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Histone Gene Switching in Murine Erythroleukemia Cells Is Differentiation Specific and Occurs without Loss of Cell Cycle Regulation

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We investigated the expression characteristics of the fully replication-dependent (FRD) and the partially replication-dependent (PRD) histone gene variants by measuring changes in steady-state mRNA levels during hexamethylene bisacetamide (HMBA)-induced differentiation of murine erythroleukemia (MEL) cells. Between 24 and 60 h after induction, there was a dramatic switch in histone gene expression, such that the ratio of PRD to FRD transcripts increased severalfold over that found in uninduced MEL cells. We demonstrated that this gene switching was not simply a partial or complete uncoupling of PRD gene expression from DNA synthesis. PRD and FRD transcript levels were regulated coordinately upon treatment of uninduced or induced MEL cells with inhibitors of DNA synthesis, protein synthesis, or both. Using several criteria, we were unable to detect any difference in PRD and FRD gene expression under any conditions except in cells undergoing differentiation. MEL cells were arrested at a precommitment stage of differentiation by induction with HMBA in the presence of dexamethasone (DEX). If DEX was subsequently removed, DNA synthesis resumed, the cells underwent commitment, and histone gene switching was observed. In contrast, if both DEX and HMBA were removed, DNA synthesis still resumed, but commitment did not occur and no gene switching was observed. These results imply that histone gene switching is intimately related to the differentiation process.

The histones play a fundamental role in organizing eucaryotic chromatin, and accordingly their structures have been highly conserved during evolution (21, 32, 59). In higher organisms, each subtype, with the possible exception of H4, is represented by from three to seven nonallelic primary sequence variants (12, 19, 25, 65, 66). In the mouse there are 10 to 20 copies of the genes for each subtype (22), the majority of which are arranged in an undetermined number of "scrambled clusters" located on at least two chromosomes (15, 27, 30, 45, 46, 64). It is presently unclear whether there is any functional significance to histone protein heterogeneity or to the multiplicity of genes encoding each variant. Nucleosomes composed of different variant histones display different physical properties (5, 43), and it has been suggested that alterations in histone variant composition contribute to the changes in chromatin conformation associated with differentiation and gene activation (7, 9, 29, 50, 56). This view is supported by the observations that, in sea urchins, distinct sets of histone genes encoding nonallelic variants are expressed in a well-defined spatial and temporal pattern during development and differentiation (31, 34). In mammals, many variants are nonrandomly distributed among adult tissues and display characteristic patterns of expression during development and differentiation (7, 9, 16, 25, 36, 61, 65, 66).

The mouse core histone variants have been described and classified according to their expression characteristics in different tissues and during murine erythroleukemia (MEL) cell differentiation (16, 65, 66). The major class, the fully replication-dependent (FRD) variants, predominate in rapidly dividing tissues, and their synthesis is tightly linked to DNA synthesis. The synthesis of partially replication-dependent (PRD) variants is tightly linked to DNA synthesis in continuously dividing cells. However, in terminally differentiating cells, the synthesis of PRD variants is induced at the beginning of S phase of the cell cycle and is not completely repressed following completion of DNA synthesis. The replication-independent (RID) variants are not linked to the cell cycle and are constitutively expressed, usually at relatively low levels (58, 62, 63). Tissue-specific variants such as those found only in spermatocytes have also been detected (41, 55).

Previously we determined the changes in steady-state mRNA levels of specific core histone variants of the FRD, PRD, and RID classes during MEL cell differentiation (6). Notably, the levels of the transcripts we detected paralleled those of the proteins for which they code, indicating that the levels of different histone variants are mediated at least in part by changes in their mRNA levels. We also found that for the genes which occur in clusters, all of the genes within the cluster belong to the same expression class, either PRD or FRD.

Perhaps the most interesting of the genes we investigated are the members of the PRD expression class. The relative levels of these transcripts appear to become transiently "uncoupled" from DNA synthesis at a particular stage of the differentiation process. The levels of these transcripts at 2 days postinduction return to their preinduction levels despite a significant decrease in the rate of DNA synthesis and cell division within the cell population. Surprisingly, the expression of these genes remains sensitive to metabolic inhibitors of DNA synthesis even at the time of maximal uncoupling. We previously proposed several models to explain the selective expression of PRD variants during the uncoupling period. In this study we addressed these models and investigated the expression characteristics of FRD and PRD histone genes in greater detail. The results indicate that histone gene switching occurs without loss of cell cycle regulation and that the switching phenomenon is clearly linked to the differentiation process. The implications of

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these findings as they relate to the functional significance of histone heterogeneity and the multiplicity of histone genes are discussed.

MATERIALS AND METHODS

Clones. Mouse genomic clones MH221, MH291, MH614, and MH143 have been described previously (15, 27, 45, 46, 52, 64). Plasmid pCR1β, a mouse β-major globin cDNA clone (40), was obtained from M. Edgell.

