Regulation of Nucleosomal Core Histone Variant Levels in Differentiating Murine Erythroleukemia Cells†

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ABSTRACT: During hexamethylenebis(acetamide)-induced terminal differentiation of murine erythroleukemia (MEL) cells in vitro, the histone variant proportions undergo changes similar to those observed in vivo in terminally differentiating cells of the young mouse. Thus, there is a rapid increase in the relative amounts of the variants H2A.1 and H2B.2 in parallel with the increase in the number of hemoglobin-producing cells and the sharp decrease in the growth rate. We show that the changes in variant proportions are not associated with slower growth per se but are most likely due to differential changes in the rates of variant synthesis as a result of commitment to terminal differentiation. In addition, we observed an inducer-specific increase in the rate of synthesis and the relative amount of the minor H2A variant 4, well before hemoglobin accumulation. We also present evidence that H2A and H2B histones are synthesized and incorporated into chromatin at a significant rate even when DNA synthesis is inhibited, suggesting turnover of these histones. H2A and H2B turnover can be detected directly even in exponentially growing cells. H2A.1 and H2B.2 have higher turnover rates than H2A.2 and H2B.1, respectively, in exponentially growing cells, a difference which is even more pronounced in induced cells. The magnitude of the differential turnover is not sufficient to account for the changes in the histone variant proportions in the short life of induced MEL cells but could explain the slow accumulation of H2A.2, H2B.1, and H3.3 in nondividing adult tissues of the mouse.

Histones, the primary structural proteins of chromatin, assemble in a specific manner with DNA to form linear arrays of nucleosomes, the basic structural units of eukaryotic chromosomes. Various nuclease digestion studies have indicated that the protection of DNA by histones is not uniform throughout the genome, especially among DNA sequences having different levels of transcriptional activity (Igo-Kemenes et al., 1982). It is not clear whether the histones play an active role in the establishment of different chromatin conformations and thereby in the regulation of gene expression. A more complex role than that of mere structural proteins may be indicated by the existence of nonallelic, primary structure variants which have been detected in four of the five histone classes (Kinkade, 1969; Zweidler & Cohen, 1972; Cohen et al., 1975; Franklin & Zweidler, 1977; Urban et al., 1979).

We have previously shown that the proportions of the variants of the histones H2A, H2B, and H3 change during postnatal development of the chicken and the mouse (Zweidler et al., 1978; Zweidler, 1980, 1984; Urban & Zweidler, 1983). These changes occur only in tissues in which the cellular growth and renewal rate is low. Thus, all embryonic tissues and the dividing cells of adult tissues contain relatively large amounts of the variants H2A.1, H2B.2, H3.1, and H3.2, whereas in nondividing adult tissues such as liver, kidney, and brain, these variants decrease and a second group of variants, including H2A.2, H2A.3, H2B.1, and H3.3, begins to accumulate after cell growth and DNA replication decrease to very low levels. These latter variants eventually represent as much as 80–90% of their respective histone classes (Zweidler, 1984). On the basis of these observations, it has been proposed that a subset of core histones, consisting largely or exclusively of variants H2A.2, H2A.3, H2B.1, and H3.3, are synthesized in the absence of DNA replication and incorporated into chromatin by replacement (Zweidler, 1980).

In this report, we examine the synthesis and turnover of the core histone variants in more detail in an in vitro model system of terminal differentiation, the inducible Friend murine erythroleukemia (MEL)[1] cells (Friend et al., 1971; Reuben et al., 1980). These cells, upon exposure to a number of chemical agents such as dimethyl sulfoxide (Me_2SO) and hexamethylenebis(acetamide) (HMBA), are induced to differentiate to a hemoglobin-producing state (Friend et al., 1971; Reuben et al., 1976). During this process, cell division slows and eventually stops after about four additional rounds of division. Previous work has shown that during differentiation of MEL cells, changes occur in the core histone variant composition which are analogous to those described in vivo (Blankstein & Levy, 1976; Grove & Zweidler, 1981). We now provide evidence which indicates that these changes are due primarily to changes in the relative synthesis rates of the variants of each class during differentiation and that some of the changes appear to be specifically related to the induction process while others are simply due to changes in the growth rate. In addition, we show that the histones turn over at low but significant rates, which are different for variants of the same histone species.

Materials and Methods

Cells and Culture Conditions. MEL cell clone 745 was obtained from the Human Genetic Cell Repository at the Institute for Medical Research, Camden, NJ. The cells were kept in continuous log-phase growth by diluting them 3–4 times per week to a density of (0.5–1.0) × 10^5 cells/mL. Cells were grown in Eagle's basal medium (Gibco) plus Earle's salts and L-glutamine; 25 mM sodium bicarbonate and 10 mM Hepes were added to this medium, and the pH was adjusted

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[1] Abbreviations: MEL, murine erythroleukemia; Me_2SO, dimethyl sulfoxide; HMBA, hexamethylenebis(acetamide); PAGEND, polyacrylamide gel electrophoresis in the presence of nonionic detergents; *ara*-C, arabinocytidine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, tosylphenylalanine chloromethyl ketone; TDG, thiodiglycol; HMG, high-mobility group; Cl_3CCOOH, trichloroacetic acid.

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to 7.2 prior to filter sterilization. The medium was supplemented with 10% fetal bovine serum (Gibco) and gentamycin sulfate (50 mg/mL, ICN). The doubling time of log-phase cells was 14–20 h. During induction experiments, cells were maintained at less than saturation density by daily dilution with fresh medium to approximately 0.5×10^6 cells/mL. Induction was accomplished by addition of Me_2SO (Fisher) to a concentration of 1.25–1.75% (v/v) or of HMBA to a concentration of 5 mM. HMBA was obtained from Dr. Paul Marks of the Memorial Sloan-Kettering Cancer Center and from Charles Pfizer and Co. Induction of hemoglobin synthesis was monitored by benzidine (Sigma) staining as described by Garrett & Kredich (1981).

