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Regulation of Histone and β^A-Globin Gene Expression during Differentiation of Chicken Erythroid Cells

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The expression of the genes for several histones and β^A-globin was examined in the chicken erythroid cell lineage. During the transition from CFU-(E) to the mature erythrocyte, histone H5 gradually increased fourfold in nuclei with little concomitant displacement of the H1 histones. This resulted in a 70% net increase in linker histone (H1 plus H5) content. The differential accumulation of H5 reflected (i) an increase in the transcriptional activity of the H5 gene occurring at the erythroblast stage, (ii) an apparent longer half-life of H5 mRNA, and (iii) a higher stability of the protein. Although the transcriptional activity of the histone genes (except H5) decreased with cell age, it was not tightly coupled to the S phase. On the other hand, the mRNA levels for these histones were tightly regulated during the cell cycle. Use of protein and DNA synthesis inhibitors indicated that the content of H5 mRNA was regulated at the posttranscriptional level by a control mechanism(s) differing from those for the other histones. Although the transcription rates of the H5 and β^A-globin genes were comparable, differential accumulation of β^A-globin mRNA led to a 30- to 170- fold-higher copy number of the β^A-globin mRNA as the cell matured.

The histone genes can be considered as belonging to one of three major classes, depending on how their expression is controlled (reviewed in references 56 and 59). The first group consists of the replication-dependent genes, the regulation of which is tightly coupled to DNA synthesis (8, 12, 19, 37). Genes in the second class are partially replication dependent (10, 58). Expression of this class of genes may be triggered by the onset of DNA replication but is not completely repressed after DNA synthesis. The third class of genes code for replication-independent variants, also termed replacement histones, due to their pattern of expression during development and differentiation (45, 52, 55, 57). In systems of low cellular turnover, these constitutively expressed variants can represent an important proportion of the histone moiety (53). This third class of histone genes, among which H5 could be considered to belong, are structurally unique (11, 18, 22), and as first shown for H5 (29), their mRNAs are polyadenylated (reviewed in reference 7).

The H5 gene (26, 39), coding for an H1-like linker (i.e., extranucleosomal) histone, is unusual in that it is only expressed in nucleated erythrocytes (31). In the chicken, H5 is an early marker of the erythroid lineage. Thus, it is already expressed in avian erythroblastosis virus (AEV)-transformed cells arrested at the colony-forming unit [CFU-(E)] stage, when globin is not detected (5, 44). Studies with transformed CFU-(E) cells have shown that the single-copy H5 gene (26, 39) is transcribed constitutively in the different phases of the cell cycle (14; unpublished observations). In the context of a dividing cell, this mode of regulation is similar to that of the replication-independent histone genes.

The content of H5 relative to H1 remains modest in proliferating precursor cells. However, H5 accumulates in the nucleus once the cells enter the final stages of maturation (3, 6, 30, 43). This pattern of behavior is emphasized in immature erythrocytes from anemic peripheral blood. A purified population of these cells contains less than 0.1% S-phase cells, yet the rate of H5 transcription is higher than that observed in proliferating preerythroblasts (1). This observation, together with the high levels of H5 in mature erythrocytes which are not matched by any of the other replacement variants (34, 53), suggests that the H5 gene may be regulated independently of all other histone genes and that it may constitute a special class.

To study this question, we have examined the transcriptional and posttranscriptional regulation of several histone genes in populations of adult erythroid cells at different stages of maturation. We show that although H5 shares some mechanisms of control with the other histone genes, its transcription is independently regulated. In this respect, H5 behaves like β^A-globin, a typical product of the differentiated state. In addition, we show that H5 accumulation is due not only to its continued expression, but also to its selective deposition in the chromatin of the maturing cells.

MATERIALS AND METHODS

Cells. Mature hen erythrocytes (ME) and immature erythrocytes from peripheral blood (IE-B) were obtained and purified by filtration through SP-Sephadex as described elsewhere (32). Erythroid cells at earlier stages of maturation were obtained from anemic bone marrow. After removal of adherent cells by filtration, the bone marrow cells were fractionated by isopycnic centrifugation in preformed Percoll (Pharmacia) gradients (36). Three pools of cells with average densities of 1.075, 1.084, and 1,095 g/cm^3 were made (IE-1, IE-2, and IE-3, respectively). Clone HD3-41/2, a chicken erythroleukemia cell line transformed by *ts*34 AEV (5), was grown at 36°C (the permissive temperature) as described previously (36). Erythroleukemia cells (5×10^8 to 1×10^9) were fractionated by sedimentation in Earle bal-

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anced salt solution containing 1% fetal calf serum and 0.2% Ficoll (Pharmacia) for 4 h at 4°C.

Histone analysis. After preincubation for 10 min in lysine-free Joklik minimal essential medium (GIBCO Laboratories), cells (2×10^8/ml) were labeled with 0.1 mCi of [4, 5-^{3}H] lysine (85 Ci/mmol; New England Nuclear Corp.) per ml for 15 min. Labeled cells were washed once in 10 volumes of medium containing a concentration of lysine fourfold higher than usual and suspended at 10^7 cells per ml in complete medium containing twice the regular amount of lysine. At the indicated times, cells were harvested in chilled medium, washed twice in buffer A (0.25 M sucrose, 20 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4], 3 mM $MgCl_2$, 0.4 mM phenylmethylsulfonyl fluoride) and stored at −80°C.

Nuclear proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (27). Nuclei were lightly digested with DNase I, treated successively with 18% trichloroacetic acid (TCA) and 0.1% HCl in acetone, and dissolved in sample buffer. The relative amounts of histones were quantified by densitometry (Chromoscan 3; Joyce Loebl) after Coomassie brilliant blue staining of the gel. Equivalent amounts of the different samples were run in the same slab. Since only relative changes were characterized, no correction for differential dye binding of the various histones was made.

To determine the relative rate of histone synthesis, two gel replicas were run. One was impregnated with En^{3}Hance (New England Nuclear) and autoradiographed. Histone bands were cut from the other replica, which had been stained with Coomassie, and labeling was determined by liquid scintillation counting. Gel slices were treated with 1 ml of 90% Protosol at 37°C for 18 h, after which time 10 μl of glacial acetic acid was added. The corrected count was normalized to the protein content in each gel lane by using the core histones as a standard.