Cell culture. The MEL cell line DS-19E5 was derived from DS-19 (35) and selected for high levels of induction. Under our growth conditions, this cell line reproducibly generates >98% differentiated cells after 4 days of induction. The dexamethasone (DEX)-responsive cell line Sc9 (23) was a gift from R. Rifkind and P. Marks. MEL cells were grown in basal Eagle medium (Gibco) plus Earle salts, glutamine, and 15% fetal bovine serum. Cultures were induced to differentiate by the addition of hexamethylene bisacetamide (HMBA) to a final concentration of 5 mM. When used, DEX (Sigma) was added to a final concentration of 4 μM. Cell density was maintained between 2×10^5 and 8×10^5 cells per ml during all experiments. Commitment to terminal cell division was measured by transferring a 10-μl portion of cells to 2 ml of growth medium lacking inducer but containing 1.0% methylcellulose. Cells were incubated in 35-mm petri dishes at 37°C for 5 days. Committed cells gave rise to small (<32-cell) colonies which were further identified by in situ staining of hemoglobin with benzidine (13). DNA synthesis was measured by pulse-labeling cells with 10 μCi of [^{3}H]thymidine (2.0 mCi/mmol) per ml for 30 min at 37°C. Cells were washed twice with isotonic saline and lysed and precipitated with 10% ice-cold trichloroacetic acid. Insoluble material was collected on Whatman GF/A filters and counted by liquid scintillation.

Mouse BALB/3T3 cells (clone A31 from the American Type Culture Collection) were grown in Dulbecco modified Eagle medium (Gibco) containing 10% fetal bovine serum. Cultures at 30% confluency were arrested in the G0 phase by incubation for 4 days in medium containing 0.5% fetal bovine serum. Cultures were induced to reenter the cell cycle by replacement of low-serum medium with fresh medium containing 10% fetal bovine serum. DNA synthesis was determined as described for MEL cells except that the labeling period was 1 h and cells were washed while attached and then removed by treatment with trypsinization solution (Gibco) for 5 min prior to trichloroacetic acid precipitation.

Assay for mRNA concentration. Total cellular RNA was prepared by hot phenol extraction at pH 5.5 as described previously (6). Steady-state levels of specific mRNAs were determined by quantitative S1 nuclease protection assays (4, 57). Probes were generated by digestion at a restriction site within the coding region of the gene, followed by end labeling at the 5′ ends with [^{32}P]ATP (ICN Pharmaceuticals) and polynucleotide kinase (New England Biolabs). The restriction sites used are indicated in the figure legends. Probes were coprecipitated with total RNA from 5×10^5 cells. All experiments were done on a per cell basis to avoid biasing the results due to changes in the levels of total RNA (predominantly rRNA) which occur during MEL cell differentiation (42). Probes and RNA were resuspended in S1 hybridization buffer (40 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), pH 6.4], 400 mM NaCl, 1 mM EDTA), denatured, and hybridized for 6 to 9 h at 56°C for histone probes or 52°C for the β-globin probe. Hybrids were digested with 750 U of S1 nuclease (Bethesda Research Laboratories) per ml of digestion buffer (30 mM sodium acetate [pH 4.5], 250 mM NaCl, 2 mM $ZnSO_4$) for 45 min at 37°C. S1 nuclease-resistant DNA was precipitated, suspended in 80% formamide dye, and run on denaturing 8% polyacrylamide sequencing gels. Gels were transferred to 3MM paper and exposed to preflashed Kodak XAR-5 film. The resulting autoradiograms were scanned with a Bio-Rad model 620 video densitometer. Multiple exposures of each gel were obtained to ensure that all bands were within the linear response range of the X-ray film. All assays were done in probe excess as confirmed by linear dependence of the signal intensity to the amount of added RNA. The identity of the protected fragments was determined by comigration with sequencing ladders of the appropriate probes as described previously (15, 45).

RESULTS

Changes in steady-state histone mRNA levels during the early stages of MEL cell differentiation. Previously we demonstrated that the major differences in FRD and PRD histone gene expression occur during the first 2 days of MEL cell differentiation (6). Here we present in greater detail the changes in the steady-state levels of these mRNAs during this period. As before, we used homologous cloned histone genes in a quantitative S1 nuclease protection assay to determine the levels of specific gene transcripts. We also present the results of measurements of the rate of DNA synthesis and the percentage of cells committed to terminal cell division. Figure 1 shows the changes in steady-state levels of two representative H3 mRNAs. The results are consistent with the previous assignment of H3.2-221 to the FRD class and H3.2-614 to the PRD class. This detailed kinetic analysis revealed that the differences in the expression classes were confined to a specific period of the differentiation process. Within the first 24 h after induction, the rate of DNA synthesis decreased rapidly and the levels of both PRD and FRD transcripts fell. This reflects the transient prolongation of the G1 phase of the cell cycle which precedes commitment to terminal cell division (53). The cell population overcame this early effect, and the rate of DNA synthesis and total histone gene expression gradually increased. It was only after 24 h that the differences between PRD and FRD expression became apparent. The PRD transcripts increased to levels equal to or even greater than those observed in uninduced cells, while the FRD transcripts decreased to 20 to 30% of their uninduced values. During this period, approximately 24 to 60 h after induction, the percentage of cells committed to terminal cell division increased from 10 to 15% to greater than 90% (Fig. 1C).