Isolation of Nuclei and Histones. MEL cells (ca. 1×10^8) were collected, washed with cold medium, pelleted, and frozen at −70 °C. To isolate nuclei, the frozen cell pellet was partially thawed by suspension with a stirring rod in 2 mL of buffer A [50 mM glycine, 5 mM sodium butyrate, 10 mM potassium–Tris–maleate, pH 7.4, 5 mM $MgCl_2$, 0.1 mM TLCK, (Sigma), 0.1 mM TPCK (Sigma), 100 units/mL Trasylol (Mobay Chemicals Co.), and 1% thiodiglycol (Pierce)]. TLCK, TPCK, and Trasylol are protease inhibitors. TDG is an antioxidant. To the cell suspension was added 2 mL of distilled water in order to swell the cells, which were then easily broken by homogenization with an Ultra-Turrax homogenizer (Tekmar) at low speed for 5 s. The nuclei were purified by centrifugation through an equal volume of buffer A containing 5% sucrose and 0.25% Triton X-100 at about 200*g* for 5 min.

Core histones were isolated from purified nuclei as follows: The nuclear pellet was resuspended in 1 mL of buffer A + 0.15 M NaCl. To this was added 1 mL of buffer A + 0.15 M NaCl + 0.15 M $MgCl_2$ while vortexing. After 10–15 min, the suspension was centrifuged at 2000*g* for 5 min. The supernatant, containing H1, HMGs, and non-histones, was removed and the pellet reextracted with 1 mL of buffer A + 0.15 M NaCl + 0.075 M $MgCl_2$ and centrifuged as before. The resulting pellet was suspended in buffer A + 1 M NaCl and homogenized with the Ultra-Turrax at full speed for 10 s to solubilize the chromatin. The solution was then centrifuged for 10 min at 8000*g* to remove the nuclear membrane and other insoluble material. DNA and other acid-insoluble material were precipitated from the supernatant by adding 0.1 volume of 3.85 M HCl and sedimented by centrifugation for 10 min at 8000*g*. From this supernatant, core histones were precipitated by addition of Cl_3CCOOH to 10% and pelleted for 10 min at 8000*g*. The histone pellet was then resuspended in 10 mM HCl + 1% TDG, reprecipitated with addition of Cl_3CCOOH to 5%, washed sequentially with acetone + 0.4% HCl + 1% TDG and acetone + 1% TDG, and finally dissolved in sample buffer (8 M urea, 100 mM dithiotheitol, 0.01% Pyronin Y, and 1% acetic acid) for electrophoretic analysis.

Electrophoresis and Quantitation of Histone Variants. Electrophoresis of histones in Triton–acetic acid–urea gels has been described in detail elsewhere (Zweidler, 1978, 1984; Urban et al., 1979; Urban & Zweidler, 1983). H2A variants were resolved in 30-cm gels containing 5% acetic acid, 7.5 M urea, 6 mM Triton, and 10% acrylamide for 8800 V·h (maximum of 220 V), while other core histones were resolved in 30-cm gels containing 5% acetic acid, 5.75 M urea, 6 mM Triton, and 12% acrylamide for 6600 V·h. Staining and quantitation were as described previously (Zweidler, 1978, 1984; Urban & Zweidler, 1983).

Radioactive Labeling of Cells and Determination of Label in Histone Variants. Histones were labeled under various conditions as described under Results with a mixture of ^3H-labeled amino acids (lysine, proline, valine, isoleucine, leucine, and arginine; ICN) which accounts for about 50% of the residues in all histone species. DNA synthesis rates were determined by incorporation of [^{14}C]thymidine.

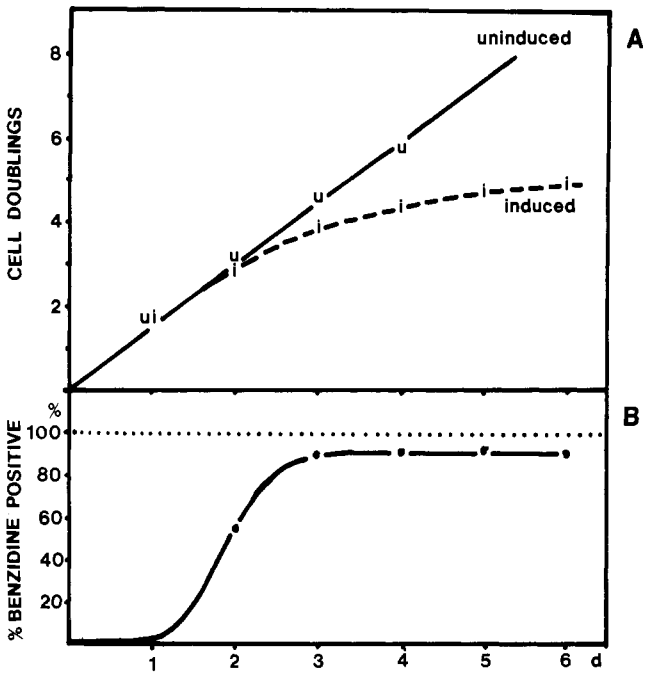

FIGURE 1: Growth kinetics and hemoglobin production during HMBA induction of MEL cells. (A) Comparison of the rates of cell division during HMBA induction and in uninduced cells. Cells were maintained well under saturation density by dilution to approximately 0.5×10^6 cells/mL with fresh medium (with or without HMBA) each day during the experiment. (B) Percent of hemoglobin-producing (benzidine positive) cells at 1-day intervals following induction [determined by the method of Garrett & Kredich (1981)].

To determine the amount of label in histones, individual bands were sliced from Amido Black stained gels and placed into scintillation vials with 200 μL of 30% hydrogen peroxide. The vials were capped tightly and kept at 60 °C overnight in order to completely hydrolyze the gel. Scintillation counting was performed with Aquassure (NEN) in an LKB Rackbeta counter with automatic quench correction.

Results

When certain lines of MEL cells are exposed to compounds such as Me_2SO or HMBA, they undergo changes which, at least in part, resemble those which occur during normal erythrocyte differentiation. This includes a commitment to limited cell division, a decrease in the cellular and nuclear volume, an accumulation of hemoglobin, and a change in the levels of a number of enzymes (Reuben et al., 1980). Figure 1 shows typical kinetics of growth and hemoglobin accumulation of MEL cells after exposure to 5 mM HMBA. After 2 days, the growth rate decreases rapidly. The number of benzidine-positive (hemoglobin-containing) cells increases to more than 90% by day 3. Because of the progressive deterioration of fully differentiated cells after 5–6 days, observations were limited to the first 6 days.