Treatment with inhibitors. The effect of DNA and protein synthesis inhibitors (aphidicolin [5 μg/ml], 0.1 mM cycloheximide, or puromycin [100 μg/ml]) was determined from the uptake of [*methyl*-^{3}H]thymidine (20 μCi/ml; specific activity, 40 Ci/mmol; Amersham Corp.) or [^{3}H]lysine (20 μCi/ml) as described above. More than 90% of the cells were viable after the different treatments, as determined by trypan blue exclusion. Control samples contained the same amount of solvent as used for the treatments (0.2% dimethyl sulfoxide for aphidicolin and 0.1% ethanol for cycloheximide). Samples of cells were diluted in water and precipitated with 20% TCA at 0°C. After being washed with 20% TCA, the pellets were incubated in 0.5 M NaOH for 30 min at 50°C. Following neutralization and precipitation with 20% TCA, the samples were washed, solubilized in Protosol (New England Nuclear), and counted.

Transcription analyses. Transcription rates were determined by run-on assays with permeabilized cells (200 to 600 μg of nuclear DNA per assay) as described (1), except that cells were washed in buffer A before freezing. RNA elongated in vitro was hybridized to equimolar amounts of the different cloned genes (about 2.5 μg of plasmid DNA) immobilized on nitrocellulose filters (BA85; Schleicher & Schuell). Semiquantitative measurements were made on autoradiographs of filters before and after treatment with a mixture of RNase A and RNase T_1 (1). Quantitative measurements (duplicate) were carried out by scintillation counting. Background hybridization (5 to 10 cpm), determined with pBR322, was substracted from the counts hybridized to the respective gene sequences. Corrected counts were normalized to the length of the genes and expressed per 100 μg of nuclear DNA present in the run-on assays. Since the only unit of transcription known is that of the H5 gene (1), it was assumed in all cases that the unit of transcription included sequences from the cap site to the processed 3′ end of the mRNAs (i.e., 880 base pairs [bp] for H5, 520 bp for H2A, and 1,525 bp for β^A-globin; see below for references).

mRNA levels. Total RNA was isolated with guanidine thiocyanate (13). A portion of the homogenate was taken to determine the amount of cellular DNA (below), and the preparations were treated with 5 μg of RNase-free DNase I (Bethesda Research Laboratories) per 100 μl. For mRNA dosages, RNA samples in 200 μl of 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–7.4% formaldehyde were heated at 65°C for 15 min. Samples were serially diluted with 15× SSC and dotted onto nitrocellulose filters (BA83; Schleicher & Schuell). Plasmid DNA containing the corresponding cloned genes was treated with 0.5 M NaOH for 10 min at 37°C. After neutralization with 1 M Tris (pH 7.4), the solution was adjusted to 1 M in ammonium acetate and absorbed onto the filters. DNA thus treated served as an internal hybridization standard. After hybridization (see below for the probes used), the radioactivity bound to the filters was determined by scintillation counting. No hybridization was observed when the RNA samples were digested with RNase A prior to absorption onto the filters. The levels of mRNA per cell were determined by comparing the RNA hybridization signals with those of the standard DNA, taking 10^9 bp as the size of the haploid chicken genome.

For Northern blots (RNA blots), RNA was denatured with formamide-formaldehyde, run in 1% agarose gels containing 6% formaldehyde (35), and transferred to Biodyne membranes (Pall). The probes used for hybridization were a 482-bp *Hha*I fragment derived from pCHV2.5B/H (H5 [39]), a 700-bp *Taq*I fragment from pCH2eS/X30 (H2A), pCH3dR8 (H4 [50]), pCH3dR1 (H3 [18]), pCH1aB1BK1 (H1 [50]), and a 530-bp *Ava*I fragment from pβ1BR15 (β^A-globin [17]). Hybridization conditions were as described (39).

The DNA probes were labeled by random priming with hexamer primers (Pharmacia) or by nick translation, as specified.

Other analytical methods. Cell cycle analyses were performed with a flow cytometer (Epics V; Coulter Electronics). Freshly prepared cells were treated with preboiled RNase A (1 mg/ml) in 0.1% sodium citrate for 10 min at 37°C. Propidium iodide was then added to a final concentration of 0.05 mg/ml, and the cells were analyzed after 10 min of incubation in the dark.

Detection of H5 in nuclei by immunofluorescence was done as described (38). DNA concentrations were determined by fluorimetry (25).

Kodak XAR-5 films and Du Pont Cronex Lightning-Plus screens were used for autoradiography.

RESULTS

Cell characterization. To obtain a wide spectrum of developmental stages, ME and three populations of immature erythrocytes (IE-1, IE-2, and IE-3) from anemic bone marrow, differing in degree of maturation, were used (see Materials and Methods). IE-1 is highly enriched in erythroblasts, whereas IE-2 and IE-3 contain changing proportions of erythroblasts and early, mid-, and late polychromatic erythrocytes. In some experiments, IE-B from the same anemic animal were also analyzed. IE-B represent a mixture of cells at the later stages of maturation, including

late polychromatic erythrocytes and approximately 20% ME. More than 99% of the cells in any given population showed a positive nuclear reaction with a monoclonal antibody specific for the globular region of H5 (5C3; results not shown; see reference 38), indicating that the vast majority of them belonged to the erythrocyte series. Since the anemic bone marrow contains few erythroid cells at very early stages of differentiation, we used a CFU-(E)-arrested cell line, transformed by *ts*34 AEV (HD3-41/2 [5]), as a model for the preerythroblast stage. We emphasize, however, that although the transformed cells can be induced to differentiate (5), they may not accurately reflect all the biochemical parameters of the natural precursors. The earliest cells of the erythroid lineage, such as BFU-(E), were not represented to any significant degree in the cell populations studied.