Figure 1 reveals an additional interesting observation. The total amount of replication-dependent H3 mRNA was determined by summing the gene-specific and ATG bands in the S1 analysis. This is a reasonable estimation, considering the conservation of sequences within the coding regions and the absence of protected fragments mapping to positions preceding the ATG codon (45, 52). The RID transcripts constitute a small percentage of the total H3 mRNA and are not sufficiently complementary to hybridize with the S1 probes used (58). Nearly identical values were obtained when either the H3.2-614 or the H3.2-221 results were summed, and the values presented are the average of the two determinations. When these values are compared with the rate of DNA synthesis, they correlate almost exactly (Fig. 1C). This suggests that what we are observing might best be described as a histone variant "switching." The total amount of H3

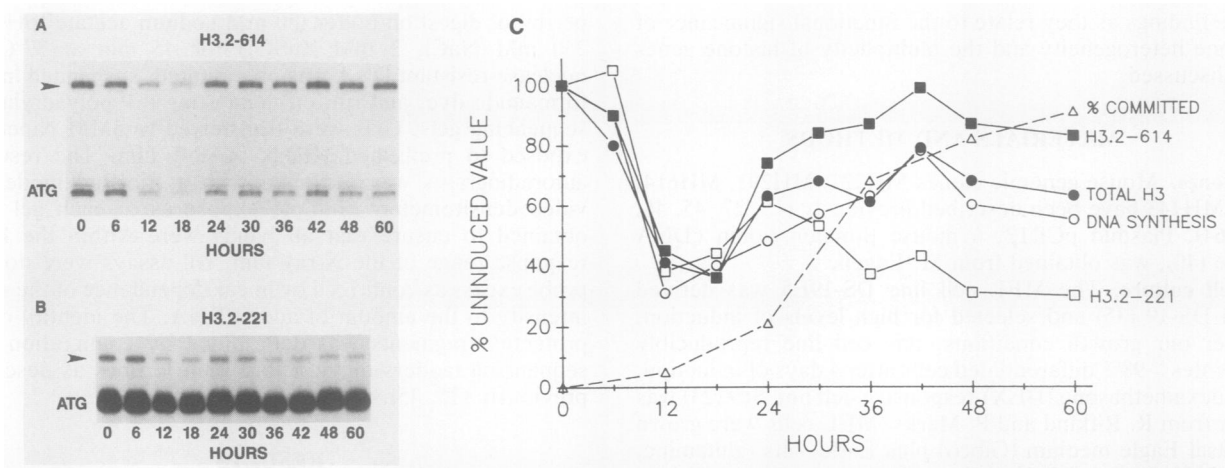

FIG. 1. Steady-state levels of FRD and PRD H3 histone mRNAs during the early stages of MEL cell differentiation. (A, B) S1 nuclease protection assays of total mRNA isolated at the indicated times following induction of DS19-E5 cells with 5 mM HMBA. The probes were labeled at the *Sal*I site in codon 58. The fragments protected by each specific mRNA are indicated by the arrows. The bands labeled ATG represent all other H3 mRNAs which are homologous within the protein-coding region only. (C) Results of densitometric scans of the S1 data and of the DNA synthesis and commitment assays. All values are the average of at least four independent determinations. No standard deviation was greater than 9.5%. Total H3 mRNA was determined as described in the text. All values except commitment are expressed as the percentage of that value obtained in uninduced, exponentially growing cells. Percent committed cells is expressed relative to the total number of viable colony-producing cells.

mRNA reflects the level of DNA synthesis, but certain H3 variants make a greater or lesser contribution to that total as differentiation proceeds.

Figure 2 shows the changes in steady-state levels of two H2a genes. The results confirmed the previous assignment of H2a.2-614 as PRD and H2a.1-291 as FRD. These genes displayed a switching pattern which coincided temporally with that observed for the H3 genes. In addition, the total amount of H2a mRNA, determined by densitometry of shorter exposures of the H2a.291 analysis, closely paralleled the rate of DNA synthesis. These results indicate that the switching phenomenon extends to at least two subtypes.

Response of FRD and PRD gene expression to inhibition of DNA synthesis by HU treatment. Previously we proposed several models to explain the differences in PRD and FRD gene expression (6). One of these suggested that the two expression classes are coupled to DNA synthesis to different extents and that as differentiation proceeds the rate of DNA synthesis falls to a point at which the expression of FRD but not PRD genes is suppressed. We had demonstrated that the expression of both classes is inhibited by treatment of cells with the DNA synthesis inhibitor hydroxyurea (HU) both before and after induction. However, in that study, prolonged treatment with a high concentration of HU was used. Here we attempted to detect a quantitative difference in the response of the two expression classes to inhibition of DNA synthesis by HU. However, the expression of representative genes of the PRD and FRD classes displayed nearly identical rates of decay when cells were treated with 5 mM HU (Fig. 3). This was true whether the cells were uninduced or had