Nucleosomal Core Histones of MEL Cells. High-resolution polyacrylamide gel electrophoresis in the presence of nonionic detergents (PAGEND; Zweidler, 1978) resolves the core histones of MEL cells into over 30 components, including 12 different polypeptides which occur in multiple forms due to posttranslational modifications (Figure 2). The core histones of MEL cells are identical with those previously described in somatic mouse tissues (Franklin & Zweidler, 1977; Blankstein

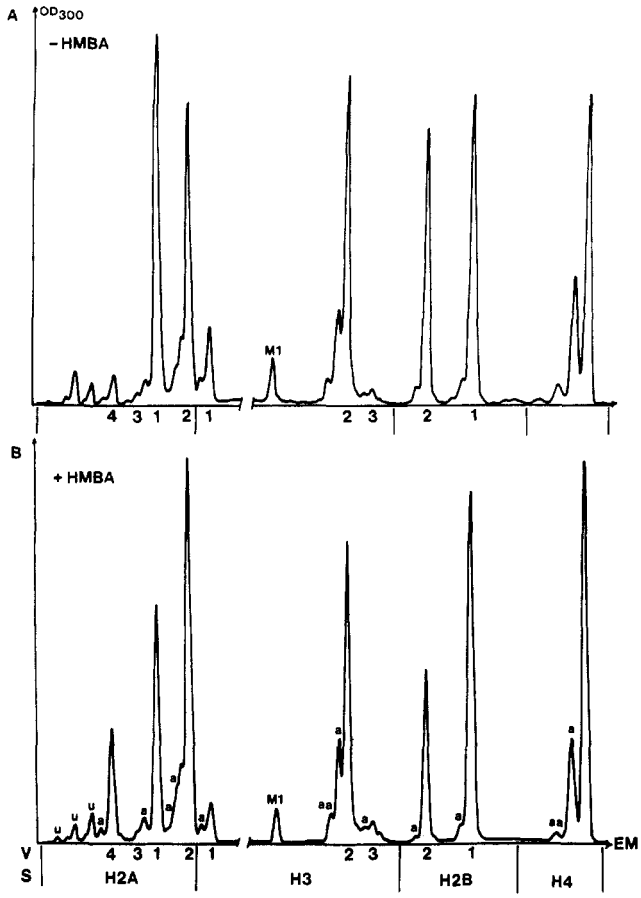

FIGURE 2: Comparison of core histone variants in uninduced MEL cells (A) and in cells harvested 4 days after HMBA induction (B). Core histones were isolated from purified MEL cell nuclei and resolved in two different electrophoretic conditions as described under Materials and Methods. The H2A region is taken from gels containing 7.5 M urea, while the remaining histones were resolved in gels containing 5.75 M urea. The gels were stained with Amido Black and scanned at 300 nm as described previously (Urban & Zweidler, 1983). Each variant is resolved into a number of components which represent posttranslational acetylation or phosphorylation (a and aa in the figure). M1 is a minor histone species distantly related to H2A; u represents covalent conjugates between H2A variants and ubiquitin.

et al., 1977; Zweidler, 1980, 1984). They include a single H4 species, two variants of H2B differing in a Ser/Gly substitution at position 60, three variants of H3 differing in a Ser/Cys substitution at position 96 or a Met/Gly substitution at position 90, four H2A variants with various degrees of primary structure differences (see below), and a minor histone species, M1.

The very complex H2A variants were further resolved in Figure 3. H2A histones undergo three types of posttranslational modification: acetylation and phosphorylation, each of which reduces the net positive charge, and covalent binding to ubiquitin (Goldknopf et al., 1977), which increases the molecular weight. Labeling with [^{3}H]acetate and [^{32}P]-phosphate allowed the identification of the charge-modified forms (m in Figure 3), which are displaced by a small increment to the left. The H2A–ubiquitin conjugates are displaced by a much larger increment and appear at the extreme left as a pattern similar to that of H2A but in about one-tenth the amount. H2A variants 1 and 2 differ primarily in a Met/Leu substitution at position 51 (Franklin & Zweidler, 1977: Blankstein et al., 1977). On very long gels, H2A.2 can be further resolved into two subcomponents (Figure 3; Zweidler, 1984) most likely differing in the presence or deletion of His^{-124} as previously shown in human H2A (Hayashi et al., 1980). Since the two forms of H2A.2 ($H2A.2^a$, $H2A.2^b$) always occur in a constant ratio in MEL cells, we have not distinguished between them for the purposes of this report. H2A.3, which differs from H2A.1 at several positions, including a Glu/His substitution at position 124 (Zweidler, 1980), is a very minor component in MEL cells and in all proliferating tissues but becomes a major H2A component in long-lived, nondividing cells (Zweidler, 1980, 1984). H2A.4 was originally described as an H2A-related minor histone species, M2 (Zweidler, 1976). It is probably identical with H2A.X (West & Bonner, 1980), as well as H2A.S, a major H2A component of mouse testes (Zweidler & Franklin, 1979; Zweidler, 1980). The latter differs from H2A.1 in a deletion of His^{-124}, several additional substitutions, and an insertion resulting in a significantly increased molecular weight. In part because of observations described below, we now consider M2 a variant of H2A (H2A.4) rather than a distinct histone species. In contrast, the minor histone M1, although related in amino acid sequence to H2A (Zweidler, 1976; Ball et al., 1983), has not seen shown to be functionally related to H2A [see below and Zweidler (1984)] and will be treated as a distinct histone species.