Figure 1 shows a flow cytometer analysis of the erythroid cells. As expected, the proportion of proliferating cells gradually decreased with increase in cell age. Essentially all cells in IE-B and ME were in G1. The decrease in proliferation was also reflected by a drop in the rate of [*methyl*-^{3}H] thymidine uptake into DNA, determined after a 30-min pulse (10-fold between IE-1 and IE-3). Note that the relative proportion of cells in G2/M was high (HD3-41/2 and IE-1 to IE-3; Fig. 1). Two-parameter analyses (cell size and DNA content) indicated that this was not due to cell doublets (a small, variable percentage of doublets and larger cells was not taken into account). In the transition from IE-1 to IE-3, the number of cells in S phase dropped by a factor of 23, whereas that in G2/M decreased only by a factor of 2.3. It

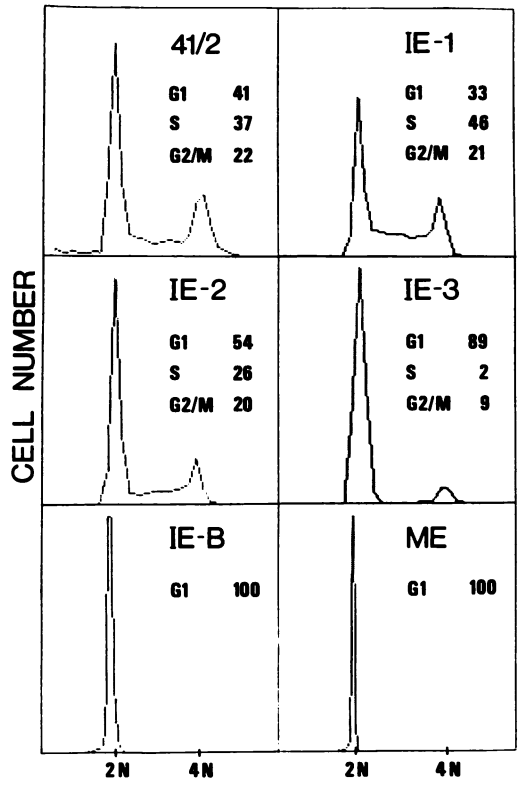

FIG. 1. DNA content of erythroid cells at different stages of maturation. In each case, 10^4 cells were analyzed by flow cytometry. The percentage of cells in different phases of the cell cycle is indicated, corrected for the presence of doublets and larger cells.

appears as if, relative to the S phase, the cells spent a long period of time in the G2/M compartment. Since there were no G2/M cells in the peripheral blood (IE-B and ME, Fig. 1), the tetraploid cells presumably entered the terminal G1 phase before being released into the bloodstream.

H5 accumulation in chromatin does not involve a concomitant loss of H1. A quantitative estimation of the relative proportions of histones H1 and H5 of the different cells is shown in Fig. 2A. With the core histones as a reference, the content of H5 gradually increased with the age of the cell, resulting in a fourfold-higher level in the ME than in the HD3-41/2 cells. On the other hand, the level of histone H1, although slightly higher in the HD3-41/2 cell line than in the more mature cells, was clearly maintained from IE-1 to ME. We do not know whether the higher H1 content of the erythroleukemia cells reflects their earlier stage of differentiation or their transformed phenotype, since no further loss of H1 was observed in the more mature cells. However, the HD3-41/2 cells and the IE cells contained different types of histone H1, and it is possible that H1 variants with lower affinity for chromatin are displaced by H5 during the earlier stages of differentiation. Consistent with the latter view is the partial loss of H1 observed in nonerythroid cells when the expression of a transfected H5 gene was induced (R. Wiaderkiewicz and A. Ruiz-Carrillo, manuscript in preparation).

An increase in H5 content with cell maturity has been noted before (3, 6, 16, 30, 42, 43, 46). However, the previous studies disagree on the extent of the increase and on the changing proportions of the other histones. The discrepancies are probably due to incomplete acid extraction of histones. As we have already shown, acid does not quantitatively remove the histones from chromatin, whereas the methods used here do (40, 43). Our results then indicate that the increase in H5 was not matched by a decrease in H1 from IE-1 to ME; that is, during maturation there was a 70% net increase in linker histone.

Interestingly, the relative increase in H5 content in IE-1 versus HD3-41/2 cells (2.6-fold) did not adversely influence the proportion of cells that entered S phase (Fig. 1). Assuming that deposition of the extra linker histone in chromatin alters the cell cycle parameters, there appears to be a threshold amount of H5 below which cell proliferation is not affected (see Discussion).

Differential synthesis and higher stability account for H5 accumulation in chromatin. Histone synthesis was examined after a 15-min pulse with [4,5-^{3}H]lysine (Fig. 2B). All cells except ME incorporated radioactivity into histones. The lack of lysine uptake by ME simply reflects the lack of mRNA and ribosomes of these cells (see reference 43 and references therein; see also below).

The relative increase in H5 synthesis was due both to higher production in absolute terms (cf. HD3-41/2 with IE-1 and IE-2) and to the decrease in synthesis of the other histones (cf. fluorography of IE-2 and IE-3, Fig. 2B). In fact, synthesis of H5 was maintained at a rather constant level, while that of the core histones and H1 dwindled as the cell matured.

Although the differential synthesis of H5 could easily explain its gradual accumulation in chromatin (Fig. 2A), it was striking that, given the low proportion of cells in S phase (Fig. 1), IE-3 still synthesized a low but significant amount of histones. Although these histones may belong to the replication-independent class of variants (34, 53), our method of analysis cannot distinguish their electrophoretic mobilities. In any event, we examined the fate of the newly synthesized histones to measure their stabilities and determine whether

