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Changes in the Levels of Three Different Classes of Histone mRNA During Murine Erythroleukemia Cell Differentiation

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We used a gene-specific S1 nuclease assay to study the changes in steady-state mRNA levels of several core histone variants during the differentiation of murine erythroleukemia cells. These studies allowed us to distinguish three distinct expression classes of histone genes. The expression of the major replication-dependent class of histone genes was tightly linked to DNA synthesis. The concentrations of these transcripts decreased rapidly as cell division slowed during the process of differentiation. In contrast, the replication-independent H3.3 transcript levels were constitutively maintained throughout differentiation and were unaffected by inhibitors of DNA or protein synthesis. We also identified among the cloned histone genes used as probes a third expression class, the partially replication-dependent variants. Expression of these transcripts became transiently uncoupled from the reduced rate of DNA synthesis accompanying the early stages of differentiation. We show that their synthesis is sensitive to the DNA synthesis inhibitor hydroxyurea but that selective uncoupling from DNA synthesis of these histone mRNAs occurs at a specific stage of differentiation. We present several hypotheses to explain how this might be accomplished. The expression characteristics of the mRNAs studied coincided with those of the proteins for which they code, indicating that changes in the relative levels of the different variants is mediated at least in part by changes in mRNA levels.

Histones are a complex family of proteins responsible for organizing the nucleosomal structure of eucaryotic chromatin (22, 27, 32). In mammals, each histone subtype, with the possible exception of H4, is represented by several nonallelic primary sequence variants which display distinct patterns of expression during differentiation (5, 6, 14, 20, 24, 49–54). Nuclease digestion studies indicate that these variants are nonrandomly distributed between actively transcribed and inactive DNA sequences, suggesting that the histones may play a role in establishing or at least maintaining different chromatin conformations (35, 47, 48).

Histone variants are classified according to their expression characteristics (53, 54). The major class, the replication-dependent variants, predominate in rapidly dividing tissues, and their synthesis is tightly linked to DNA synthesis (7, 9, 16, 34, 49). The mRNAs for these variants begin to accumulate near the G_1-S border of the cell cycle, reach maximum levels at mid-S phase, and then rapidly decline as DNA synthesis is completed (1, 2, 8, 11, 21, 33). Regulation of replication-dependent histone mRNAs occurs at the level of transcription and stability (21, 40).

Considerably less is known about the regulation of expression of the other classes of histones. In studies of regenerating liver, partially replication-dependent histones have been identified as a group of variants which are induced with the onset of DNA synthesis but not completely repressed after cessation of DNA synthesis (53, 54). The expression of another class of histones, the replication-independent variants, is not linked to DNA synthesis (49, 52, 54). In previous work we have shown, using a human-processed H3.3 gene (48a) as a probe, that the steady-state levels of corresponding mRNAs are not affected by DNA synthesis inhibitors (40). Tissue-specific and other, minor variant histones also exist, but little is known concerning their regulation (52, 54).

We chose the murine erythroleukemia (MEL) cell system to study developmental regulation of histone gene expression. These cell lines are transformed erythroid precursors arrested at a relatively late stage of the differentiation pathway (15, 25). They can be induced by a number of chemical agents to resume erythropoiesis (13, 25, 31). In this process a limited number of cell divisions occurs, and specific gene products are expressed, including the well-characterized globins (28, 37, 38).

Grove and Zweidler (20) have described the changes in the steady-state levels of core histone variants in MEL cells induced to differentiate with hexamethylene bisacetamide (HMBA). They have demonstrated moderate increases in the relative amounts of H2a.2 and H2b.1. They also have reported smaller but significant changes in the different H3 variants.

In this study we used an S1 nuclease assay to monitor the changes in steady-state levels of specific histone gene transcripts during MEL cell differentiation. The changes we observed in discrete histone mRNA levels were consistent with the changes seen in the levels of the protein variants for which they code. Synthesis of the partially replication-dependent class of histones was illustrated by the expression of two of our cloned genes. During the first 2 days of differentiation the levels of these transcripts became transiently uncoupled from the reduced rate of DNA synthesis accompanying differentiation. We present several hypotheses to explain this selective uncoupling of histone synthesis from DNA synthesis which results in histone switching.

MATERIALS AND METHODS

Cell culture. MEL cell line DS-19 (29) was a gift from R. Rifkind and P. Marks. These cells and the derived subclone DS-19E5 were grown in Eagle basal medium plus Earle salts and glutamine supplemented with 15% fetal bovine serum. Cultures were induced by the addition of HMBA to a final concentration of 5 mM. During induction experiments cell density was maintained between 1.0×10^5 and 5.0×10^5 cells per ml. Induction was monitored by benzidine staining (17)

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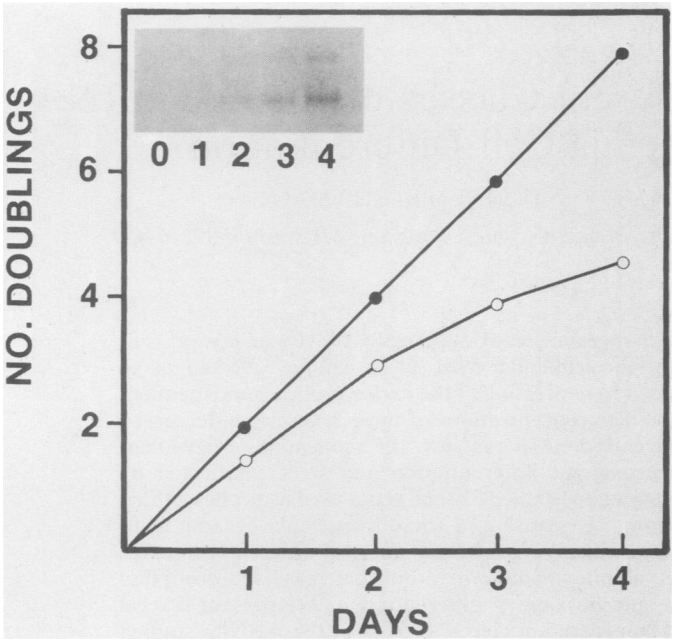

FIG. 1. Growth characteristics and globin mRNA accumulation during HMBA-induced differentiation of MEL cells. Control (●) and induced (○) cells were counted daily with a hemocytometer, and the total number of doublings from day 0 was plotted. Induced cells were treated at day 0 with 5 mM HMBA. Both cultures were diluted daily with fresh medium. Accumulation of β-major globin mRNA in the induced culture was determined by S1 nuclease protection of 275- and 300-nucleotide (nt) fragments of the pCR1β cDNA clone (36) 3′ labeled at the unique *Bam*HI site in exon 2 (inset).

and was always greater than 98%. Cell viability was measured by exclusion of trypan blue dye.

