Journal of Cellular Biochemistry 51:479–487 (1993)

Differential Expression of the Chromosomal High Mobility Group Proteins 14 and 17 During the Onset of Differentiation in Mammalian Osteoblasts and Promyelocytic Leukemia Cells

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Abstract The expression of chromosomal proteins HMG 14 and HMG 17 during proliferation and differentiation into the osteoblast and monocyte phenotypes was studied. Cellular levels of HMG 14 and HMG 17 mRNA were assayed in primary cultures of calvarial-derived rat osteoblasts under conditions that (1) support complete expression of the mature osteocytic phenotype and development of a bone tissue-like organization; and (2) where development of osteocytic phenotypic properties are both delayed and reduced in extent of expression. HMG 14 and HMG 17 are preferentially expressed in proliferating osteoblasts and decline to basal levels post-proliferatively at the onset of extracellular matrix mineralization. In contrast, under conditions that are not conducive to extracellular matrix mineralization, HMG 14 is maximally expressed following the downregulation of proliferation. Consistent with previous reports by Bustin and co-workers [Crippa et al., 1990], HMG 14 and HMG 17 are expressed in proliferating HL-60 promyelocytic leukemia cells and downregulated post-proliferatively following phorbol ester-induced monocytic differentiation. However, differentiation into the monocyte phenotype is accompanied by reinitiation of HMG 17 gene expression. The results indicate that the levels of HMG 14 and HMG 17 mRNA are selectively down-regulated during differentiation.

Key words: HL-60, gene regulation, HMG14, HMG17, proliferation, osteoblasts, chromosomal proteins

The high-mobility group (HMG) proteins are among the most abundant chromosomal proteins present in a broad spectrum of eukaryotic organisms. The HMG 14 and 17 proteins have been postulated to play a role in modulating the structure of active chromatin [Einck and Bustin, 1985; Bustin et al., 1990; Johns, 1982]. Several lines of evidence suggest that these proteins may in part be responsible for rendering actively transcribed genes susceptible to nuclease digestion [Weintraub and Groudine, 1976; Weisbrod, 1982; Weisbrod and Weintraub, 1979; Swerdlow and Varshavsky, 1983; Stein and Townsend, 1983]. The HMG 14 and 17 proteins undergo post-translational modifications which may influence the manner in which interactions with nucleosomal components is mediated [Einck and Bustin, 1985; Allfrey, 1982; Spaulding et al., 1991].

Generally, the development of the differentiated phenotype is associated, initially with active cell proliferation, and followed by a complex and interdependent series of events necessitating modifications in gene expression, many of which are transcriptionally regulated. Expression of tissue-specific phenotypic properties is often accompanied by modifications in chromatin structure, of a magnitude which can be directly visualized by light microscopy. However, while sequence-specific interactions of transcription factors with modularly organized promoter regulatory elements that mediate alterations in gene expression associated with differentiation have been determined, our understanding of higher-order changes in chromatin organization

Received July 24, 1992; accepted November 18, 1992.

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that contribute to transcriptional control is minimal.

To address mechanisms by which chromatin structure associated with transcriptional regulation is modified during the onset and progression of differentiation, we have examined the expression of the HMG 14 and HMG 17 nonhistone chromosomal proteins during development of the osteoblast and monocyte phenotypes. Our results indicate that both HMG 14 and HMG 17 are actively expressed in proliferating osteoblasts and promyelocytic leukemia cells. Following the completion of proliferative activity, selective expression of the HMG proteins occurs during specific periods of the osteoblast and monocyte development.

MATERIALS AND METHODS

Rat Osteoblast Cell Growth and Differentiation

Osteoblasts were isolated from the calvaria of 21-day gestation fetal rats by trypsin/collagenase digestion as described [Aronow et al., 1990; Owen et al., 1990a]. Osteoblasts were plated in minimal essential medium (MEM, GIBCO) supplemented with 10% fetal calf serum (FCS) in 100-mm dishes at a density of 5×10^5 cells/dish. Cells maintained under conditions to support the complete development of the osteoblast phenotype were maintained on the following cell feeding schedules: On day 4 after plating, the cells were fed with MEM/FCS supplemented with ascorbic acid (25 μg/ml). Beginning 7 days after plating and on every second day throughout a 30-day differentiation time course, cells were fed with BGJb medium (GIBCO) supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate (Sigma, St. Louis, MO), and 10% FCS. For experiments where there was a delay in the onset and reduction in the extent of extracellular matrix mineralization, the cells were maintained in the absence of β-glycerol phosphate. These protocols within the context of osteoblast differentiation are schematically illustrated in Figure 1A.