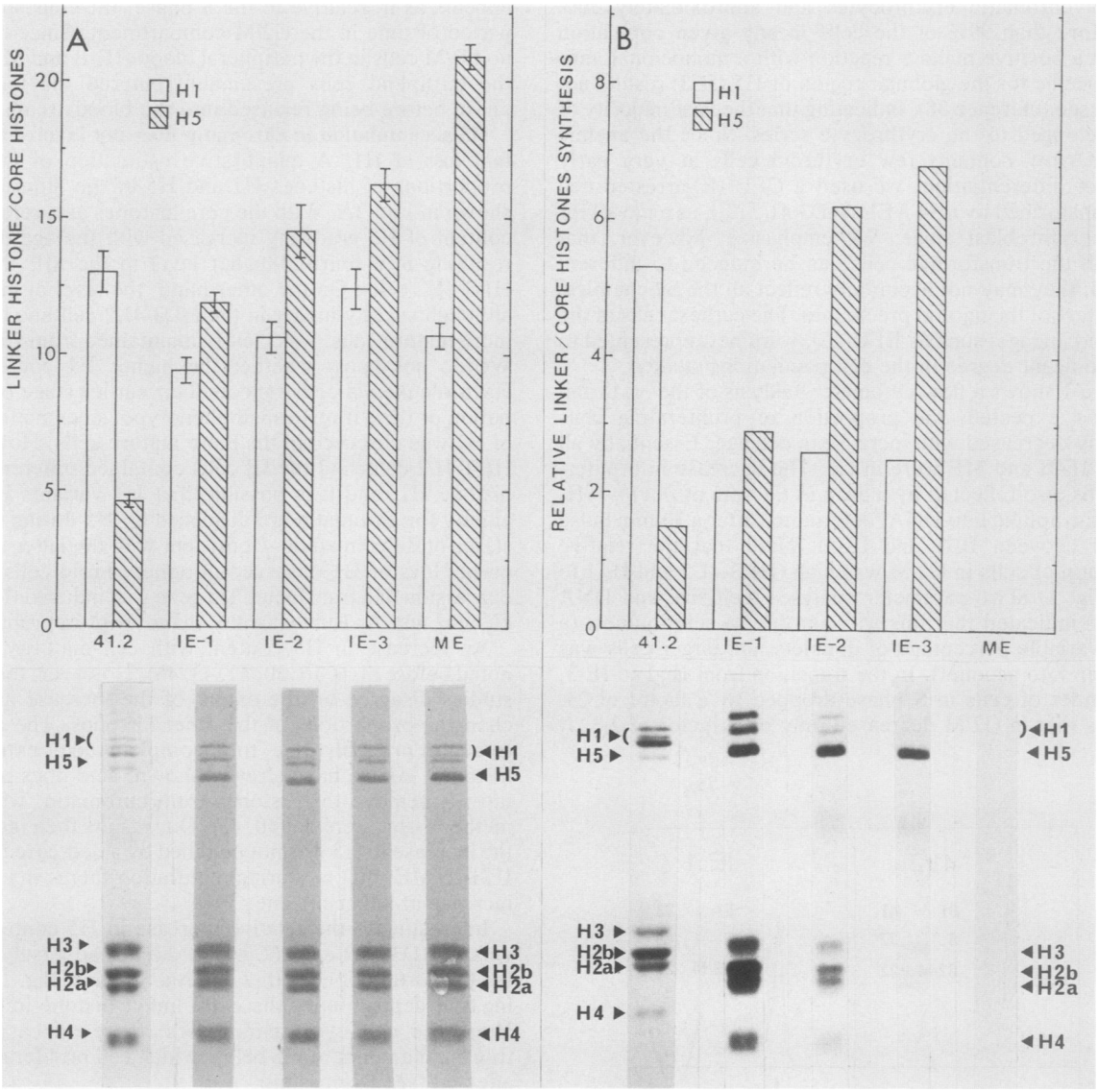

FIG. 2. Remodeling of chromatin during erythroid cell differentiation. (A) Relative changes in the content of H1 and H5 of erythroleukemia cells (HD3-41/2), immature erythrocytes of increasing degrees of maturation (IE-1 to IE-3), and mature erythrocytes (ME). The values are given as percentages of the areas obtained by densitometry of stained gels (shown below) and are averages of four independent determinations. (B) Relative synthesis of H1 and H5. The histograms show the ratios of [^{3}H]lysine radioactivity in H1 and H5 relative to that in the core histones after a 15-min pulse. The radioactivity was measured by scintillation counting of gel slices. A fluorograph of a duplicated gel is shown below.

changes in half-life also contributed to the relative increase of H5.

Results from pulse-chase experiments (Fig. 3) showed that most of the [^{3}H]lysine radioactivity incorporated into histones by HD3-41/2, IE-1, and IE-2 following a 15-min pulse was rather stable. However, some reduction in the amount of label was evident (about 20%), the rate of loss being relatively higher at shorter chase times. This suggests that the effect is not merely a consequence of dilution of the histone pool due to chromatin replication, but of a shorter half-life of a significant number of the newly synthesized histones. This phenomenon was even more accentuated in the most mature bone marrow cells (IE-3), where 35 and 60% of the newly synthesized H1 and core histones, respectively, were degraded within the first 2 h and 60 to 70% after 12 h. In contrast, the relative amount of H5 retained in the nucleus did not vary appreciably with the stage of cell differentiation.

Therefore, H5 accumulation in erythroid cells reflects two selective mechanisms, increased expression at the earlier stages of maturation and higher stability of the protein compared with the other histones at the later stages. This result is at variance with an earlier report (3) in which, by using a more indirect method of analysis, H5 was found to be the most unstable. A possible reason for this discrepancy is that in the previous work (3), H5 was contaminated with rapidly turning-over nonhistone proteins. This potential problem is unlikely to affect our results (see the fluorography in Fig. 2B), which are in accordance with the observed net increase of H5 in chromatin.

Transcriptional regulation of the histone genes during

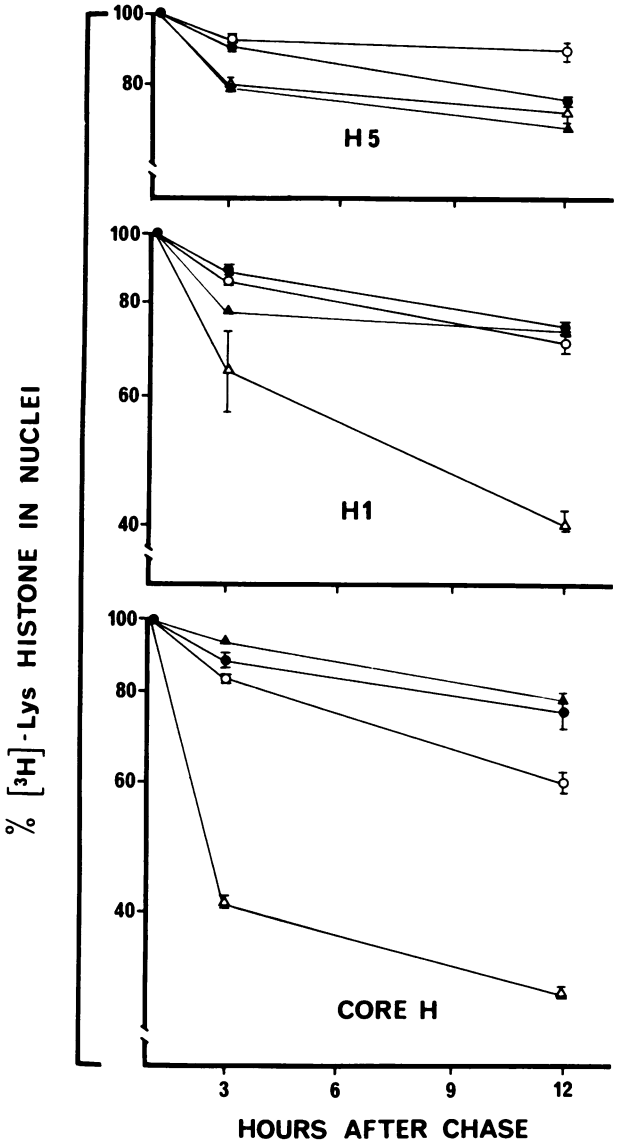

FIG. 3. Fate of newly synthesized histones. Erythroid cells were pulsed for 15 min with [^{3}H]lysine and chased for the indicated periods of time in complete medium containing excess cold lysine. The radioactivity incorporated into each histone was measured after electrophoretic separation (see Fig. 2B). The values are normalized to the amount of core histones in each gel track, as determined by densitometry of stained gels. The radioactivity of histones was higher (10 to 15%) after 1 h than at the beginning of the chase. The 1-h values are therefore taken as 100%. The values are the averages of two independent determinations. Symbols: ●, IE-1; ○, IE-2; △, IE-3; ▲, HD3-41/2.