Clones. Mouse genomic DNA clones mm221, mm291, and mm614 have been described previously (19a, 41). Clone mm1011 was recently isolated from genomic DNA and contains an apparent H3.3 processed gene (S. E. Wellman, unpublished data). Clone Hu3.3 contains a human H3.3 processed gene (48a). Plasmid pCR1β, a mouse β-major cDNA clone (36), was obtained from M. Edgell.

Assay for mRNA concentration. Total cellular RNA was prepared by lysis of cells in phosphate-buffered saline containing 1% sodium dodecyl sulfate and 10 mM EDTA followed by extraction with phenol at 55°C and pH 5.5 (26). Steady-state levels of specific mRNAs were determined by a quantitative S1 nuclease assay with end-labeled DNA probes (4, 46). Probes were generated by digestion at a restriction enzyme site within the coding region of the cloned gene followed by end labeling either at the 5′ ends with [γ-^{32}P]ATP and polynucleotide kinase or at the 3′ ends with [α-^{32}P]dCTP and the Klenow fragment of *Escherichia coli* DNA *pol*I. Restriction sites used are indicated in the figure legends. Probes were hybridized with total RNA from 10^5 cells for 6 to 9 h at the optimal temperature empirically derived for each probe. Hybrids were digested with 750 U of S1 nuclease per ml for 1 h at 37°C. S1 nuclease-resistant DNA was extracted with phenol-chloroform, ethanol precipitated, suspended in 99% formamide, and run on denaturing 8% polyacrylamide sequencing gels. Gels were exposed to preflashed XAR-5 film, and the resulting autoradiograms were scanned with a Transidyne model 2510 densitometer. That all assays were done in DNA excess was confirmed by linear dependence of added RNA.

Materials. Cell culture supplies were obtained from GIBCO Laboratories, Grand Island, N.Y. Chemical reagents were from Sigma Chemical Co., St. Louis, Mo. Enzymes were obtained from New England Biolabs, Inc., Beverly, Mass., or from Bethesda Research Laboratories, Gaithersburg, Md. Radioactive nucleotides were from ICN Pharmaceuticals Inc., Irvine, Calif.

RESULTS

Growth and induction characteristics of MEL cells. Any effect which reduces the rate of DNA synthesis may reduce the synthesis of the replication-dependent histone variants. Therefore, in these experiments care was taken to ensure that the reduction in DNA synthesis during HMBA-induced cell differentiation was not due to the growth conditions. By dilution with the appropriate medium all cultures were kept at a concentration which would support linear growth of uninduced cells. Figure 1 shows a plot of the number of doublings of control and induced cells which were diluted to the same concentration daily during the time course of a typical induction experiment. It was essential that the number of cells undergoing differentiation approximated 100%, as even a small percentage of rapidly dividing, uninduced cells could have resulted in an aberrantly high level of replication-dependent histone mRNAs. The original DS-19 cell line displayed 100% induction when we first received them; however, under continued propagation an increasing percentage of the population was refractory to induction. We therefore clonally selected a cell line, DS-19E5, which under our growth conditions generated 98 to 99% differentiated cells after 4 days as measured by in situ staining of hemoglobin with benzidine. The 1 to 2% of the population that was not stained probably represented nonviable cells, as the same percentage of cells was unable to exclude trypan blue. We limited the period of induction to 4 days since the percentage of nonviable cells increased beyond this point.

The inset to Fig. 1 shows the induction of β-globin mRNA accompanying HMBA-induced differentiation as measured by an S1 nuclease assay with a cDNA clone of the mouse β-major globin mRNA (36). The level of S1 nuclease-resistant β-globin mRNA showed a significant increase during the course of differentiation over the undetectable level of uninduced cells.

Qualitatively similar changes in β-globin and histone mRNA levels were obtained during induction of another MEL cell line, CV-39, induced with dimethyl sulfoxide. We did not continue to study extensively expression in CV-39 cells because we could not induce more than 70 to 80% of the cells to differentiate. However, we concluded from studying differentiation of two cell lines induced with different compounds that the changes in histone mRNA metabolism shown for DS-19E5 were the result of the differentiation process and were not due to a direct effect of the chemical inducing agent, HMBA.

Quantitation of gene-specific histone transcripts. We used a quantitative S1 nuclease assay to measure the steady-state levels of specific histone mRNA transcripts during the HMBA-induced differentiation of MEL cells. The probes used were derived from three different mouse genomic DNA clones, mm221, mm291, and mm614, containing a variety of different histone genes (19a, 39, 41; S. E. Wellman, unpublished data). We also used a cloned human H3.3 processed gene (40, 48a). Each gene was protected in an S1 nuclease assay by two major mRNA populations. A diagram of the