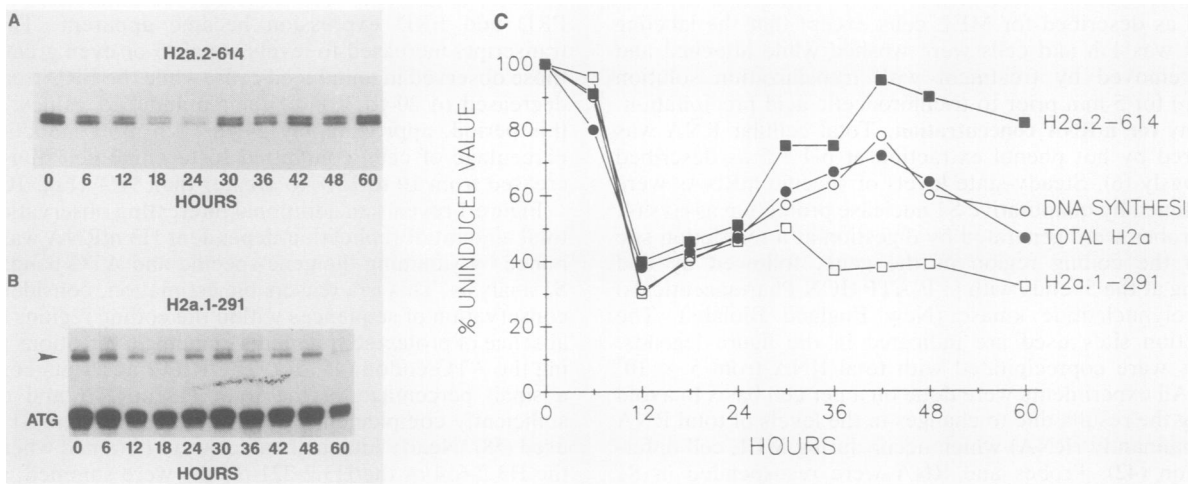

FIG. 2. Steady-state levels of FRD and PRD H2a histone mRNAs during the early stages of MEL cell differentiation. (A, B) S1 nuclease protection assays of total mRNA isolated at the indicated times following induction of DS19-E5 cells with 5 mM HMBA. The H2a.2-614 probe was labeled at the *Ava*I site in codon 20. The H2a.1-291 probe was labeled at the *Xho*II site in codon 61. (C) Results of densitometric scans of the data in panels A and B and of the DNA synthesis assay. Total H2a mRNA was determined as described in the text. All values are expressed as the percentage of that value obtained in uninduced, exponentially growing cells.

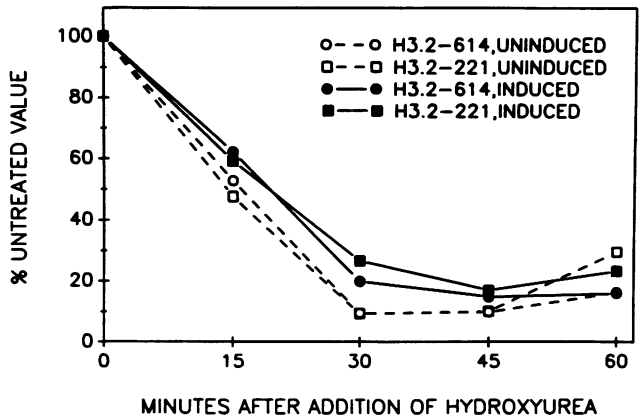

FIG. 3. Effect of HU on the steady-state levels of FRD and PRD histone mRNAs. Uninduced and induced (48 h) DS19-E5 cells were treated with 5 mM HU for the indicated times prior to the isolation of total RNA. S1 assays were performed with the H3.2-221 and H3.2-614 probes. The results of densitometric scans of the specific protected fragment from each probe are presented.

been allowed to differentiate for 48 h, the period of maximal gene switching, prior to treatment with HU. We obtained identical results with concentrations of HU of 0.01 to 10 mM (D. T. Brown, Ph.D. thesis, University of Mississippi, Jackson, 1988).

Response of FRD and PRD gene expression to CH-induced uncoupling. We also investigated the possibility that PRD and FRD genes are differentially responsive to alterations in protein synthesis. Inhibition of protein synthesis with any of a number of compounds uncouples histone gene expression from DNA synthesis and leads to elevated levels of histone mRNA (47, 49, 51). During MEL cell differentiation the rate of total protein synthesis decreases significantly (38, 42). We therefore investigated the effect of treating uninduced or induced MEL cells with cycloheximide (CH). The levels of both PRD and FRD transcripts increased severalfold upon CH treatment (Fig. 4). The rate of change in the presence of CH was essentially the same for the two expression classes. We also found that for both PRD and FRD transcripts, the

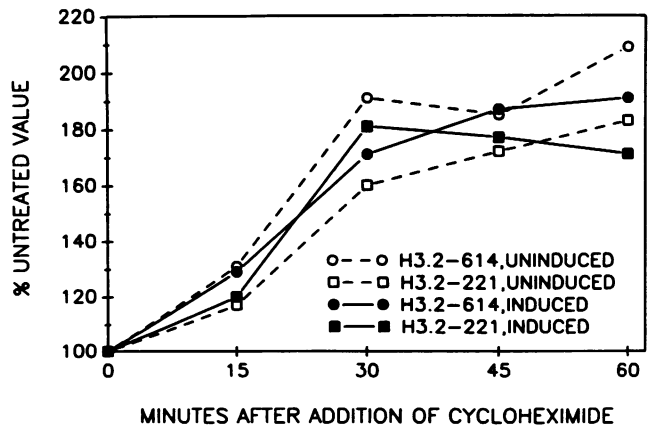

FIG. 4. Effect of CH on the steady-state levels of FRD and PRD histone mRNAs. Uninduced and induced (48 h) DS19-E5 cells were treated with 0.1 mM CH for the indicated times prior to the isolation of total RNA. S1 assays were performed with the H3.2-221 and H3.2-614 probes. The results of densitometric scans of the specific protected fragment from each probe are presented.

rate of change with simultaneous treatment with CH and HU gave results similar to those obtained by treatment with CH alone (Brown, thesis).