HL-60 Cell Growth and Differentiation

HL-60 human acute promyelocytic leukemia cells were grown in suspension culture in RPMI 1640 Medium (GIBCO, Grand Island, NY) supplemented with 5% FCS (GIBCO) in T-25 flasks in a 37°C incubator with a humidified atmosphere containing 10% CO_2. Cells were maintained in logarithmic growth by serial subcultivation to 10^5 cells/ml at 3-day intervals. HL-60 cells (1×10^6 cells/ml) were induced to differentiate along the monocyte/macrophage lineage by the addition of 12-0-tetradecanoylphorbol 13-acetate (TPA) (Sigma) from a 100-μg/ml stock in acetone to a final concentration of 100 ng/ml. The down-regulation of proliferation following TPA addition was monitored by pulse labeling triplicate samples of 10^6 cells with 10 μCi of ^{3}H-thymidine (20 Ci/mmol, Amersham, Arlington Heights, IL) for 0.5 h. Incorporated ^{3}H-thymidine was measured as trichloroacetic acid precipitable radioactivity. The multiple differentiation pathways of pluripotent HL60 promyelocytic leukemia cells are diagrammed in Figure 1B.

Preparation and Analysis of RNA

Total cellular RNA was extracted, from 2.5×10^7 HL-60 cells at each time point, by lysis in guanidinium isothiocyanate followed by cesium chloride centrifugation [Chirgwin et al., 1979]. Cytoplasmic RNA was prepared from 5×10^7 rat osteoblasts, at each time point, by the LiCl/urea precipitation method after removal of the nuclei [Tushinski et al., 1977]. RNA (10–20 μg) was size-fractionated by electrophoresis in 1.2% (w/v) agarose–formaldehyde gels. The integrity and quantitation of RNA was confirmed by visualization of ethidium bromide staining prior to transfer to ZetaProbe membrane (BioRad, Richmond, CA) in 20× SSC.

Hybridization Conditions

Probes used for Northern blot analysis of cellular mRNA levels of specific gene transcripts included human histone H4 [Plumb et al., 1983; Sierra et al., 1982], rat alkaline phosphatase [Noda et al., 1987], rat osteocalcin [Lian et al., 1989], human HMG 14 [Landsman et al., 1986b], human HMG 17 [Landsman et al., 1986a], and rat osteopontin [Oldberg et al., 1986]. DNA probes were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol; New England Nuclear, Boston, MA) by the random primer technique [Feinberg and Vogelstein, 1983]. The blots were prehybridized in 50% formamide, 5× SSC, 5× Denhardt's solution, 50 mM phosphate buffer (pH 6.5), 1% w/v SDS, 250 μg/ml salmon sperm DNA at 43°C for 2 h. For hybridization, 10^6 cpm/ml of denatured DNA probe was added and incubated at 43°C for 18 h. Following hybridization, blots were washed three times in 2× SSC/0.1% SDS at room temperature for 15 min each and twice