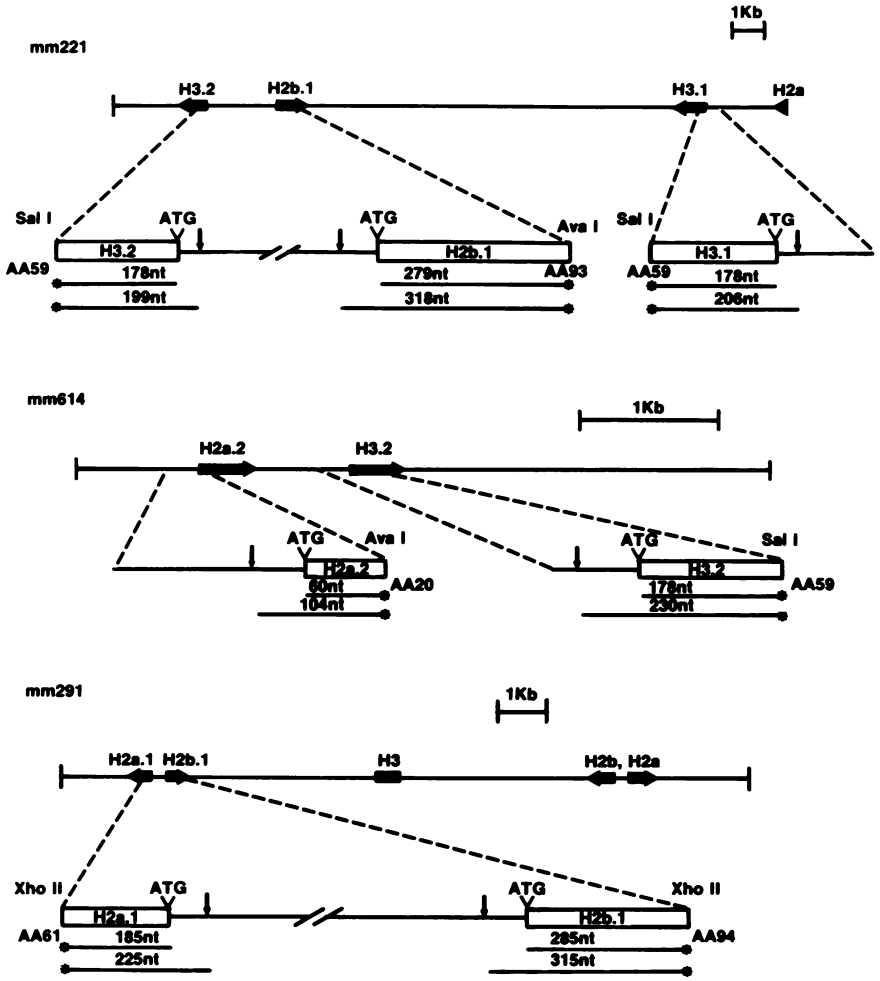

FIG. 2. Genomic organization of cloned mouse histone genes. Closed arrows indicate coding regions and directions of transcription. Expansions indicate DNA fragments used as probes in S1 nuclease-protection assays. Open boxes represent coding regions. Vertical arrows indicate the transcriptional start site. Lines below the expansion indicate sizes and identities of DNA fragments protected from S1 nuclease digestion. Kb, kilobase.

genes, probes, and S1 nuclease-protected fragments of the three mouse clones with linked genes is shown in Fig. 2. The identity of the protected fragments was determined previously by comigration with DNA sequencing ladders (19a, 41). For each of the genes one of the protected fragments represented a collection of RNAs homologous to the probe only within the protein-coding region (ATG in Fig. 2). The other, longer fragment represented protection of the probe by transcripts, the sequence homology of which extends through the 5'-untranslated portion of the mRNA (vertical arrow in Fig. 2). These latter transcripts originated from the gene from which the probe was derived. For the results presented here we compared only the levels of these gene-specific transcripts; the S1 nuclease assays were optimized for protection of these fragments.

Changes in H3 mRNA levels. MEL cells express three major H3 proteins. Two of these, H3.1 and H3.2, are classified as replication-dependent variants (53, 54). They differ only in a Cys-Ser substitution at amino acid position 96. The third, H3.3, is a replication-independent variant which differs from H3.2 by having Ile and Gly at positions 89 and 90 instead of Val and Met (53). During MEL cell differentiation the synthesis rates of all the histones decrease as the cells reduce their division rate. The magnitude of the decrease in synthesis is different for each variant such that the relative levels of the variants change. After 4 days of differentiation H3.1 decreased from 18% of all H3 variants to just under 8%, H3.3 approximately doubled from 5 to 10%, while H3.2 remained at about 75% (20).

Figure 3 shows the change per cell in steady-state levels of four different H3 transcripts. We detected three distinct patterns of expression. Two genes, H3.1-221 (Fig. 3A) and H3.2-221 (Fig. 3B), gave rise to transcripts which decreased in abundance very rapidly during day 1 of differentiation, to approximately 30 to 40% of the uninduced level (Fig. 3E). Their levels then either stayed the same or increased slightly during the day 2 of differentiation. During the days 3 and 4 they again decreased, reaching levels that were 10 to 15% of those of uninduced cultures by day 4.

The H3.2-614 gene encodes an H3.2 variant identical in amino acid sequence to that of H3.2-221 but has different 5'- and 3'-flanking sequences. Transcript levels from this gene dropped only slightly during the first 2 days of differentiation and then declined to levels approximately 50% of those of uninduced cultures by day 4 (Fig. 3C and E). During MEL cell differentiation this transcript exhibited the behavior

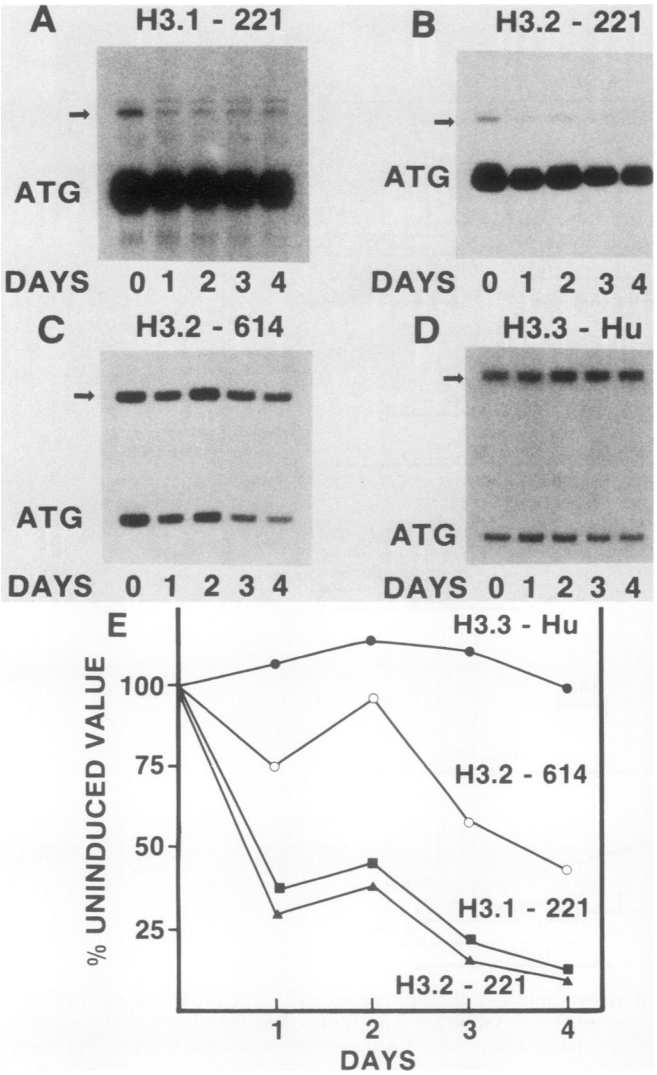

FIG. 3. Steady-state H3 mRNA levels during MEL cell differentiation. (A through D) S1 nuclease protection by specific H3 mRNA transcripts in total RNA isolated daily following HMBA induction. Probes from the H3.1 and H3.2 genes are depicted in Fig. 2. The H3.3 probe was generated by 5′ labeling at a *Bgl*II site in codon 80. The additional top-most band observed in the H3.1-221 assay is present in control assays containing no added RNA. (E) Results of densitometric scans of the specific transcripts in panels A through D. Values are presented as the percentage of uninduced (day 0) levels.

expected of a partially replication-dependent histone mRNA. Transcripts from this gene made an even larger contribution after differentiation than before to the total H3 mRNA population.