Changes in Histone Variant Accumulation and Synthesis Rates during MEL Cell Differentiation. Figure 2 shows the histone variant profiles of MEL cells prior to and 4 days after induction with HMBA. There are obvious changes in the proportions of the H2A and H2B variants and less pronounced changes in the proportions of the H3 variants. Similar but less extensive changes had been observed previously after induction with Me_2SO (Blankstein & Levy, 1976; Grove & Zweidler, 1981). The observed increases in the relative amounts of H2A.2, H2B.1, and H3.3 and the corresponding decreases in the relative amounts of H2A.1, H2B.2, and H3.1 are similar to the histone variant changes observed in terminally differentiated, normal tissues of the mouse during postnatal development (Zweidler, 1980, 1984). The substantial increase in H2A.4 after HMBA induction of MEL cells has not been observed in any somatic tissue. However, a similar increase in H2A.4 does occur during early stages of spermatocyte differentiation (Zweidler & Franklin, 1979; Trostle-Weige et al., 1982). Since the stoichiometric relationship of H2A to the other core histones does not change after HMBA induction, we conclude that H2A.4 increases at the cost of the major H2A variants and therefore can replace the latter in the nucleosome core complex.

Figure 4 shows the kinetics of the changes in the relative amounts of the different variants after induction with 5 mM HMBA. In the case of H2A, all four variants and M1 behave differently. The two major variants, H2A.1 and H2A.2, show reciprocal changes, with the relative amount of H2A.1 decreasing between day 1 and day 4 to about half the initial value and H2A.2 increasing correspondingly. H2A.4 also increases, but earlier than H2A.2, similar to the rapid, early increase in H1.0 (Keppel et al., 1977). H2A.3 and M1 remain essentially constant although M1 may increase slightly in the late stages of differentiation. The two H2B variants behave similarly to the major H2A variants, with H2B.2 decreasing after day 1 to about 75% of its initial relative amount and H2B.1 increasing correspondingly. The relative amount of the major H3 variant, H3.2, remains essentially constant, with H3.1 decreasing rapidly to about half its initial value and H3.3 increasing slightly.

In general, the changes in the relative amounts are paralleled by changes in the relative rates of synthesis (Figure 4). The absolute synthesis rate of each variant declined after induction

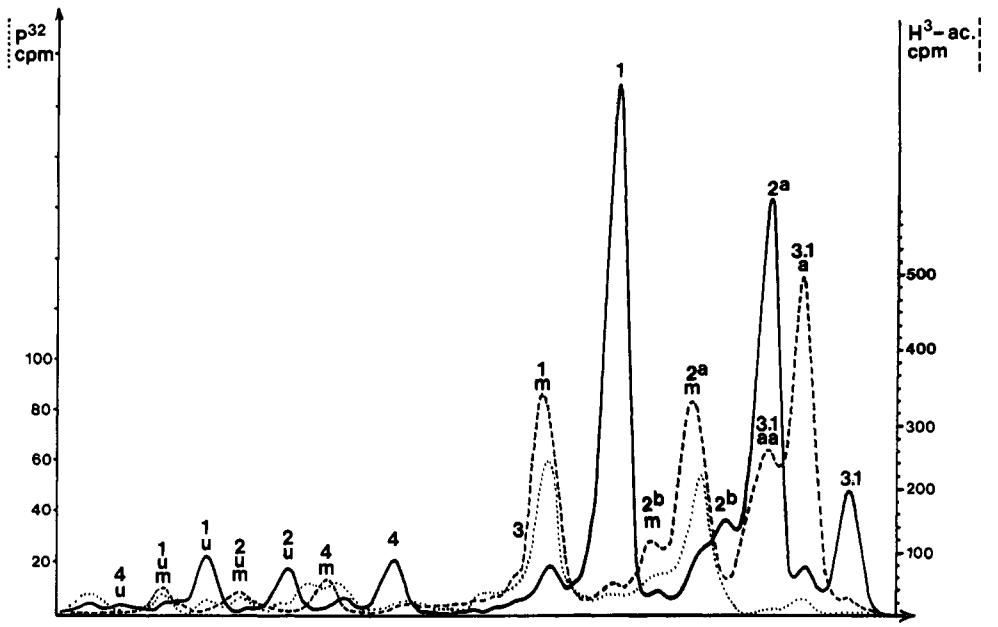

FIGURE 3: Resolution of MEL H2A variants and their modified forms. Core histones were extracted as described under Materials and Methods from uninduced cells labeled either with sodium [^{3}H]acetate (NEN, 12.5 μCi/mL, 0.5 h) in the presence of 10 μg/mL cyclohexamide or with [^{32}P]phosphate (NEN, 20 μCi/mL, 1 h). The solid line represents the protein profile (scanned at 300 nm). H2A variants were analyzed in 30-cm polyacrylamide (10%) gels containing 6 mM Triton, 5% acetic acid, and 7.5 M urea. Electrophoresis was for 8800 V·h (maximum of 220 V). Variants H2A.1, H2A.2^a, H2A.2^b, H2A.3, and H2A.4 are indicated. m denotes modification by acetylation (---) or by phosphorylation (···). u denotes H2A molecules covalently bound to ubiquitin. H3.1 and its mono- and diacetylated forms (a and aa, respectively) are also present in this portion of the gel (far right).