erythrocyte differentiation. In an erythroleukemia cell line originally derived from the HD3 clone as the HD3-41/2 cells used here (5), it has been shown that H5 mRNA levels do not change appreciably with the phase of the cell cycle (14). Although these results were obtained with cells synchronized with aphidicolin, we made a similar observation with the subclone HD3-41/2 by using sedimentation to separate cells at different phases of the cell cycle (unpublished results; see below). This pattern has been correlated with constitutive transcription of the H5 gene throughout the cell cycle (14). In view of these properties, we examined the

TABLE 1. Transcriptional activity of erythroid cells during differentiation

Cells	Activity[a] (cpm hybridized/100 μg of nuclear DNA per 100 bp of probe)		
	H2A	H5	β^A-globin
HD3-41/2	942 (27) [75]	37 (64) [97]	1
IE-1	960 (38)	114 (88)	112 (100)
IE-2	942 (63)	235 (82)	181 (100)
IE-3	767 (56)	271 (94)	398 (100)
IE-B	374	210	613
ME	31	238	316

[a] Residual transcriptional activity (percent of activity without treatment) after aphidicolin (5 μg/ml, 3 h) or cycloheximide (0.11 mM, 3 h) treatment is shown in parentheses and in brackets, respectively.

temporal relationship between the transcription of several histone genes (H1, H3, H2A, and H5) and that of a gene for a differentiated cell product (β^A-globin).

Since the results of run-on analyses (1) for the H1, H3, and H2A genes were comparable, only those obtained with H2A are shown (Table 1). Also, the H2A gene will be referred to in the singular, even though the assays measured transcription from all active genes of the H2A family (at least nine copies per haploid genome [39]). Although the homology of the probe with the transcribed H2A genes will influence the levels of hybridization, no appreciable change in the strength of the signals was detected before or after RNase (RNase A plus RNase T_1) treatment of the filters.

Qualitatively, the data in Table 1 indicate a clear distinction between the transcription rates of H5 and H2A. While transcription of H2A did not change appreciably during the earlier stages of maturation (HD3-41/2 and IE-1 to IE-3), that of H5 gradually increased by a factor of 6. The same trend, albeit more dramatic, was displayed by the β^A-globin gene, the rate of transcription of which increased by a factor of about 400 between the same stages of differentiation. Furthermore, the transcriptional activity of the H2A gene declined to very low levels in the ME, whereas that of the H5 and β^A-globin genes was maintained. Clearly, the pattern of transcription of the H5 gene parallels more closely that of the β^A-globin gene than that of the other histone genes.

The finding that the transcription rates of the H5 and β^A-globin genes in the ME were comparable to those of more immature bone marrow cells was somehow unexpected. However, it is in accord with the persistence and lack of modulation of the majority of the DNase I-hypersensitive sites on these genes during development (36, 54). We argue, moreover, that the run-on assays measure the transcriptional activity of the genome (that is, the actual levels of transcription in vivo) rather than its transcriptional capacity (that is,

TABLE 2. Molecules of mRNA per cell[a]

Cells	No. of mRNA molecules/cell[b]		
	H2A	H5	β^A-globin
HD3-41/2	96	31	3
IE-1	153 (12)	91 (50)	2,806 (2,948)
IE-2	118 (9)	80 (49)	3,314 (3,018)
IE-3	19 (2)	54 (22)	3,340 (3,226)
IE-B	2	18	3,083
ME	0	0	0

[a] Estimated from hybridization values for the standard DNA (see Materials and Methods).

[b] Numbers in parentheses indicate values after a 3-h treatment with aphidicolin (5 μg/ml).

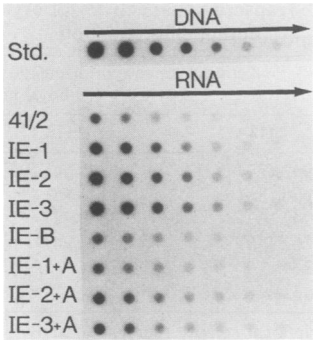

FIG. 4. H5 mRNA levels in erythroid cells at different stages of maturation. Samples of total RNA, corresponding to 2.5 μg of cellular DNA (leftmost dots), and serial twofold dilutions were dotted onto nitrocellulose filters and hybridized with a 482-bp *Hha*I fragment from pCHV2.5B/H labeled by random priming. RNA in rows labeled +A was from cells treated with aphidicolin (5 μg/ml for 3 h). The internal standard (Std.) Contained 0.6 ng of pCHV2.5B/H (corresponding to 0.05 ng of the *Hha*I fragment, leftmost dot) and serial twofold dilutions.

its readiness to be transcribed). This is because the observed drop in the total rate of in vitro transcription between the most active cells and the ME (12-fold with respect to HD3-41/2 and 6-fold with respect to IE-1) was of the same order as that determined in vivo by incorporation of radioactive phosphate into RNA (43). Since the ME contained no mRNA (Table 2), it is likely that the nuclear transcripts were not transported into the cytoplasm (see Discussion).

Our results point to differential transcriptional regulation of the H5 gene, among the histone genes, as one of the elements in independent H5 expression and accumulation during maturation of erythrocytes. Furthermore, they provide clear evidence for selective and efficient repression of the S-phase histone genes in the most mature cells, a phenomenon only partially observed in the G1 phase of proliferating cells (2, 4, 15, 23, 45; see also below). In spite of this, the inactivation of the H2A gene during maturation was not tightly coupled to DNA replication. Thus, the IE-3 and the IE-B cell populations, which have 2 and <0.1% of their cells in the S phase, respectively (Fig. 1), nevertheless maintained a high transcription rate of the H2A gene.