A third pattern of expression was observed for H3.3 transcripts. A human-processed H3.3 gene was used as a probe in these experiments. The steady-state level of H3.3 mRNAs detected with this probe did not change during HMBA-induced differentiation (Fig. 3D and E).

Changes in H2a mRNA levels. MEL cells contain at least five H2a variants with a variety of primary sequence differences (5, 6, 20). The bulk of the H2a population is made up of two species: H2a.1, a replication-dependent variant which decreases from 47 to about 23% of all H2a's after 4 days of differentiation, and H2a.2, a partially replication-dependent variant which increases in relative abundance from 48 to 58% (20). Each of the three mouse genomic clones used in this study contains H2a sequences. Figure 4 shows the changes in the level of the specific transcripts from two of these. Transcripts from the H2a.1-291 gene rapidly decreased during day 1 of differentiation. They stayed at approximately the same level through day 2 of differentiation and then decreased at a rate paralleling the reduction in cell division to approximately 25% of their initial level (Fig. 4A and C). S1 nuclease analysis of the transcripts from another H2a.1 gene (H2a.1-221) gave a pattern of expression identical to that of H2a.1-291 (data not shown).

In contrast to H2a.1-291, transcript levels from the H2a.2-614 gene changed in a manner similar to that seen for H3.2-614, except that there was virtually no decrease at day 1, and a slight increase in the level of this mRNA was detected after 2 days of differentiation relative to that of uninduced cultures (Fig. 4B and C). This observation was consistent with the pattern expected for a partially replication-dependent variant.

Changes in H2b mRNA levels. MEL cells contain two major species of H2b, the replication-dependent variant H2b.2 and the partially replication-dependent variant H2b.1 (20). The H2b.2 histone decreases from about 40 to 30% of H2b's after 4 days of differentiation. In contrast the H2b.1 increases from 60 to 70% during 4 days of differentiation (20). The H2b.1-221 gene encodes an H2b.1-like variant which has a single amino acid substitution at position 18 (41). Figures 5A and C show that the level of H2b.1-221 mRNA changed during differentiation in a manner similar to that of the other replication-dependent transcripts. H2b.1-291 encodes a protein containing the expected valine at position 18 for an H2b.1 (19a). Figure 5B shows that the decrease in the level of H2b.1-291 transcripts was intermediate between that of replication-dependent and partially replication-dependent variants during the first 2 days of differentiation. Its levels then rapidly fell to approximately 25% that of the uninduced levels (Fig. 5C).

Effect of hydroxyurea on histone mRNA levels. The results presented above indicate that the changes in the relative amounts of the individual histone variants during the differentiation process are reflected in the steady-state levels of their respective mRNAs. This may be mediated at least in part by a total or partial uncoupling from DNA synthesis of the expression of some transcripts. We next determined the effect of the DNA synthesis inhibitor hydroxyurea (HU) on mRNA levels in histones in both uninduced and induced MEL cells. For the latter we used cultures that had been allowed to differentiate to a point at which the levels of the replication-dependent transcripts were significantly reduced but levels of the partially replication-dependent transcripts were still at or near those found in exponentially growing cultures (day 3 in this experiment). Figure 6 shows S1 nuclease analysis of mRNA levels for representatives of each expression class. The results of densitometric scans of these data are compiled in Table 1.

The levels of the replication-dependent H2b.1-221 and H3.2-221 transcripts were reduced in parallel with the reduction in DNA synthesis-accompanying differentiation; a 50% reduction in these mRNAs accompanied a 50% reduction in the incorporation of [^{3}H]thymidine. H2b.1-221 and H3.2-221 expression was completely sensitive to HU in both uninduced and induced cultures. The expression of their mRNAs appears to be tightly coupled to the level of DNA synthesis.

The results for the replication-independent H3.3 variant were as expected. This experiment was also done with a recently isolated mouse H3.3 gene, H3.3-1011 (S. E. Wellman, unpublished data). The concentrations of H3.3 transcripts detected by either the human or mouse probe were unaffected by the reduction in DNA synthesis, whether as a result of differentiation or HU treatment.

It is interesting that the level of the partially replication-dependent H3.2-614 mRNA in exponentially growing MEL cells is sensitive to HU (Table 1) and to other DNA synthesis inhibitors in other mouse cell lines (10, 19, 19a). In differentiating cultures H3.2-614 transcripts remained at preinduction levels despite an approximately 50% reduction in DNA synthesis (Table 1). This suggests that during the differentiation process the expression of the partially replication-dependent genes becomes uncoupled from replication. However, in these differentiating cultures the mRNAs of H3.2-614 were completely sensitive to HU indicating that some degree of coupling to DNA synthesis remained.

Effect of cycloheximide on histone gene expression. We and others have shown previously that inhibition of protein synthesis with any of a number of compounds effectively uncouples histone gene expression from DNA synthesis (3, 40, 42–44). This has been interpreted as evidence for a labile protein responsible for mediating the cell cycle regulation. We investigated the effect of cycloheximide on the level of transcripts from representative genes of each of the three expression classes. Table 2 shows that in both exponentially growing and fully differentiated cultures cycloheximide completely prevented the effect of HU on the steady-state level of mRNAs for both replication-dependent and partially replication-dependent variants. Treatment with cycloheximide alone resulted in a 1.5- to 6-fold increase in the level of these transcripts. Although a greater relative increase was observed in differentiated cultures, the absolute level of each transcript was higher in exponentially growing, undifferentiated cells. In both cultures H3.2-221 exhibited a slightly higher relative increase in response to cycloheximide than did H3.2-614 or H2a.2-614. The significance of this observation is not clear. The level of the replication-independent H3.3 mRNA was unaffected by either HU or cycloheximide, consistent with the constitutive expression of this transcript.