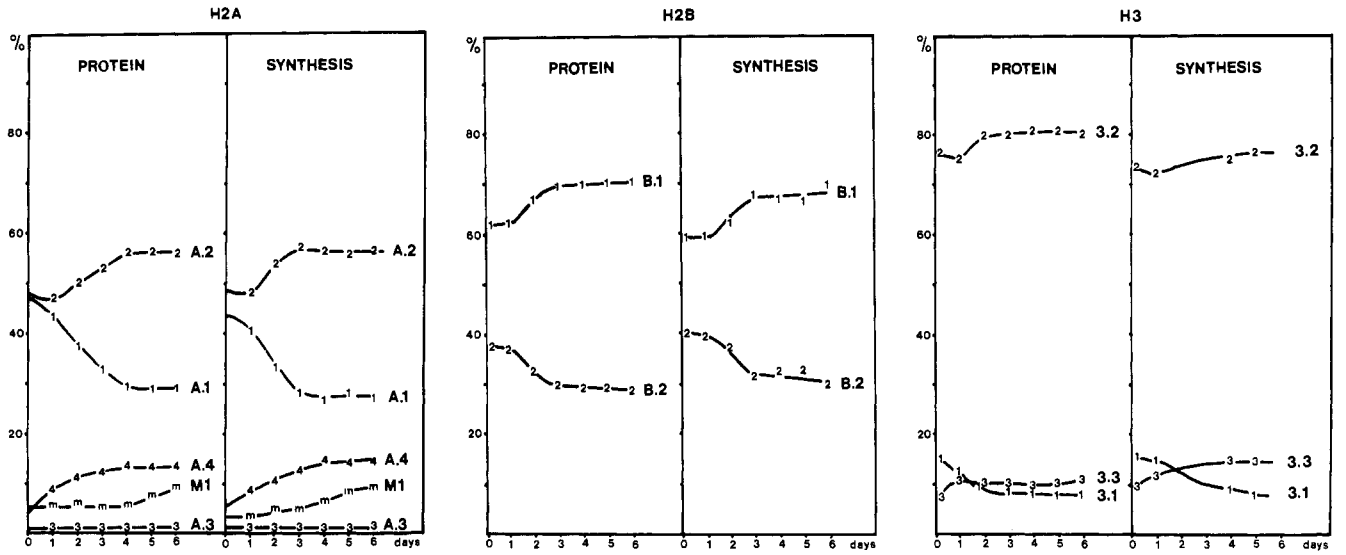

FIGURE 4: Changes in variant protein and synthesis ratios during HMBA induction. Histones were isolated from 10^8 cells collected each day during induction by HMBA (5 mM). After separation in Triton gels, scans of the gels were used to quantitate the amount of each variant. For synthesis rates, aliquots (10^8 cells) of an induced culture were pulse labeled at each time point for 90 min with 150 μCi of ^{3}H-labeled amino acids. Radioactivity in electrophoretically resolved variants was determined as described under Materials and Methods. In each part of the figure, the portion of protein and the synthesis represented by each variant within a species (i.e., H2A, H2B, and H3) are shown. H2A variants (left panels) 1, 2, 3, and 4 are expressed as the percent of total H2A. M1 (m) is expressed relative to total H2A. H2B variants 1 and 2 (center panels) and H3 variants 1, 2, and 3 (right panels) are expressed as a fraction of their respective classes.

(Figure 5), essentially paralleling the declining rate of cell division. The rate of decline and the ultimate level of residual synthesis, however, were different for the variants within each histone species, giving rise to the observed changes in relative synthesis rates. Since HMBA treatment results in a reduced fraction of the cell population traversing S phase at any given time, the change in the relative rates of synthesis of different variants after HMBA induction may result either from a change in their relative rates of synthesis during S phase of induced cells or from a greater contribution of basal histone synthesis rates between S phases. That histones can be synthesized in the absence of DNA synthesis is indicated by the *ara*-C experiments shown in Figure 5. The relative rates of variant synthesis in the absence of DNA replication (Figure 5, Table I), however, could account only for the accumulation of H3.3 in HMBA-induced cells but not for the accumulation of H2A.2, H2A.4, and H2B.1 or the decrease in H3.1. Thus, most of the changes in relative variant synthesis rates are apparently related to the induction of differentiation rather than to changes in cell cycle parameters. M1 is synthesized at a high rate in the absence of DNA synthesis but does not accumulate significantly during induction, indicating rapid turnover. Residual synthesis of M1 (H2A.Z) and H3.3 in the absence of DNA synthesis has also been observed in other cells

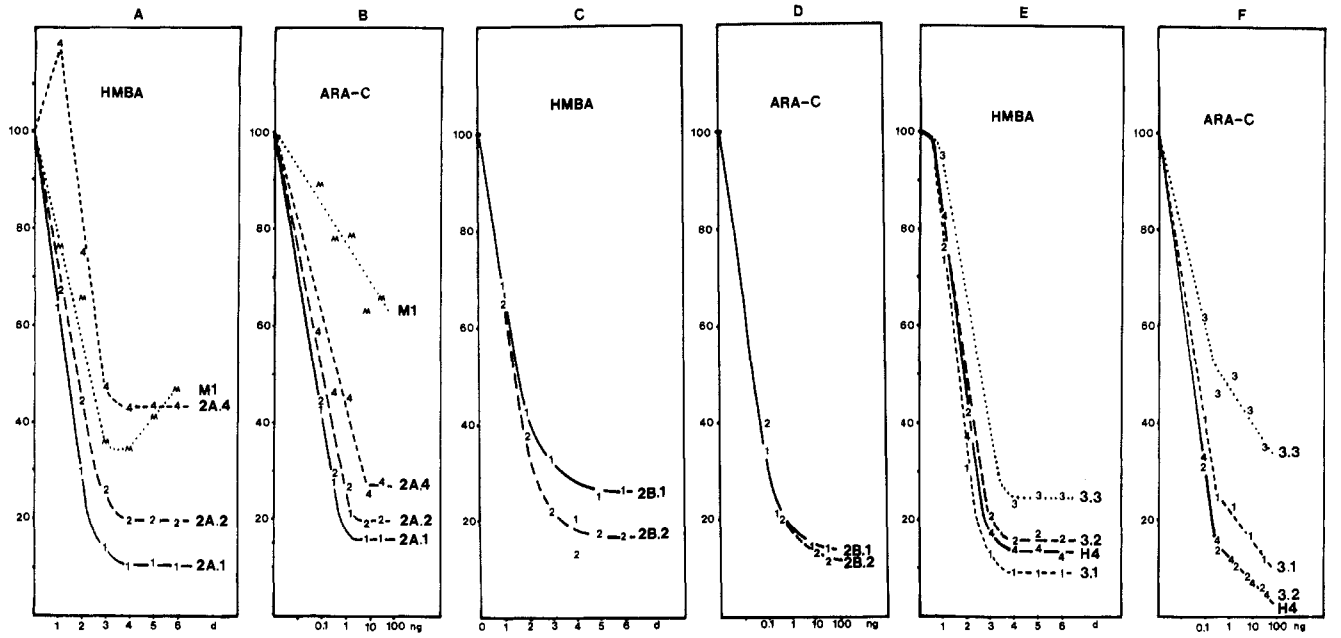

FIGURE 5: Comparison of synthesis rates of histone variants during HMBA induction and during inhibition of DNA synthesis by arabinocytidine. Histones were pulse labeled and analyzed for radioactivity as described under Materials and Methods. The left half of each pair of graphs (panels A, C, and E) shows the relative amount of label incorporated by each histone variant (from 10^8 cells) at each time point during HMBA induction. The amount incorporated at time zero has been normalized to 100%, and each subsequent time point has been expressed as the percent of the zero time point. The right half of each pair of graphs (panels B, D, and F) shows the effect of *ara*-C on individual histone variant synthesis. Cells were treated with various concentrations of *ara*-C (0–33.3 ng/μL) for 1 h and then labeled in the continued presence of *ara*-C for 1.5 h with 2 μCi/mL ^{3}H-labeled amino acids. The label incorporated into control, untreated cells has been normalized to 100% for each variant. The amount of label incorporated at each concentration of *ara*-C has been expressed relative to the control.