Relationship of PRD and FRD gene expression to entry into S phase. We also considered that perhaps the expression of PRD genes is initiated earlier in the cell cycle than that of the FRD genes. This might not be apparent in rapidly cycling cells, but the extended G1 phase of differentiating cells might accentuate the difference enough to generate differential gene expression. Although the data in Fig. 1 and 2 suggest that this is not true, we attempted to investigate this directly in uninduced MEL cells synchronized by density arrest. We found no difference between FRD and PRD gene expression in growth-arrested cells or following dilution of arrested cells into fresh growth medium (Brown, thesis). However, upon suspension in fresh medium, the cells rapidly reinitiated DNA synthesis, possibly obscuring subtle differences between the expression classes. We therefore investigated histone gene expression during the extended G1 phase which occurs when 3T3 cells are released from serum starvation. FRD and PRD gene expression was initiated simultaneously with the onset of DNA synthesis following refeeding of starved cultures (Fig. 5).

Collectively, the data presented to this point indicate that models based solely on differences in cell cycle regulation will not account for histone gene switching during MEL cell differentiation.

Histone gene expression in MEL cells synchronized by DEX treatment. Our attempts to study the relationship of histone gene switching to other aspects of differentiation were hindered by the asynchronous nature of the induction process. Recently a method for synchronizing the differentiation process by treatment of cells with DEX has become available (23, 33). When responsive cell lines are treated with HMBA and DEX, differentiation is arrested at an apparently non-rate-limiting stage prior to commitment to terminal cell division. Upon removal of DEX, the cells resume the differentiation process in a rapid and fairly synchronous manner.

We investigated the expression of H3 genes in the DEX-responsive cell line Sc9 induced to differentiate with HMBA alone (Fig. 6). We included in the analysis the FRD genes H3.1-221, H3.1-291, and the recently isolated H3-143 (64). The differences between the expression classes were not as dramatic as observed for the DS19 line, but the particular DS19 subline we used was selected for maximal induction, whereas the Sc9 line was selected for DEX responsiveness. However, histone gene switching was observed in the Sc9 cell line. The H3.2-614 gene was expressed in a PRD manner, while all others were of the FRD class.

The Sc9 cell line was synchronized by treatment for 48 h with HMBA and DEX. The culture was then split in half, washed, resuspended in fresh growth medium containing HMBA or fresh growth medium alone, and incubated for an additional 48 h. The steady-state levels of H3 transcripts (Fig. 7 and 8) and of β-globin mRNA (Fig. 9) were measured both prior to and following removal of DEX. The rate of DNA synthesis and the percentage of cells committed to terminal cell division were also measured. The results of this and additional experiments are compiled in Table 1. After 48 h of treatment with HMBA and DEX, the rate of DNA synthesis and the levels of all histone transcripts measured were reduced to 10 to 20% of their values in untreated cells. Only a small percentage of the cells were committed, and globin mRNA levels were low, indicating that the differentiation process was interrupted by DEX treatment. Upon

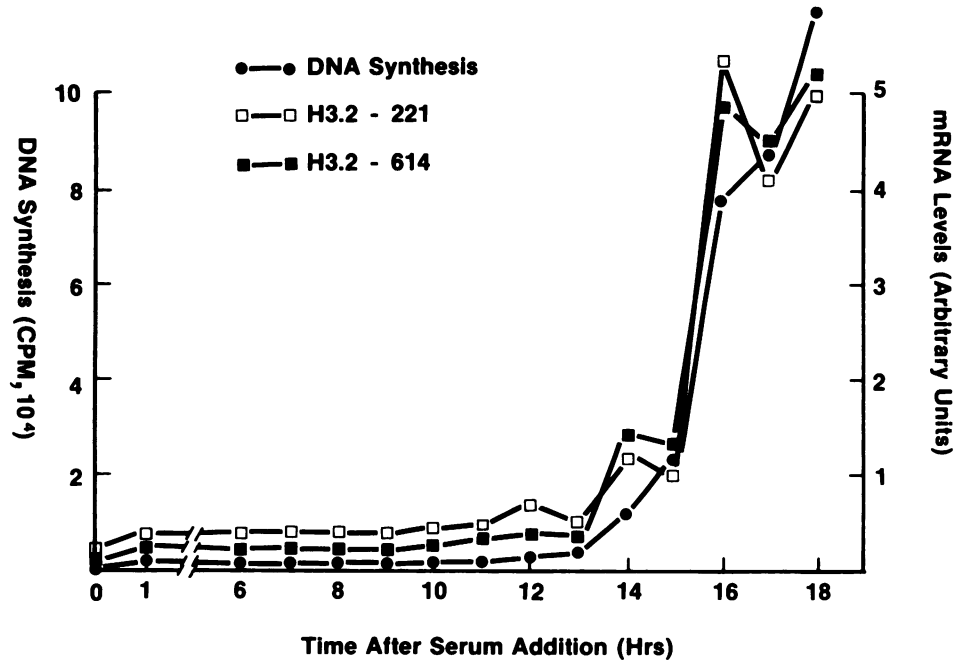

FIG. 5. Steady-state levels of FRD and PRD histone mRNAs following serum stimulation of arrested 3T3 fibroblasts. Total RNA was isolated at the indicated times following release from serum starvation. S1 assays were performed with the H3.2-221 and H3.2-614 probes. The results of densitometric scans of the specific protected fragments from each probe are presented.

resuspension into fresh medium containing HMBA, the culture rapidly displayed almost complete commitment to terminal cell division, and as DNA synthesis resumed, the PRD gene H3.2-614 was preferentially expressed. All FRD genes assayed displayed only minor increases in expression. As a result of this gene switching, transcripts from H3.2-614 constituted as much as 80% of the total H3 mRNA. This is the largest degree of switching we have yet detected and probably reflects the synchronization of the culture with respect to both the cell cycle and the differentiation process. In contrast, when the synchronized culture was resuspended in fresh medium lacking HMBA, commitment to terminal