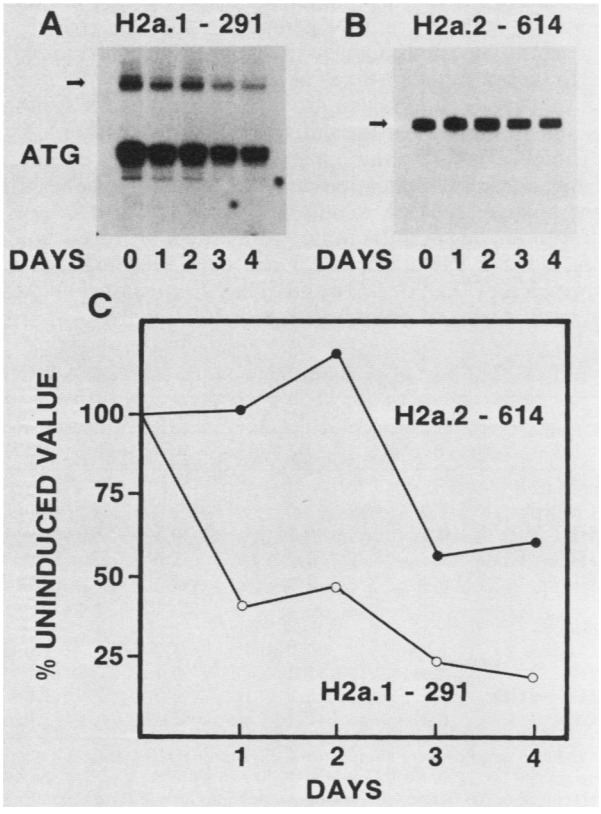

FIG. 4. Steady-state H2a mRNA levels during MEL cell differentiation. (A and B) S1 nuclease analysis of specific H2a transcripts. Probes are depicted in Fig. 2. (C) Results of densitometric scans.

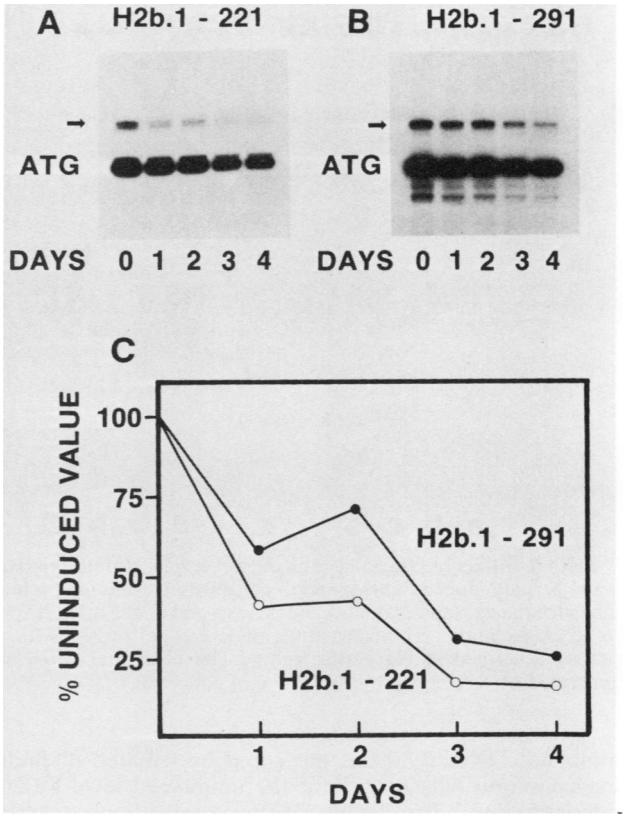

FIG. 5. Steady-state H2b mRNA levels during MEL cell differentiation. (A and B) S1 nuclease analysis of specific H2b transcripts. Probes are depicted in Fig. 2. (C) Results of densitometric scans.

DISCUSSION

We studied the changes in the steady-state levels of mRNAs for several different core histone variants during the differentiation of MEL cells. We detected three distinct expression classes which coincide with the classification scheme described by Grove and Zweidler (20).

The mRNA levels of the replication-dependent variants H3.1-221, H3.2-221, H2b.1-221, and H2a.1-291 were tightly coupled to DNA synthesis. As the doubling rate and DNA synthesis decreased during differentiation, the levels of the RNA products of these genes fell. The largest decrease was seen during day 1 of differentiation. Their levels seemed to decrease to a greater extent than would be expected from the reduced doubling rate. Studies of the early stages of differentiation of DS-19 cells by Terada et al. (45) have shown that during day 1 after induction the G_1 phase of the cell cycle is prolonged and entry of cells into the S phase is transiently

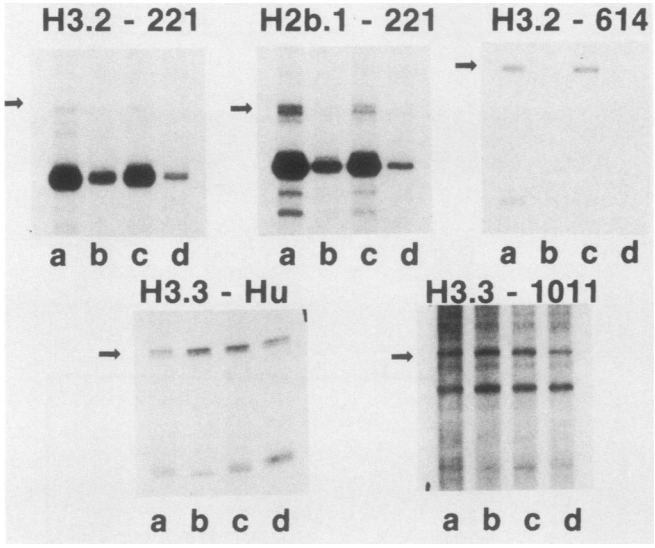

FIG. 6. Effect of HU on histone mRNA levels. Uninduced (lanes a and b) and induced (lanes c and d) cells were treated for 1 h in the presence (lanes b and d) or absence (lanes a and c) of 5 mM HU prior to RNA isolation. S1 nuclease assays were performed with the probes described in Fig. 2 through 4. The H3.3-1011 probe was generated by 5′ labeling the *Bgl*II site in codon 80.

inhibited. DNA synthesis measured by tritiated thymidine incorporation falls to 25% of the uninduced level by 20 h postinduction. The levels of the replication-dependent histone mRNAs appeared to decrease rapidly with this rapid decrease in DNA synthesis. By day 2 of differentiation the cells had overcome this early block, and the levels of these transcripts paralleled the decrease in cell doubling rate throughout the later stages of differentiation.

The levels of the replication-independent H3.3 transcripts were constitutive throughout differentiation.

The third histone expression class was represented by the H3.2-614 and H2a.2-614 products. The H3.2-614 mRNA decreased somewhat during day 1 of differentiation and then returned to preinduction levels by day 2, and the levels of H2a.2-614 stayed at 100% or greater throughout the first 2 days. Both transcripts then decreased during the later stages of differentiation to approximately 50 to 60% of their preinduced levels.