Aphidicolin, an inhibitor of DNA polymerase α (reviewed in reference 24), is known to influence, as well as other inhibitors of DNA synthesis, both the transcription rate of the histone genes and the levels of histone mRNA (14, 23). We examined to what extent these events were coupled in the erythroid cells by performing run-on assays with cells treated with aphidicolin. Relative to the β^A-globin gene (internal standard), aphidicolin reduced transcription of the H2A gene in all cells tested (Table 1). Although the degree of inhibition was a little greater in cells with higher proliferative potential (HD3-41/2 and IE-1), the effect was, by and large, independent of the absolute rate of DNA synthesis. The rate of transcription of the H5 gene was also affected, although to a lower degree than that of the H2A gene (Table 1).

Histone and β^A-globin mRNA levels during erythroid cell differentiation. The lack of correlation between the rate of H2A gene transcription and the levels of histone H2A synthesis (i.e., IE-3; cf. Table 1 and Fig. 2B) suggested that posttranscriptional regulation must play the most significant role in the control of histone production. Hence, we examined the steady-state levels of mRNA. This was accomplished by dot hybridization of total RNA, extracted under denaturing conditions, with fragments of several cloned genes labeled by random priming. The radioactivity bound to the filters was quantified by scintillation counting and corrected for probe length. As an example, Fig. 4 shows the titration of H5 mRNA from the different cells, except ME, that gave negative results at every input tested, and Table 2 summarizes the quantitative data for H5, H2A, and β^A-globin, expressed as number of mRNA molecules per cell.

Although the amount of H5 mRNA clearly increased in IE-1 relative to the less differentiated HD3-41/2 cells, it was not maintained at the higher level during subsequent stages of maturation. The gradual decrease in H5 mRNA content was, however, offset by a more rapid decline in the content of H2A mRNA, so that in the IE-2 to IE-3 transition, when most of the cells entered terminal G1, the level of H5 mRNA was higher than that of H2A mRNA. The mRNA variations (Fig. 5) were reflected in the rates of histone synthesis (Fig. 2B), suggesting that during erythrocyte maturation there is no significant change in regulation at the level of mRNA translation. However, the same cannot be said of other levels of posttranscriptional regulation, since variations in the levels of mRNA did not parallel the rates of transcription. Thus, the eightfold drop in H2A mRNA (Table 2) from IE-1 to IE-3 can only be compared with an estimated 1.25-fold decrease in the transcription rate of the gene (Table 1). During the same transition, the H5 mRNA level dropped by a factor of 1.7 despite a 2.3-fold increase in the transcription rate of the H5 gene. These results indicate that mRNA stability and/or processing and transport of the nuclear transcripts varies with cell maturity or with the loss in proliferative potential (see Discussion). The results in Table 2 and Fig. 5 also emphasize a dramatic difference in the posttranscriptional control of the H5 and β^A-globin genes. Thus, while the rate of transcription of both genes was similar (Table 1), the level of β^A-globin mRNA built up 30-fold over that of H5 mRNA (Table 2) as the cells reached the erythroblast stage (IE-1). Moreover, while the level of β^A-globin mRNA was maintained, that of H5 mRNA dropped during maturation.

Posttranscriptional regulation of the histone genes. The differences between the transcriptional activities of the H5 and H2A genes and the respective mRNA levels suggest that they do not have the same posttranscriptional control mechanism(s). Since inhibition of DNA synthesis resulted in a drastic decrease in the mRNAs for the main histones, we examined the effect of DNA synthesis inhibitors on H5

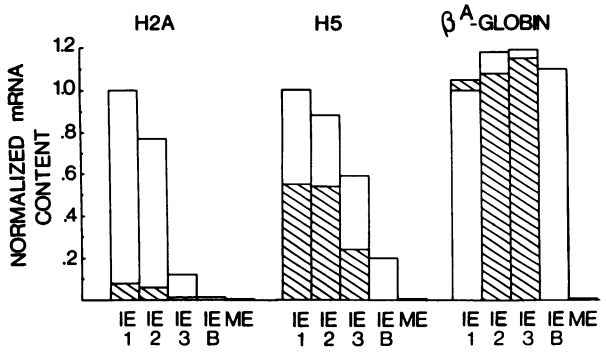

FIG. 5. Variation in the specific cell content of several mRNAs during erythroid cell differentiation (open bars). The level of each mRNA species was normalized to 1 for IE-1. The shaded bars show the relative mRNA content after aphidicolin treatment (IE-1, IE-2, and IE-3, 5 μg/ml, 3 h).