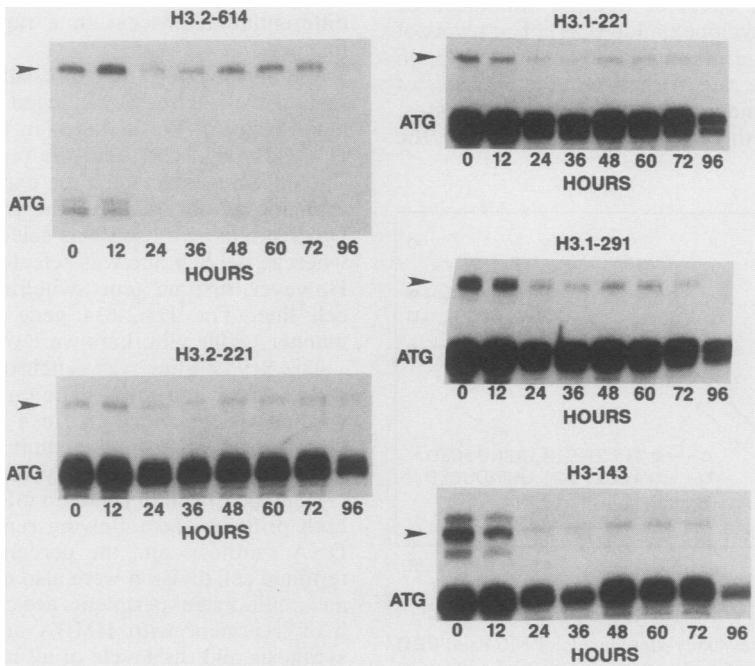

FIG. 6. Steady-state levels of H3 histone mRNAs during differentiation of Sc9 MEL cells. S1 nuclease protection assays of total mRNA isolated at the indicated times following induction of Sc9 cells with 5 mM HMBA. The probes were labeled at the *Sal*I site in codon 58. The fragments protected by the specific mRNAs are indicated by the arrows. The bands labeled ATG represent all other H3 mRNAs homologous within the protein-coding region only.

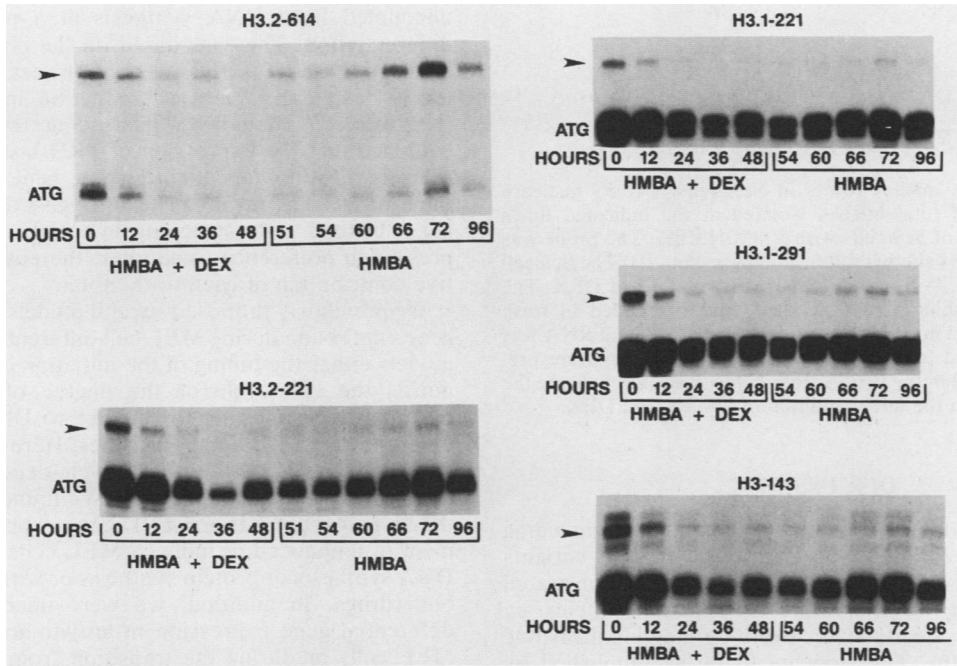

FIG. 7. Steady-state levels of H3 histone mRNAs in synchronized Sc9 cells induced to differentiate. The Sc9 cell line was treated for 48 h with 5 mM HMBA and 4 μM DEX. The culture was then split in half, washed, and suspended in fresh medium containing 5 mM HMBA but lacking DEX. Total RNA was isolated prior to and following resuspension. S1 assays were performed with the H3 probes labeled at the *Sal*I site. Values below the lanes indicate the time (in hours) from the initial addition of HMBA and DEX.

cell division was not observed. The rate of DNA synthesis and the levels of total H3 gene expression increased with kinetics similar to those observed for cultures resuspended in medium containing HMBA. However, in the absence of inducer, no gene switching was detected; all H3 genes tested were regulated coordinately. This indicates that treatment with HMBA and DEX is reversible and implies that histone gene switching is related to the differentiation process. In addition, under all conditions used in this protocol, the total amount of H3 mRNA reflected the rate of DNA synthesis, reinforcing the concept that we are detecting a switching rather than an uncoupling phenomenon.