During MEL cell differentiation the synthesis rates of all the histones decrease (20). However, the magnitude of the decrease differed for different variants such that the relative levels of the variants within their respective subtypes changed. Overall, the mRNAs of the histone genes studied had expression characteristics which reflected those of the proteins for which they coded. The H3.2-221 mRNA was an exception; its levels decreased drastically during differentiation, whereas the relative level of the H3.2 protein has been shown to change very little (20). This is probably due to the fact that the partially replication-dependent H3.2-614 mRNAs comprise 40% of the H3 mRNAs and the H3.2 mRNAs comprise only 5 to 10% of the H3 mRNAs in mice (19a). The level of mRNAs from the two H2b.1-like genes used in this study, H2b.1-221 and H2b.1-291, went down in a replication-dependent manner and in a manner intermediate between replication-dependent and partially replication-dependent, respectively, while the relative level of H2b.1 protein increased (20). It is likely that these are genes for minor variants and that the major H2b.1 gene(s) have yet to be isolated. We have looked at the expression of only a few of the mouse histone genes (19a, 23). However, results of our studies do suggest that the changes in the relative levels of different histone variants are mediated at least in part by changes in mRNA levels.

Histone switching during differentiation probably occurs by virtue of having genes for different histone variants with different regulatory capacities. For example, one group of genes is tightly coupled to DNA synthesis (replication-dependent variants), and as DNA synthesis is reduced during differentiation so are the levels of the mRNAs that they encode. Another group of genes, replication-independent variants, maintain a basal level of mRNA by constitutive synthesis. In the absence of specific translational or posttranslational control, the relative percentage of this type of histone will increase accordingly as the others decrease. A third group of genes, the partially replication-dependent variants, increase the ratio of their histone product to that of other histones of the same subtype by becoming transiently uncoupled from their cell cycle synthesis linkage during part of the differentiation process.

The observation of an apparent uncoupling of expression of the partially replication-dependent histone genes from tight linkage to DNA synthesis during the first 2 days of differentiation is intriguing. We suggest three major, nonexclusive explanations for the continued high level of expression of the H3.2-614 and H2a.2-614 transcripts during the early stages of differentiation of MEL cells.

TABLE 1. Effect of HU on histone mRNA levels

Condition	Percentage of uninduced control on the following probes[a]					
	H2b.1-221	H3.2-221	H3.2-614	H3.3-Hu	H3.3-1011	DNA synthesis[b]
Uninduced						
Control	100	100	100	100	100	100
HU	20	20	27	105	100	26
Induced						
Control	52	50	105	108	98	50
HU	9	8	16	106	97	13

[a] Data are from Fig. 6.
[b] DNA synthesis was measured as trichloroacetic acid-insoluble counts incorporated during a 10-min pulse with tritiated thymidine.

TABLE 2. Effect of cycloheximide on histone mRNA levels

Condition[a]	Levels of the following probes[b]:			
	H3.2-221	H3.2-614	H2a.2-614	H3.3-Hu
Uninduced				
Control	1.0	1.0	1.0	1.0
HU	0.1	0.1	0.1	1.0
HU + CH	1.8	1.2	1.8	1.0
CH	3.4	1.6	2.5	1.0
Induced				
Control	1.0 (0.2)	1.0 (0.4)	1.0 (0.4)	1.0 (1.0)
HU	0.1	0.1	0.1	1.0
HU + CH	4.6	1.8	3.5	1.0
CH	6.2	2.1	3.6	1.0

[a] Induced cultures were assayed for 4 days after HMBA addition. Cultures were treated for 30 min with 5 mM HU, 40 min with 20 μg of cycloheximide (CH) per ml or for 10 min with cycloheximide plus an additional 30 min in the presence of both drugs prior to RNA isolation.
[b] Values are reported relative to the levels of untreated controls. Numbers in parentheses indicate ratio to uninduced culture.

First, it is known that the early stages of differentiation of MEL cells involve a lengthening of the G_1 phase with no change in the length of S or G_2 phase (18, 25). It has also been shown that histone gene expression precedes S phase (21). It is possible that the point of initiation of expression may vary for different histone genes. During day 1 of the differentiation of MEL cells, the G_1 phase may be lengthened to a point at which partially replication-dependent genes are expressed and replication-dependent genes are not. Later in differentiation an extended G_1 phase could account for a larger amount of activity of the partially replication-dependent genes as compared with that of the replication-dependent genes. The observation that the expression of these transcripts is severely reduced by treatment with HU even in differentiating cells is not inconsistent with this model. DNA synthesis inhibitors are known to affect most histone synthesis which occurs in G-phase cells (40) and may act directly on the coupling mechanism.

Second, there may be a cell-specific differentiation signal which overrides the coupling of partially replication-dependent gene expression to DNA synthesis during the early stages of differentiation. In studies of regenerating liver H3.2 appeared to be a replication-dependent rather than a partially replication-dependent histone (53). Also in a number of nondifferentiating, growth-inhibitied cell lines, including uninduced MEL cells, expression of the H3.2-614 gene appears to be dependent on DNA synthesis. These two facts circumstantially argue in favor of a differentiation-specific signal.

Third, partially replication-dependent and replication-dependent histone gene expression may be differentially responsive to a regulatory molecule, the level of which is linked to the cell cycle. These levels could change during differentiation to a point at which replication-dependent gene expression is reduced and partially replication-dependent gene expression is unaffected. As differentiation proceeds, the levels of these regulatory molecules may change to a point at which expression of the partially replication-dependent genes is also affected. This model could incorporate aspects of either of the previous models. The level of the regulatory molecule could be controlled temporally during the cell cycle such that the extended G_1 phase of early differentiation extends the period of differential sensitivity of the two expression classes. Alternatively, there may be a differentiation-specific signal which transiently modulates the level of the regulatory molecule or its interaction with its target.

Our results also indicate that linked histone genes may be regulated in a coordinate manner. Clones mm221 and mm291, which contain replication-dependent variants, are both located on chromosome 13, whereas mm614, containing partially replication-dependent variants, has been mapped to chromosome 3 (19a). Histone gene regulation is mediated at least in part at the transcriptional level, and it is conceivable that entire clusters are activated by a single signal. Whether there is independent regulation of individual genes within a cluster is unknown at this time. A component of the regulation of histone gene expression is at the level of mRNA stability (21, 40), so at least at the mRNA level, specific regulation of unique transcripts would not be surprising.