Table I: Variant Synthesis Ratios during HMBA Induction and during Inhibition of DNA Synthesis by Arabinocytidine

	ratio[a]		
	control	*ara*-C[b]	HMBA[c]
H2A.2/H2A.1	1.05 (1.00)	1.26	1.95
H2A.4/H2A.1	0.10 (1.00)	1.71	4.19
M1/H2A.1	0.10 (1.00)	4.16	4.19
H2B.1/H2B.2	1.45 (1.00)	1.07	1.42
H3.1/H3.2	0.18 (1.00)	2.22	0.78
H3.3/H3.2	0.12 (1.00)	5.33	1.67
residual DNA synthesis[d]	100	<1	10–20[e]

[a] Ratios were determined from the data in Figure 5. [b] At 33 μg/mL (see Figure 5 legend). [c] Four days after initiation of induction. [d] Values are percentages. [e] This value is an approximation. Because of changes in the thymidine pool specific activity during the several day duration of the experiment, precise measurement of the relative levels of [^{14}C]thymidine incorporation was not possible.

(Wu & Bonner, 1981). A striking observation in cells treated with high levels of *ara*-C is the 3–4-fold higher level of residual incorporation into chromatin of H2A + H2B relative to H3 + H4 (compare panels B, D, and F of Figure 5). This indicates that in *ara*-C-treated cells differential accumulation or turnover of histones occurs in the absence of DNA replication and raises the possibility of differential turnover of histones in growing cells.

Histone Turnover. It is difficult to determine absolute rates of turnover for very stable proteins such as the histones because of the reutilization of label released from proteins with shorter half-lives. It is, however, possible to determine relative rates of turnover among similar proteins, such as the histone variants, under steady-state conditions where the rates of synthesis and the relative amounts of proteins remain constant. This condition is met in exponentially growing cells and in induced cells after about 3 days. Figure 6A shows that after removal of the labeled precursors from exponentially growing cells, the incorporated radioactivity is lost at a similar rate for H3 and H4 but at a higher rate for H2B and H2A, as predicted if the latter were turning over preferentially. The minor histone species M1 turns over at a much higher rate than any of the other core histone components. Among the H2A variants, H2A.1 has the highest relative rate of turnover and H2A.2 the lowest, while H2A.4 is intermediate (Figure 6B). The H2B variants differ only slightly in their rate of turnover, and no significant difference is detectable for the turnover rates of the H3 variants (Figure 6C,D). The difference in turnover rates between H2A and H2B variants is increased significantly after induction of terminal differentiation with HMBA (A.1i and B.2i in Figure 6B,C) and appears not to be influenced by the changes in the relative rates of synthesis. Thus, the increased difference in turnover rates between H2A.1 and H2A.2, as well as between H2B.1 and H2B.2, is noticeable within the first day of induction, when the cellular growth rate is still high and the relative rates of variant synthesis are still unchanged, and continues at the same level after the third day, when the rate of histone synthesis has dropped to about 20% of control and the relative rates of synthesis have stabilized at the level of fully differentiated cells (Figures 4 and 5).

One of the major pathways of protein turnover requires ATP and involves formation of protein–ubiquitin intermediates (Hershko & Ciechanover, 1982). A significant fraction of all H2A histones occurs in conjugates with ubiquitin (Goldknopf et al., 1975; Wilkinson et al., 1980). The level of H2A–ubiquitin conjugates is a function of three possible reactions: (a) formation; (b) reversal or cleavage; and (c) removal from chromatin and degradation. If a significant portion of the H2A–ubiquitin conjugates led to protein breakdown, it might be expected that the level of these conjugates would be different for two variants differing in their rate of turnover and that this difference should increase when the difference in their relative turnover rates increases after induction of differentiation. We therefore carefully quantitated the level of ubiquitin conjugates for the H2A variants 1 and 2 before and after