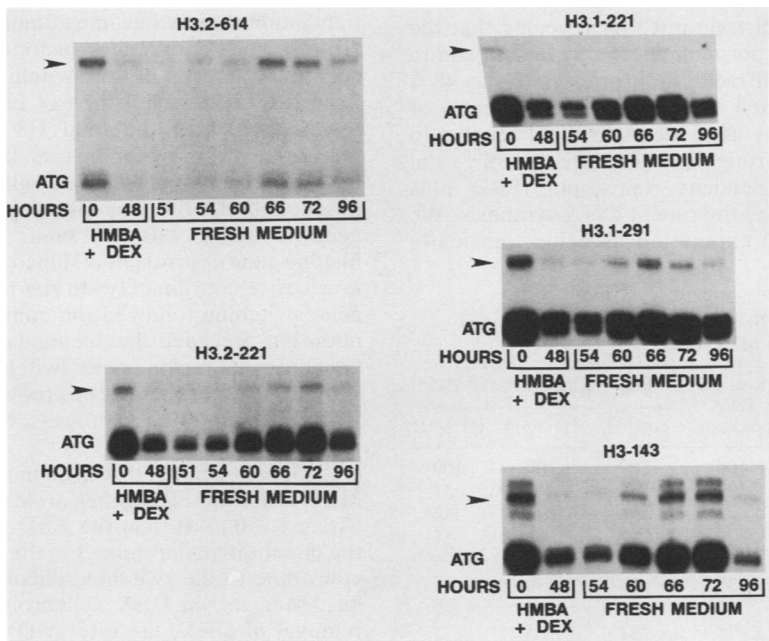

FIG. 8. Steady-state levels of H3 histone mRNAs in synchronized Sc9 cells released from induction of differentiation. The Sc9 cell line was treated for 48 h with 5 mM HMBA and 4 μM DEX. The culture was then split in half, washed, and suspended in fresh medium without inducer or DEX. Total RNA was isolated prior to and following resuspension. S1 assays were performed with the H3 probes labeled at the *Sal*I site. Values below the lanes indicate the time (in hours) from the initial addition of HMBA and DEX.

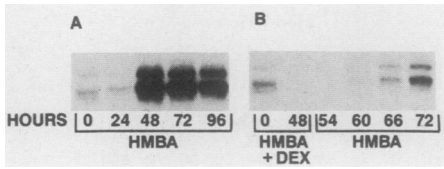

FIG. 9. β-Globin mRNA levels in Sc9 cells. (A) S1 nuclease protection assay of total mRNA isolated at the indicated times following induction of Sc9 cells with 5 mM HMBA. The probe was labeled at the *Bam*HI site within the coding region. (B) The Sc9 cell line was treated for 48 h with 5 mM HMBA and 4 μM DEX. The culture was then split in half, washed, and suspended in fresh medium containing 5 mM HMBA but lacking DEX. Total RNA was isolated prior to and following resuspension. S1 assays were performed as described in the text. Values below the lanes indicate the time (in hours) from the initial addition of HMBA and DEX.

DISCUSSION

During differentiation of a number of cell types, including MEL cells, the relative levels of histone protein variants change (16, 17, 36, 61, 65, 66). We previously demonstrated that for the core histones, this is mediated in part by alterations in the steady-state levels of individual histone gene transcripts (6). This appears to be accomplished by differences in the expression characteristics of individual gene clusters during the intermediate stages of differentiation. From these differences, we were able to classify a number of cloned histone genes into three distinct expression classes. However, it was unclear whether and how the differences among the expression classes were related to the differentiation process. Here we have investigated the differences, both before and during terminal differentiation, between the expression characteristics of the previously defined PRD and FRD classes of histone variants. Together these two classes make up the bulk of mouse core histones in most tissues (65).

A detailed kinetic analysis of the changes in the steady-state levels of individual histone mRNAs revealed that the differences between the expression classes were confined to a specific period of the differentiation process (24 to 48 h after induction) and resulted in alterations of the ratio of PRD to FRD transcripts by as much as fourfold relative to that in uninduced cells. Throughout differentiation, the total amount of replication-dependent transcripts (PRD plus FRD) very closely paralleled the rate of DNA synthesis. We previously described PRD expression as being transiently uncoupled from DNA synthesis at a particular stage of differentiation. This was based on the observation that the levels of these transcripts were at or near their preinduction levels despite a significant reduction in DNA synthesis. "Uncoupled" may be a misleading term. We have no evidence that the expression of PRD histone genes or any other replication-dependent histone gene occurs outside of the S phase. The results instead suggest that we are observing a histone gene switching in which one variant is expressed in preference to another, thereby altering the relative contribution of each to the total.

TABLE 1. Histone mRNA levels in DEX-synchronized Sc9 MEL cells

Conditions[a]	% Committed	Relative DNA synthesis[b]	Relative mRNA level[b] (%)		
			Total H3	H3.2-614	H3.2-221
Uninduced	<5	100	100	100	100
D-48	<5	47	56	45	51
H-48	78	47	51	98	39
H,D-48	<5	27	12	17	11
H,D-48; H-12	50	33	24	30	7
H,D-48; H-24	92	85	79	150	23
H,D-48; FM-12	<5	37	31	37	39
H,D-48; FM-24	<5	74	81	65	72

[a] D, Dexamethasone; H, hexamethylene bisacetamide; FM, fresh medium alone. Numbers are hours of treatment.

[b] Values are expressed as percentage of uninduced value (set at 100%). Each value is the average of at least three independent determinations.