In high eucaryotes there are approximately 10 to 20 replication-dependent genes for each subtype (19a, 23). Results of our studies point out that there is no reason to believe they are all regulated in a similar manner. It is possible that the multiple genes of a particular isotype have different regulatory capacities. This, coupled with the fact that there are variants of each type of histone, allows for a considerable diversity of histone expression. It is not known whether histone variants are due to nonfunctional evolutionary drift or if the changes are functional. It is probable that examples of both will be found. If the H3.3 replication-independent genes are typical of other replication-independent genes, then this group of histone genes will differ considerably from replication-dependent genes, both in their structure and regulation. Chicken and human H3.3 genes have been isolated which have introns and which may be poly(A)$^+$ (12, 48a). Results of our studies indicate that H3.3 expression is not linked to DNA synthesis. Histone genes are a multigene family, the expression and products of which are more complex and interesting than they initially appeared (30).

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LITERATURE CITED

1. **Alterman, R. M., S. Ganguly, D. H. Schulze, W. F. Marzluff, C. L. Schildkraut, and A. I. Skoultchi.** 1984. Cell cycle regulation of mouse H3 histone mRNA metabolism. Mol. Cell. Biol. **4**:123–132.
2. **Artishevsky, A., A. M. Delegeane, and A. S. Lee.** 1984. Use of a cell cycle mutant to delineate the critical period for the control of histone mRNA levels in the mammalian cell cycle. Mol. Cell. Biol. **4**:2364–2369.
3. **Baumbach, L. L., F. Marashi, M. Plumb, G. Stein, and J. Stein.** 1984. Inhibition of DNA replication coordinately reduces cellular levels of core and H1 histone mRNAs: requirement for protein synthesis. Biochemistry **23**:1618–1625.
4. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
5. **Blankstein, L. A., B. D. Stollar, S. G. Franklin, A. Zweidler, and S. B. Levy.** 1977. Biochemical and immunological characterization of two distinct variants of histone H2A in Friend leukemia. Biochemistry **16**:4557–4562.
6. **Blankstein, L. A., and S. B. Levy.** 1976. Changes in histone f2a2 associated with proliferation of Friend leukaemic cells. Nature (London) **260**:638–640.
7. **Borun, T. W., F. Gabrielli, K. Ajiro, A. Zweidler, and C. Baglioni.** 1975. Further evidence of transcriptional and translational control of histone messenger RNA during the HeLa S3 cycle. Cell **4**:59–67.
8. **Borun, T. W., M. D. Scharff, and E. Robbins.** 1967. Rapidly labeled, polyribosome-associated RNA having the properties of histone messenger. Proc. Natl. Acad. Sci. USA **58**:1977–1983.
9. **Butler, W. B., and G. C. Mueller.** 1973. Control of histone synthesis in HeLa cells. Biochim. Biophys. Acta **294**:481–496.
10. **DeLisle, A. J., R. A. Graves, W. F. Marzluff, and L. F. Johnson.** 1983. Regulation of histone mRNA production and stability in serum-stimulated 3T6 fibroblasts. Mol. Cell. Biol. **3**:1920–1929.
11. **Detke, S., A. Lichter, I. Phillips, J. Stein, and G. Stein.** 1979. Reassessment of histone gene expression during cell cycle in human cells using homologous H4 histone cDNA. Proc. Natl. Acad. Sci. USA **76**:4995–4999.
12. **Engel, J. D., B. J. Sugarman, and J. B. Dodgson.** 1982. A chicken histone H3 gene contains intervening sequences. Nature (London) **297**:434–436.
13. **Fibach, E., R. C. Reuben, R. A. Rifkind, and P. A. Marks.** 1977. Effect of hexamethylene bisacetamide on the commitment to differentiation of murine erythroleukemia cells. Cancer Res.

