comparatively little when cell division was inhibited, and its turnover was slow. In contrast, H1c did not decrease its synthesis in parallel to that of the core histones when cell division was inhibited by serum deprivation, thus clearly indicating that this H1 subfraction continued to be made in nondividing cells.

The turnover of H1 in the absence of DNA replication might represent a repair mechanism in which the chromatin is indifferent to the exact amino acid sequence of the subfractions being exchanged. A different set of H1 genes could be used to the repair process, which requires a relatively low level of H1 gene expression, than is used during DNA replication when the demand for histone production is very high. Alternatively, rather than merely repair, the replacement of replication-linked H1's by different subfractions or variants might be part of a design for the regulation of function in particular parts of the chromatin.

Processes other than transcription could affect the pattern of H1 variants present on chromatin. Melli et al. (1977) found transcripts of histone genes in the nucleus at all stages of the cell cycle, even though histone mRNA occurred in the cytoplasm only during the S phase. Since in our studies it was the accumulation of H1 onto chromatin that was measured, rather than transcription, the specificity for the observed H1 patterns could in fact have occurred at the level of RNA processing or for that matter at the levels of translation or transport (Groppi & Coffino, 1980). Once in the nucleus the replacement H1's might differ from one another in their ability to dislodge damaged H1's, or the pattern of subfractions replaced might depend on the accessibility of replacement sites. The diversity of H1 turnover rates show that some processes after transport of histone into the nucleus contribute to the changing subfraction recipe.

The idea of a segregation of histone genes into one category that is linked to replication, and another that is not, is included

by Zweidler (1980) in a model that also includes separate categories of histone genes for meiosis and oogenesis. Moreover Zweidler's model includes alterations in recipes of core histone variants, which he has observed in several biological systems. His results seem to show sets of histone variants whose tissue levels rise and fall approximately in coordination during various developmental processes. As Zweidler recognized, the imperfections in coordination make it seem likely that posttranscriptional modulations are superimposed on the setwise activation of histone genes. Clearly our observation of diverse turnover rates of H1 subfractions and variants in homogeneous cell lines gives emphasis to posttranscriptional processes in the alteration of H1 patterns.

With the data currently available, the physiological significance of changing H1 patterns is unclear. The two processes, random repair and specific design, both have attractions, and they are not mutually exclusive. The diversity in metabolic behavior of H1a,b, H1c, and $H1^0$, in which each form differs from the others in one way during synthesis and in a different way during the degradative phase, seems to favor the specific design of histone patterns rather than merely a repair process, but further experimentation is required to test this postulate more compellingly.

Acknowledgments

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