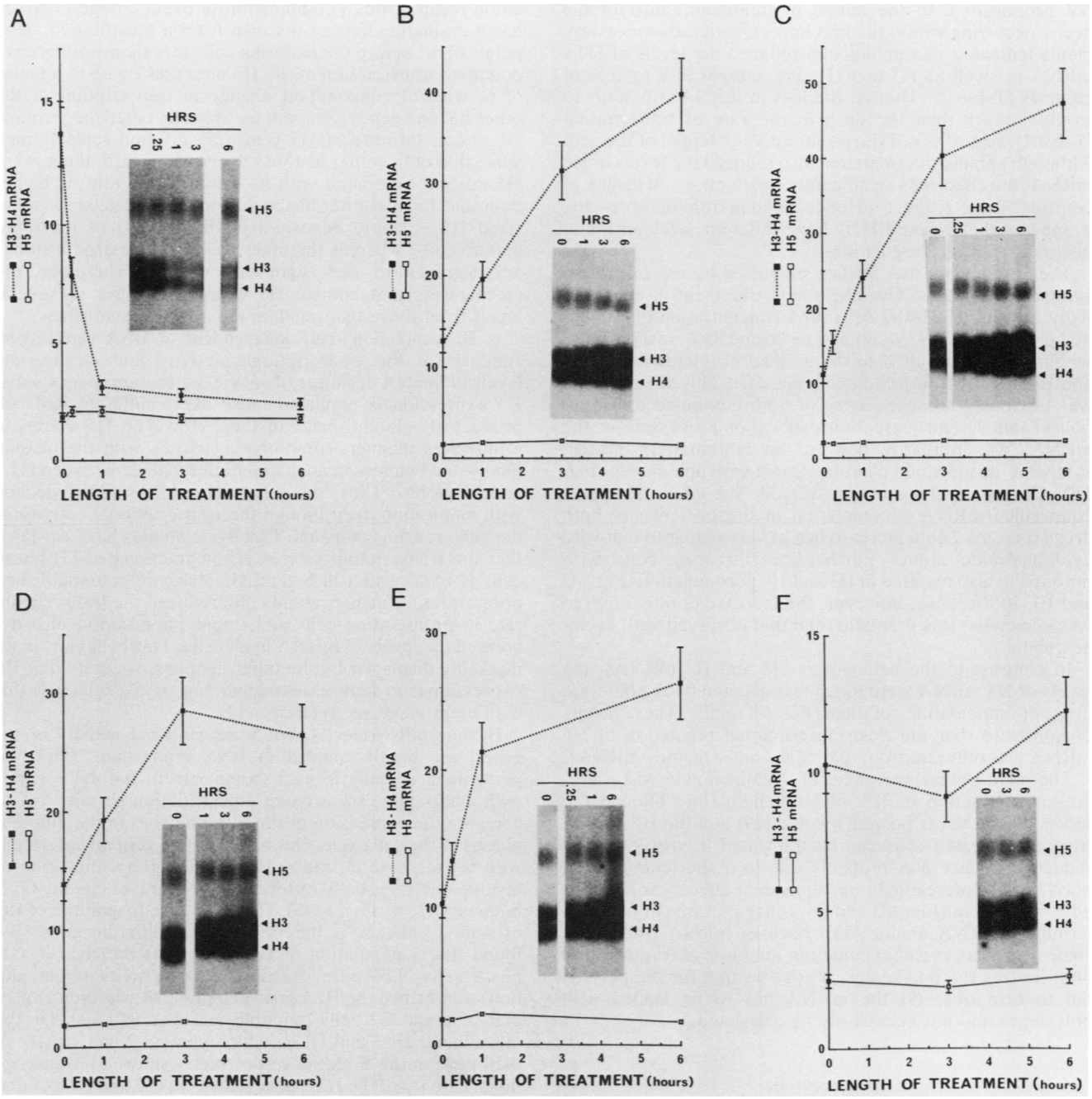

FIG. 6. Differential posttranscriptional regulation of H5 and core histone mRNA. HD3-41/2 cells (1.5×10^6/ml) were treated with either aphidicolin (5 μg/ml), cycloheximide (0.1 mM), puromycin (100 μg/ml), or combinations thereof for the indicated periods of time. Total RNA was isolated and analyzed by electrophoresis (4 μg/lane) in a 1% agarose denaturing gel slab. The blotted RNA was hybridized with a mixture of nick-translated H5, H3, and H4 gene probes. The signals, quantified by densitometry of less-exposed autoradiographs, are expressed as the integrals of the respective peak areas. The values were corrected by the amount of RNA in each track, using the rRNA as a standard (determined by densitometry of ethidium bromide-stained gels). Drug treatment: (A) aphidicolin, (B) aphidicolin plus cycloheximide, (C) cycloheximide, (D) aphidicolin plus puromycin, (E) puromycin, (F) none. Maximal DNA (97%) and protein (96%) synthesis inhibition was obtained within 30 min of treatment. At the concentrations used, cycloheximide and puromycin inhibited [*methyl*-^{3}H]thymidine incorporation into DNA by 88 and 86%, respectively.

mRNA levels. This was determined semiquantitatively by hybridization of Northern blots and quantitatively by dot hybridization.

Figure 6A shows the data for the H3, H4, and H5 mRNAs. Aphidicolin treatment, at a concentration that reduced DNA synthesis by 97% within 30 min, resulted in a 5.5-fold decrease in the amounts of H3 and H4 mRNA without a noticeable effect on the level of H5 mRNA (estimated by densitometry; see Table 2 for quantitative measurements). Further, the reduction in the H3 and H4 mRNA levels was

not proportional to the length of treatment, most of the decay occurring within the first hour. Quantitative measurements indicated that aphidicolin reduced the levels of H2A mRNA (as well as H3 and H1; not shown) to 8 to 10% of controls (Table 2). That is, the loss in mRNA was four- to fivefold larger than the drop in the rate of transcription (Table 1), regardless of the proliferative potential of the cell. Although aphidicolin treatment also reduced the levels of H5 mRNA, the effect was significantly smaller (i.e., 40 to 60% of controls; Table 2, Fig. 5). Hence, judging from these results, it appears that H5 and H2A have different mechanisms of posttranscriptional regulation.

The above view was further supported by use of protein synthesis inhibitors. Cycloheximide treatment of exponentially growing HD3-41/2 cells, at a concentration that inhibited protein and DNA synthesis by 96 and 88%, respectively, within 30 min, resulted in the gradual accumulation of H3 and H4 mRNA (fourfold in 6 h; Fig. 6C). This effect, which was obviously a consequence of both continued transcription (Table 1) and increased stability or processing of the mRNA, was primarily due to the inhibition of protein synthesis, as indicated by cotreatment with aphidicolin (Fig. 6B). Clearly, cycloheximide overrode the effect of aphidicolin since mRNA accumulation in the presence of both drugs (i.e., a 3.2-fold increase in 6 h) was similar to that with cycloheximide alone. Furthermore, treating cells with puromycin also resulted in H3 and H4 accumulation (Fig. 6D and E). In this case, however, the increase (2.6-fold in 6 h) was somewhat less dramatic than that observed with cycloheximide.

In contrast to the behavior of H3 and H4 mRNAs, the levels of H5 mRNA were much less affected by the different drugs or combinations of them (Fig. 6B to 6E). These results demonstrate that the posttranscriptional regulation of H5 mRNA was different from that of the other histone mRNAs.

The main observable effect of cycloheximide was a progressive reduction of H5 mRNA length (Fig. 6B and C). Since H5 mRNA is polyadenylated (29) and the H5 mRNA from drug-treated cells can be translated in vitro (32), the reduction in size was probably due to a shortening of the poly(A) tail. Interestingly, an equivalent effect was not seen with puromycin (Fig. 6D and E). Since puromycin (a chain-terminator tRNA analog [33]) releases mRNA from polysomes whereas cycloheximide (an inhibitor of translocation [48]) freezes the polysomes, it appears that for the poly(A) tail to turn over (9) the mRNA has to be loaded with polysomes and not necessarily be translated.

DISCUSSION

Regulation of H5 expression. The chicken erythroid lineage provides a natural system to examine the regulation of the histone genes during terminal differentiation. Inasmuch as the earliest precursor cells amenable for biochemical analysis [CFU-(E)] already express H5, we have not addressed the question of the primary activation of the gene but rather whether and how its expression is regulated during the more advanced stages of maturation. Because the H5 content in the chromatin of the maturing cell increases with respect to that of the other histones, a selective mechanism(s) must exist to allow an unscheduled production of H5. In particular, we wished to distinguish the levels at which the expression of H5 and the other histone genes is differentially controlled.