37:440–444.
14. **Franklin, S. G., and A. Zweidler.** 1977. Non-allelic variants of histones 2a, 2b and 3 in mammals. Nature (London) **266:** 273–275.
15. **Friend, C., W. Scher, J. G. Holland, and T. Sato.** 1971. Hemoglobin synthesis in murine virus-induced leukemic cells *in vitro*: stimulation of erythroid differentiation by dimethyl sulfoxide. Proc. Natl. Acad. Sci. USA **68:**378–382.
16. **Gallwitz, D., and G. C. Mueller.** 1969. Histone synthesis *in vitro* on HeLa cell microsomes. J. Biol. Chem. **244:**5947–5952.
17. **Garrett, C., and N. M. Kredich.** 1981. Induction of hemoglobin synthesis by xylosyladenine in murine erythroleukemia cells. Metabolism of xylosyladenine and effects on transmethylation. J. Biol. Chem. **256:**12705–12709.
18. **Geller, R., R. Levenson, and D. Housman.** 1978. Significance of the cell cycle in commitment of murine erythroleukemia cells to erythroid differentiation. J. Cell Physiol. **95:**213–222.
19. **Graves, R. A., and W. F. Marzluff.** 1984. Rapid reversible changes in the rate of histone gene transcription and histone mRNA levels in mouse myeloma cells. Mol. Cell. Biol. **4:**351–357.
19a. **Graves, R. A., S. E. Wellman, I. M. Chiu, and W. F. Marzluff.** 1985. Differential expression of two clusters of mouse histone genes. J. Mol. Biol. **183:**179–194.
20. **Grove, G. W., and A. Zweidler.** 1984. Regulation of nucleosomal core histone variants in differentiating murine erythroleukemia cells. Biochemistry **23:**4436–4443.
21. **Heintz, N., H. L. Sive, and R. G. Roeder.** 1983. Regulation of human histone gene expression: kinetics of accumulation and changes in the rate of synthesis and in the half-lives of individual histone mRNAs during the HeLa cell cycle. Mol. Cell. Biol. **3:**539–550.
22. **Igo-Kemenes, T., W. Hörz, and H. G. Zachau.** 1982. Chromatin. Annu. Rev. Biochem. **51:**89–121.
23. **Jacob, E.** 1976. Histone-gene reiteration in the genome of mouse. Eur. J. Biochem. **65:**275–284.
24. **Lennox, R. W., and L. H. Cohen.** 1984. The H1 subtypes of mammals: metabolic characteristics and tissue distribution, p. 373–395. *In* G. S. Stein, J. L. Stein, and W. F. Marzluff (ed.), Histone genes: structure, organization, and regulation. John Wiley & Sons, Inc., New York.
25. **Marks, P. A., and R. A. Rifkind.** 1978. Erythroleukemic differentiation. Annu. Rev. Biochem. **47:**419–448.
26. **Marzluff, W. F., Jr., E. C. Murphy, Jr., and R. C. C. Huang.** 1973. Transcription of ribonucleic acid in isolated mouse myeloma nuclei. Biochemistry **12:**3440–3446.
27. **McGhee, J. D., and G. Felsenfeld.** 1980. Nucleosome structure. Annu. Rev. Biochem. **49:**1115–1156.
28. **Nudel, U., J. Salmon, E. Fibach, M. Terada, R. Rifkind, P. Marks, and A. Bank.** 1977. Accumulation of α- and β-globin messenger RNAs in mouse erythroleukemia cells. Cell **12:**463–469.
29. **Ohta, Y., M. Tanaka, M. Terada, O. J. Miller, A. Bank, P. A. Marks, and R. A. Rifkind.** 1976. Erythroid cell differentiation: murine erythroleukemia cell variant with unique pattern of induction by polar compounds. Proc. Natl. Acad. Sci. USA **73:**1232–1236.
30. **Old, R. W., and H. R. Woodland.** 1984. Histone genes: not so simple after all. Cell **38:**624–626.
31. **Reuben, R. C., R. L. Wife, R. Breslow, R. A. Rifkind, and P. A. Marks.** 1976. A new group of potent inducers of differentiation in murine erythroleukemia cells. Proc. Natl. Acad. Sci. USA **73:**862–866.
32. **Richmond, T. J., J. T. Finch, B. Rushton, D. Rhodes, and A. Klug.** 1984. Structure of the nucleosome core particle at 7Å resolution. Nature (London) **311:**532–537.
33. **Rickels, R., F. Marashi, F. Sierra, S. Clark, J. Wells, J. Stein, and G. Stein.** 1982. Analysis of histone gene expression during the cell cycle in HeLa cells by using cloned human histone genes. Proc. Natl. Acad. Sci. USA **79:**749–753.
34. **Robbins, E., and T. W. Borun.** 1967. The cytoplasmic synthesis of histones in HeLa cells and its temporal relationship to DNA replication. Proc. Natl. Acad. Sci. USA **57:**409–416.
35. **Roche, J., C. Gorka, P. Goeltz, and J. J. Lawrence.** 1985. Association of histone H1° with a gene repressed during liver development. Nature (London) **314:**197–198.
36. **Rougeon, F., and B. Mach.** 1977. Cloning and amplification of α and β mouse globin gene sequences synthesized *in vitro*. Gene **1:**229–239.
37. **Salditt-Georgieff, M., M. Sheffrey, K. Krauter, J. E. Darnell, Jr., R. Rifkind, and P. A. Marks.** 1984. Induced transcription of the mouse β-globin transcription unit in erythroleukemia cells. Time-course of induction and of changes in chromatin structure. J. Mol. Biol. **172:**437–450.
38. **Sheffery, M., P. A. Marks, and R. A. Rifkind.** 1984. Gene expression in murine erythroleukemia cells. Transcriptional control and chromatin structure of the α1-globin gene. J. Mol. Biol. **172:**417–436.
39. **Sittman, D. B., I.-M. Chiu, C.-J. Pan, R. H. Cohn, L. H. Kedes, and W. F. Marzluff.** 1981. Isolation of two clusters of mouse histone genes. Proc. Natl. Acad. Sci. USA **78:**4078–4082.
40. **Sittman, D. B., R. A. Graves, and W. F. Marzluff.** 1983. Histone mRNA concentrations are regulated at the level of transcription and mRNA degradation. Proc. Natl. Acad. Sci. USA **80:** 1849–1853.
41. **Sittman, D. B., R. A. Graves, and W. F. Marzluff.** 1983. Structure of a cluster of mouse histone genes. Nucleic Acids Res. **11:**6679–6697.
42. **Sive, H. L., N. Heintz, and R. G. Roeder.** 1984. Regulation of human histone gene expression during the HeLa cell cycle requires protein synthesis. Mol. Cell. Biol. **4:**2723–2734.
43. **Stahl, H., and D. Gallwitz.** 1977. Fate of histone messenger RNA in the absence of initiation of protein synthesis. Eur. J. Biochem. **72:**385–392.
44. **Stimac, E., V. E. Groppi, Jr., and P. Coffino.** 1984. Inhibition of protein synthesis stabilizes histone mRNA. Mol. Cell. Biol. **4:**2082–2090.
45. **Terada, M., J. Fried, U. Nudel, R. A. Rifkind, and P. A. Marks.** 1977. Transient inhibition of initiation of S-phase associated with dimethyl sulfoxide induction of murine erythroleukemia cells to erythroid differentiation. Proc. Natl. Acad. Sci. USA **74:**248–252.
46. **Weaver, R. F., and C. Weissman.** 1979. Mapping of RNA by a modification of the Berk-Sharp procedure: the 5′ termini of 15S β-globin mRNA precursor and mature 10S β-globin mRNA have identical map coordinates. Nucleic Acids Res. **7:**1175–1193.
47. **Weintraub, H.** 1984. Histone-H1-dependent chromatin superstructures and the suppression of gene activity. Cell **38:**17–27.
48. **Weisbrod, S.** 1982. Active chromatin. Nature (London) **297:**289–295.
48a. **Wells, D., and L. Kedes.** 1985. Structure of a human histone cDNA: evidence that basally expressed histone genes have intervening sequences and encode polyadenylated mRNAs. Proc. Natl. Acad. Sci. U.S.A. **82:**2834–2838.
49. **Wu, R. S., and W. M. Bonner.** 1981. Separation of basal histone synthesis from S-phase histone synthesis in dividing cells. Cell **27:**321–330.
50. **Wu, R. S., S. Tsai, and W. M. Bonner.** 1982. Patterns of histone variant synthesis can distinguish G0 from G1 cells. Cell **31:**367–374.
51. **Wu, R. S., S. Tsai, and W. M. Bonner.** 1983. Changes in histone H3 composition and synthesis pattern during lymphocyte activation. Biochemistry **22:**3868–3873.
52. **Wu, R. S., M. H. P. West, and W. M. Bonner.** 1984. Patterns of histone gene expression in human and other mammalian cells, p. 457–483. *In* G. S. Stein, J. L. Stein, and W. F. Marzluff (ed.), Histone genes: structure, organization, and regulation. John Wiley & Sons, Inc., New York.
53. **Zweidler, A.** 1980. Nonallelic histone variants in development and differentiation. Dev. Biochem. **15:**47–56.
54. **Zweidler, A.** 1984. Core histone variants of the mouse: primary structure and differential expression, p. 338–371. *In* G. S. Stein, J. L. Stein, and W. F. Marzluff (ed.), Histone genes: structure, organization, and regulation. John Wiley & Sons, Inc., New York.