We previously proposed several models to explain histone gene expression during MEL cell differentiation (6). In these models either the timing of the initiation of gene expression during the cell cycle or the degree of coupling to the mechanism responsible for linkage to DNA synthesis was different from PRD and FRD genes. Here we attempted in a number of ways to detect cell cycle-dependent differences between the expression classes. We found no differences in the response of PRD and FRD gene expression upon treatment of uninduced or induced MEL cells with inhibitors of DNA synthesis or protein synthesis or with a combination of both drugs. In addition, we were unable to detect any differential gene expression in growth-arrested, uninduced MEL cells or during the transition from the extended G1 phase of starved 3T3 cells as they recommenced growth after refeeding. Collectively these results indicate that the switching observed during MEL cell differentiation is not due simply to a different degree or timing of coupling to a replication-dependent regulatory mechanism, which becomes evident only when cell metabolism slows.

We were able to synchronize MEL cells at a particular stage of differentiation by treatment with HMBA and DEX. After 48 h of treatment, the rate of DNA synthesis and the levels of all replication-dependent histone mRNAs, both FRD and PRD, were low, but the cells were not committed to terminal cell division. If DEX was then removed and the cells were resuspended in the presence of HMBA, the population rapidly became committed, and as DNA synthesis resumed, a dramatic histone gene switching was observed. As a result of this switching, the levels of transcripts from the PRD gene H3.2-614 increased to account for as much as 80% of the total H3 mRNA population. Most importantly, this switching was dependent on treatment with inducer. When synchronized cells were resuspended in the absence of HMBA, commitment did not occur and histone gene switching was not seen. DNA synthesis and total histone gene expression resumed, but PRD and FRD mRNA levels rose coordinately. In the protocol used here, histone gene switching followed the commitment step of differentiation but preceded the accumulation of globin mRNA. We conclude that histone gene switching is not simply a circumstantial result of the loss of proliferative capacity associated with the differentiation process, but is, in fact, a component of differentiation.

It should be noted that in all mouse cells studied, including MEL cells, the PRD genes are expressed to a much greater extent (>10-fold) than the FRD genes. The possibility that the quantitative differences in the degree of expression could contribute to the switching phenomenon is unlikely. This is best seen in the DEX synchronization experiment. Upon removal of DEX, the rate of DNA synthesis and total H3 mRNA increased to the same level in the presence and absence of HMBA, yet only in the presence of the inducer did switching occur. If quantitative differences between the classes were responsible for switching, then a similar effect

should have been observed in the absence of inducer. Even if the switching phenomenon were partially due to differences in the overall expression level or promoter strength between PRD and FRD genes, the conclusion would be the same: a significant, differentiation-specific alteration in the pattern of gene expression within a family of histone subtype genes.

It is still not clear how histone gene switching is accomplished mechanistically. Cell cycle regulation of histone gene expression is both transcriptional and posttranscriptional (14, 20, 44). The mechanism which regulates histone mRNA stability appears to recognize (26, 28, 37) and act upon (39) the stem-loop structure at the 3′ terminus of the mRNA. These sequences are highly conserved in all replication-dependent histone transcripts (59) and the mRNA degradation systems probably do not discriminate between FRD and PRD transcripts. If histone gene switching is then mediated solely by preferential transcription of the PRD genes (or repression of the FRD genes), the relative levels of the variants could be modulated without loss of cell cycle regulation. The nondiscriminatory posttranscriptional mechanism would ensure that the total amount of mRNA for each subtype reflects the level of DNA synthesis. Although we have no direct evidence for differential transcription of the histone genes studied here, several recent reports suggest that sequences flanking histone genes in chickens (54, 60) and sea urchins (3) are capable of interacting with cellular factors to confer tissue-specific regulation. These sequences are outside the transcribed regions of the gene and presumably act at the transcriptional level. Transcription of human histone genes involves the interaction of multiple cellular transcription factors with distinct promoter-proximal sequence elements (8, 18, 48). While a subset of these interactions are clearly responsible for the induction of histone gene transcription during S phase (11), others may represent regulatory interactions specific to particular cell types. A cell type-specific human H1 gene has recently been described (24). This gene is expressed to high levels in KB cells but at low or undetectable levels in several other cultured cell lines. Interestingly, in KB cells this gene is cell cycle regulated and in this respect differs from other strictly tissue-specific histone genes and more closely resembles the genes in this study.

Mouse core histones are encoded by gene families present at 10 to 20 copies per haploid genome for each subtype (22, 30). Within each subtype, except for H4, there are multiple nonallelic primary sequence variants (12, 25, 65), and each variant is often encoded by two or more genes (15, 27, 45, 52, 59). It is unclear whether there is a functional role for histone heterogeneity or why there are multiple gene copies for each variant. It is also unclear whether there is any functional significance to the clustered arrangement of histone genes. If entire clusters are regulated coordinately, as appears to be true for the clones in this study, the expression characteristics of the cluster might be dictated by a single subtype within the cluster, presumably the subtype of greatest functional importance. The less-divergent subclasses, such as H3s and H4s, might exist within the clusters and be coordinately regulated in order to maintain a stoichiometry reflecting that of the nucleosome. An alternative explanation is that multiple clusters of histone genes with different expression characteristics have been maintained due to the need to express high levels of histones in a variety of cell types (24). Due to inherent differences in diverse cell types, with potentially different growth signals, different clusters of histone genes may exist to provide diverse control elements that can respond to different cell type-specific signals. Histone gene switching, then, may reflect the need for different control elements capable of responding to the different factors present in different cell types.

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