We have shown that the gradual process of H5 accumulation results from a combination of events, none of which has a dramatic effect on its own from a quantitative viewpoint. First, before the maturing cells lose their proliferative potential, transcription of the H5 increases by up to a factor of 6, without concomitant change in transcription of the other histone genes. Second, as the cells enter the terminal G1 phase, the rate of H5 gene transcription remains high while that of the other histones decreases. Third, the level of H5 mRNA, compared with its transcription rate, is higher than that for the other histones. Finally, the newly synthesized H5 is stably deposited in the nucleus of the more mature cells, whereas the other newly synthesized histones are degraded to very significant extents. Therefore, the mechanisms that control H5 expression differ at several levels from those that regulate the other histone genes.

Is H5 expression truly independent of DNA replication? Analysis of the transcriptional activity and steady-state levels of mRNA during erythrocyte maturation suggests that H5 expression is regulated more like a differentiated cell product (β^A-globin) than a histone. However, H5 shares, in a mitigated manner, some characteristics with the histone family and behaves in an manner intermediate between H2A and β^A-globin. Thus, the cell levels of H5 mRNA decline with maturation even though the transcriptional activity of the gene remains constant. Cell cycle studies have revealed that the transcription rate of H5 in fractionated G1-phase cells is 65% of that in S- and G2-phase cells (unpublished observation). Further, aphidicolin reduced the transcription rate in proliferating cells and, more importantly, also decreased the levels of mRNA in all cells. This behavior, more markedly displayed by the other histones, suggests that H5 expression is to some extent depending on the cell cycle (for a different view, see reference 14).

Histone mRNA levels, not transcriptional activity of the genes, are tightly coupled to DNA replication. Since the percentage of cells in each compartment naturally varies with maturation, erythrocyte differentiation permits one to compare the expression of the histone genes in the different phases of the cell cycle. Our results are in general agreement with the increase in transcription rate and accumulation of histone mRNA usually observed in cells traversing the G1/S boundary (2, 4, 15, 23, 45). However, the magnitude of the estimated changes is different for the different cells. We found that a population of HD3-41/2 cells enriched in G1-phase cells (93% pure, fractionated by gravity sedimentation) transcribed the H2A gene at a rate that was only 18% of that of S and G2 cells (unpublished observations). On the other hand, IE-3 and IE-B, which contain 2 and <0.1% of their cells in the S phase, respectively, showed a transcriptional activity of the H2A gene which was 62 and 28% of that of the IE-1 and HD3-41/2 cells, respectively. A similar observation was made by using aphidicolin, which consistently reduced histone gene transcription to lower levels in exponentially growing than in quiescent cells. In any case, even if H1 and core histone gene transcription is eventually repressed in ME, transcription of the same genes is clearly not coupled to DNA synthesis at the earlier stages of maturation, regardless of the state of cell growth. This situation contrasts, however, with the levels of histone mRNA, which are tightly related to the state of cell proliferation. Thus, there was a 76-fold decrease in the H2A mRNA content between HD3-41/2 (or IE-1) and IE-B, compared with only a 2.5-fold drop in transcription of the gene between the same cell populations. Our results indicate that the most significant regulatory level of histone gene expression is posttranscriptional.

The analysis of cells that naturally leave the cell cycle has permitted us to compare the transcript utilization (T.U.) of the different genes without the use of inhibitors or changes in culture conditions. For the purpose of this discussion, we define T.U., on a cell basis, as the arbitrary ratio between the number of steady-state mRNA molecules and the transcriptional activity of the corresponding genes, as determined from the extent of hybridization of the nuclear transcripts elongated in vitro, normalized to the length of the genes. Although one of the main factors influencing the value of T.U. is most likely the half-life of the mRNA, the T.U. may also reflect other factors, such as processing and transport of the primary transcripts. In IE-1, the T.U. for H2A, H5, and β^A-globin was 6.0×10^6, 27.9×10^6, and 880×10^6, respectively. Assuming that processing and transport in IE-1 are equally efficient, these values would predict that β^A-globin mRNA is 146 times more stable than H2A mRNA, in accordance with the long half-life of globin mRNAs (47). During the transition from IE-1 to IE-B, the T.U. for H2A drops by a factor of 32, whereas that for H5 and β^A-globin drops by a factor of 9 and 5, respectively.

Although our data cannot distinguish which of the different parameters reflected in the T.U. is responsible for its variation, it is likely that the drop during maturation is due to a combination of them (21, 28). On the one hand, the decrease in the T.U. of H2A following aphidicolin treatment could be interpreted in terms of reduced mRNA stability. This view is supported by the fact that cycloheximide (or puromycin) together with aphidicolin resulted in mRNA accumulation, suggesting that the loss in mRNA is not due to alterations in processing or transport (see also reference 49). The mechanism(s) that reduces histone mRNA stability when DNA synthesis is inhibited could also decrease the half-life of the mRNA in response to changes in cell proliferation. On the other hand, it is clear that the T.U. of β^A-globin, probably not modulated during the cell cycle, also decreases during maturation, despite the high levels of transcription. Hence, it is likely that the rate of processing and transport of the nuclear transcripts decreases with cell age. In this regard it is worth pointing out that the more mature the IE-B population, the higher the level of H5 pre-mRNA (1). The lack of replenishment might explain why at the end of the maturation pathway there is no mRNA in the cytoplasm of ME.

Possible function of H5. Although the accumulation of H5 in the nucleus of the maturing cell is a gradual phenomenon, the highest relative increase occurs before the cell loses its proliferative potential, between CFU-(E) and the erythroblast stage. This is interesting in view of the proposed role for H5 in the inactivation of the erythrocyte nucleus. Consistent with this view is the higher affinity of H5 than of H1 for chromatin, which results in increased stability of the higher-order structure (34, 41). However, our results suggest that if H5 is instrumental in chromatin inactivation, there should be mechanisms (i.e., phosphorylation [42, 51]) that prevent the function of H5 from being fully expressed at the earlier stages (IE-1 and IE-2) or that there is a threshold amount of H5 below which its effect is not manifested or is reversible. Moreover, the effect, if any, on transcription is not general, since the transcriptional activity of the H5 and β^A-globin genes is little affected by the higher H5 content of ME.

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