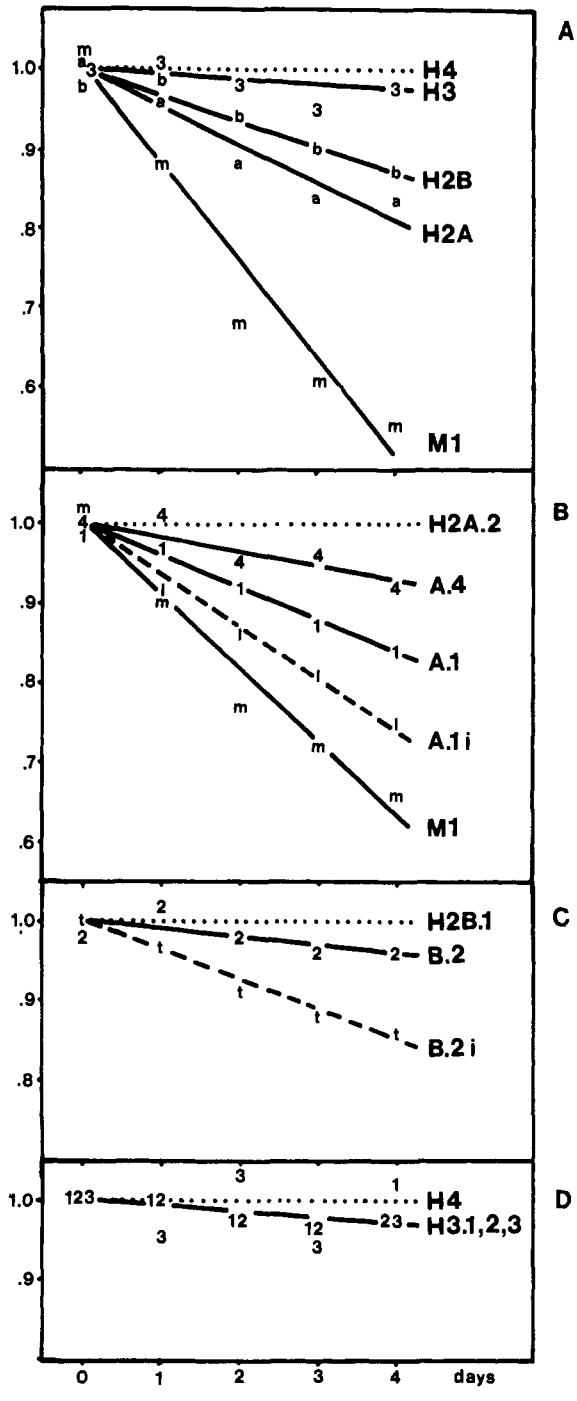

FIGURE 6: Turnover rates of MEL cell histones. Histones in an exponentially growing culture were labeled for 10 h with 5 μCi/mL ^{3}H-labeled amino acids. The cells were then pelleted, resuspended into fresh medium without label, and grown for an additional 24 h before the first duplicate samples (zero time) were collected. Subsequent samples (10^8 cells) from exponentially growing or induced (5 mM HMBA) cultures were collected at 24-h intervals. The data in each panel in the figure represent the average of two or more experiments. (A) Relative turnover rates among the four classes of core histones and M1 in exponentially growing MEL cells. For each time point, the amount of label remaining in H3, H2B, H2A, and M1 has been expressed relative to H4. The same order of stability and approximate extent of difference in the relative turnover rates was also obtained in several experiments with induced cells. Panels, B, C, D show the relative rates of turnover among the H2A variants (B), the H2B variants (C), and H4 and the H3 variants (D). In each case, the label remaining in the most stable species, i.e., H2A.2, H2B.1, H4, has been normalized to 1.0, and all other variants in each class have been expressed relative to these. Except where indicated (i), the data are taken from uninduced, exponentially growing cells.

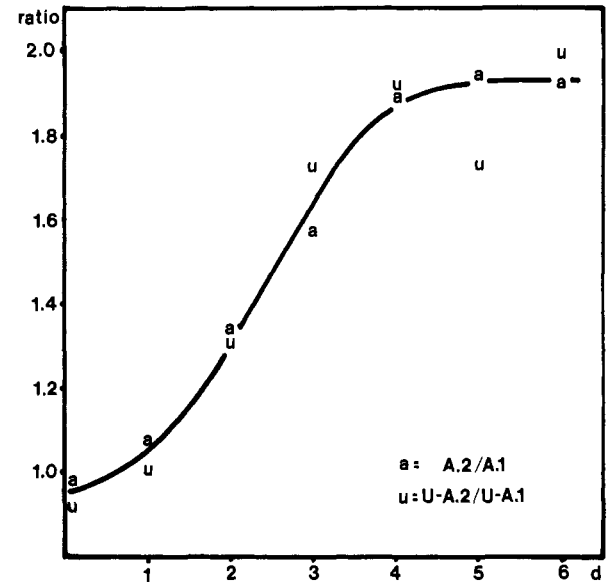

FIGURE 7: Change in the ratio of ubiquitinated H2A variants during HMBA induction. The relative amounts of H2A.1 and H2A.2 found in covalent association with ubiquitin at 1-day intervals during HMBA induction have been expressed as the ratio H2A.2/H2A.1 (u). Also shown is the ratio of the nonubiquitinated H2A variants (a). Amounts were determined from Triton gel scans as described under Materials and Methods.

induction of terminal differentiation in MEL cells with HMBA. Figure 7 shows that the ratio of H2A.2–ubiquitin to H2A.1–ubiquitin conjugates changes in parallel to the ratio of the free H2A variants despite their difference in turnover rates (Figure 6). It is, therefore, unlikely that the H2A–ubiquitin conjugates are intermediates in turnover. Whether they have any other functional significance is not known.

Discussion

During induction of terminal differentiation of murine erythroleukemia cells, there are changes in the histone variant proportions which are similar to those occurring in most differentiating tissues of the young mouse (Zweidler, 1980, 1984; Grove & Zweidler, 1981). A detailed analysis of histone metabolism in the differentiating MEL cells revealed that several different mechanisms affect the relative amounts of the different histone variants. These include the following:

(*1*) *Early Changes in the Relative Rates of Synthesis.* Relatively small changes in the histone complement are observed in the first 24 h after MEL cells are exposed to an inducing agent, before a significant number of cells exhibit the differentiated phenotype of hemoglobin accumulation. It had previously been observed that H1°, an outer histone, is induced and accumulates early (Keppel et al., 1977). We now show that the core histone variant H2A.4 is induced and accumulates with kinetics similar to those of H1° (Figure 4). H2A.4 accumulation is partially inducer specific in that the extent of accumulation is severalfold higher with HMBA than with Me_2SO (Grove & Zweidler, 1982). Although H2A.4 accumulation has not been observed during terminal differentiation of normal somatic cells, a similar increase in H2A.4 is observed as an early event in spermatocyte differentiation (Zweidler & Franklin, 1979; Trostle-Weige et al., 1982).

(*2*) *Late Changes in the Relative Rates of Synthesis.* Most of the molecular changes associated with the differentiated phenotype develop between 24 and 72 h after induction, as a consequence of the commitment to terminal differentiation of increasing numbers of cells. During this period, the proportions of H2A.1/H2A.2, H2B.2/H2B.1, and H3.1/H3.2

incorporated into newly synthesized nucleosomes change with kinetics similar to those of hemoglobin accumulation (compare Figures 1B and 4). This could be due either to quantitative regulation of expression of all the involved histone genes or to a reduction in the number of H2A.1 and H2B.2 genes which are induced at the beginning of each S phase. The latter possibility is more likely in view of the recent observations on the expression of H2A.1 mutants in terminally differentiating cells in vivo (Zweidler, 1984).

(*3*) *Replication-Independent Synthesis and Incorporation of Histone Variants.* Replication-independent incorporation of histones into chromatin has been postulated on the basis of changes in the histone variant proportions in nondividing tissues of adult mice (Zweidler et al., 1978; Zweidler, 1980, 1984) as well as in cells synchronized in G1 (Wu & Bonner, 1981) or blocked during S phase (Zlatanova, 1980; Grove & Zweidler, 1982; Leffak, 1983). The differential incorporation of newly synthesized histone variants in the absence of DNA replication in *ara*-C-treated MEL cells (Figure 5) could be due to differences either in the rates of their residual, replication-independent synthesis or in the rates of their exchange with histones from nucleosome core complexes, or both. Our data indicate that incorporation by exchange into old nucleosomes may occur, although at a very low rate. Thus, one would have expected those histone variants which are synthesized at the highest relative rate in the absence of DNA replication, such as H3.3 and H2A.4, to accumulate most rapidly after the cellular growth rate had dropped to a very low level, 3–4 days after induction. However, this was not observed. It is possible that replication-independent incorporation of histones is inhibited in the condensed chromatin of fully differentiated MEL cells (Friend et al., 1971). In addition, the high rate of replication-dependent synthesis of histones prevents the measurement of small amounts of replication-independent incorporation during the early stages of induction. We therefore directly measured the rate of loss of old histones due to exchange, or histone turnover.

(*4*) *Differential Turnover of Histone Variants and Histone Species.* In this context, we were primarily interested in possible differences in the relative, rather than the absolute, rates of turnover among histones. By simply measuring the ratios of counts remaining after the different chase periods, we could eliminate most sources of variability, and even small differences were reliably measured. The observation that H2A and H2B turn over significantly faster than H3 and H4 histones agrees with the higher level of H2A and H2B synthesis in the absence of DNA replication (Figure 5). Unexpectedly, these experiments also revealed a significant difference in the rate of turnover between the variants of H2A and H2B, a difference that was even more pronounced after induction of differentiation. A similar difference was not observed among the H3 variants, possibly because the overall rate of turnover of H3 is too low to allow its detection. The significance of the observed differences in relative turnover rates is not clear at this time, but it could possibly reflect a difference in the stability of nucleosome core complexes of different variant compositions (Simpson, 1981).

The minor histone M1 exhibits unusual behavior in several respects. M1 synthesis is almost completely independent of DNA replication (Figure 5; Wu & Bonner, 1981). It does not, however, accumulate in nondividing cells (Zweidler, 1984). This is most likely due to a high rate of turnover (Figure 6) which compensates for the high rate of replication-independent synthesis. Thus, although M1 is related to H2A in amino acid sequence (Ball et al., 1983), its metabolic properties have diverged substantially. Other lines of evidence which support this conclusion are the following: (1) M1 may not participate in the formation of typical nucleosome core complexes because it can be dissociated individually from native nucleosome preparations under conditions where H2A + H2B is dissociated as dimers (A. Zweidler, unpublished results); (2) Allis et al. (1982) have shown that a protein, designated hv 1 and apparently identical with M1, is enriched in the nucleoli of mammalian cells and of the transcriptionally active macronucleus of *Tetrahymena*; and (3) a chicken gene coding for a protein, H2A.F, with an amino acid sequence identical with that of M1 (Harvey et al., 1983) produces a polyadenylated mRNA, while other somatic histone mRNAs are not polyadenylated.

Our observations with the MEL cells support and extend the classification of somatic histones into at least four expression types, proposed on the basis of observations made with normal tissues during terminal differentiation and liver regeneration (Zweidler, 1984):

(1) One expression type consists of strictly replication-dependent histones induced at the beginning of S phase and repressed when replication stops (e.g., H2A.1, H2B.2, H3.2, and H3.1). These histones occur in the highest amounts in rapidly dividing cells. The extent of induction may vary with the rate of replication.

(2) A second expression type is partially replication-dependent histones, which are also induced at the beginning of S phase but are not completely repressed when replication stops (e.g., H2A.2 and H2B.1). These histones occur in high amounts in dividing cells and increase even further in nondividing cells.

(3) Replication-independent histones which are constitutively expressed at a low rate (e.g., H2A.3 and H3.3) constitute the third expression type. These histones occur in very low amounts in rapidly dividing cells but accumulate slowly in nondividing cells.

(4) Histones whose synthesis and relative amounts are determined by factors other than growth rate (e.g., H2A.4, H1°, and M1) are the fourth expression type. These histones usually occur in low amounts but can be induced, often under tissue-specific circumstances.

Histone synthesis is regulated at least at two levels, namely, the rate of transcription and mRNA stability (Hereford et al., 1981; Rickles et al., 1982; Heintz et al., 1983). Thus, metabolic differences between replication-dependent and replication-independent histones are based both on the regulation of gene expression and on the properties of their mRNAs (Sittman et al., 1983). While replication-dependent histone mRNA does not contain poly(A), replication-independent histone mRNA contains either oligo(A) (Borun et al., 1977; Engel et al., 1982) or poly(A) (Harvey et al., 1983).

Histone turnover is generally very low with half-lives of weeks or months (Piha et al., 1966; Commerford et al., 1982). That there is significant turnover, however, is indicated by the changes in histone variant proportions in nondividing cells (Zweidler, 1980, 1984) as well as the differential decay of histone species and variants in exponentially growing MEL cells as reported here (Figure 6). The significance of this asymmetric turnover is not known. It may be related to different requirements for nucleosome stability in long-lived nondividing cells, since the rate of turnover is generally lower for those histone variants which accumulate in nondividing cells (H2A.2 and H2B.1).

The significance of the observed changes in histone variant synthesis, in terms of the process of commitment to terminal

differentiation of MEL cells, is difficult to assess. Further experiments are required in order to establish whether they are necessary, for example, for the decrease in proliferation potential or for the altered state of transcription. We have determined that the inhibition of hemoglobin accumulation by exposure to procaine or dexamethasone (Scher et al., 1978; Tsiftsoglou et al., 1981) does not inhibit the changes in the histone metabolism (G. W. Grove and A. Zweidler, unpublished results). The relationship between alterations in transcriptional patterns, growth inhibition, and histone variant changes remains to be established.

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