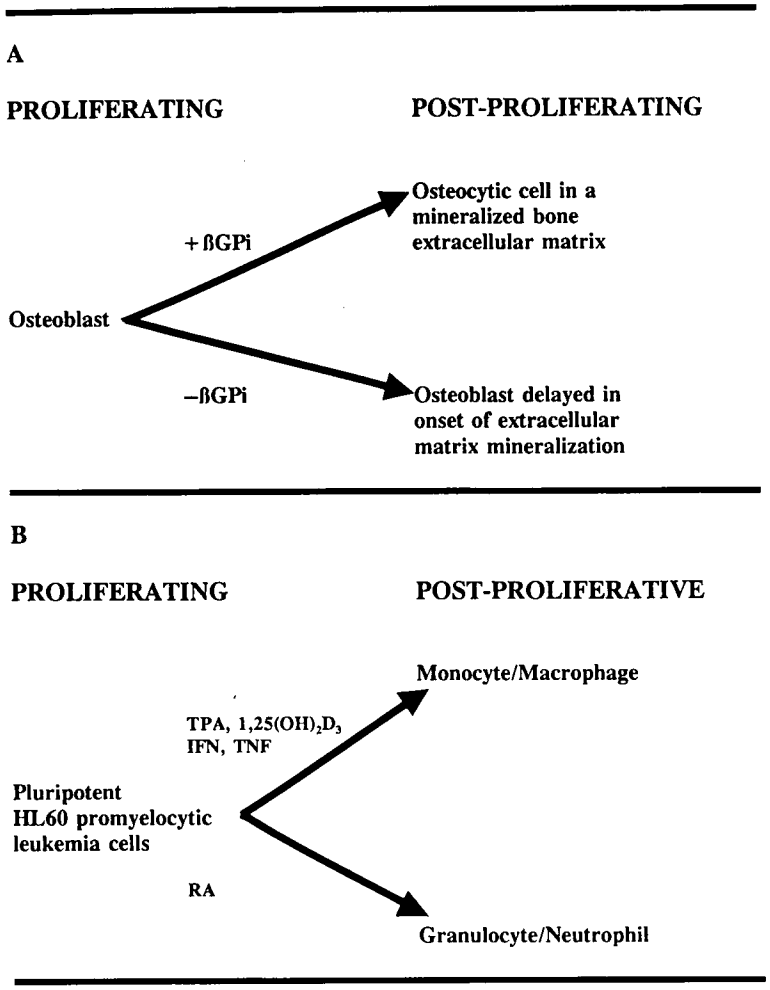

Fig. 1. Differentiation pathways of rat osteoblasts and HL60 promyelocytic leukemia cells. A: Influence of β-glycerol phosphate (βGPi) in culture media on promoting the time course and extent of development of the mature bone cell phenotype in primary cultures of normal diploid osteoblasts. B: Induction of promyelocytic leukemia cell differentiation to the monocyte macrophage lineage or the granulocyte neutrophil lineage by multiple factors. TPA, 12-0-tetradecanoylphorbol 13-acetate; $1,25(OH)_2D_3$, 1,25 dihydroxyvitamin D_3; IFN, interferon γ; TNF, tumor necrosis factor; RA, retinoic acid; βGPi, β-glycerol phosphate.

in 1× SSC/0.1% SDS at 65°C, for 30 min each. Blots were exposed to Kodak XAR film at −70°C and the resulting autoradiographs were quantitated with an LKB Ultrascan XL Laser Densitometer. Results were normalized to 28S ribosomal RNA to account for any variations in RNA quantitation. Each time point represents the average of three assays from at least two independent experiments.

RESULTS

HMG 14 and HMG 17 Gene Expression During Progressive Development of the Osteoblast Phenotype

The extent to which expression of the HMG 14 and HMG 17 genes is related to proliferation and post-proliferative development of the osteoblast phenotype was examined in primary cultures of calvarial-derived rat osteoblasts [Owen et al., 1990a]. These cells were maintained under two types of growth conditions. The first was in the presence of 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate, which supports the post-proliferative expression of genes that promote extracellular matrix mineralization. The second was in the absence of β-glycerol phosphate, which delays the onset and dramatically reduces the extent of extracellular matrix mineralization.

As previously reported, when cultured under conditions that support proliferation, extracellular matrix biosynthesis and extracellular matrix

mineralization, normal diploid osteoblasts undergo a three-stage developmental sequence over a 35-day period resulting in progressive development of nodules with a mineralized extracellular matrix and with a tissue-like organization similar to embryonic bone [Aronow et al., 1990; Owen et al., 1990a; Stein et al., 1990]. Figure 2A–C shows that primary osteoblast cultures, maintained in ascorbic acid and β-glycerol phosphate, proliferate for the initial 12 days in culture, during which times genes associated with cell growth control and extracellular matrix biosynthesis are actively expressed (Fig. 2A: histone, type I collagen, TGFβ, fibronectin). At a key transition point (days 10–12) early during osteoblast phenotype development, the down-regulation of proliferation is accompanied by and functionally related to initiation of gene expression associated with extracellular matrix maturation and organization (e.g., alkaline phosphatase). The third period of the osteoblast developmental sequence, initiated on approximately day 12 of primary culture, supports the ordered deposition of mineral within the orthogonally organized bundles of type I collagen fibrils associated with osteocytic cells in the bone-like nodules. At this time, genes expressed that are related to the osteoblast phenotype include osteopontin and osteocalcin.

Results presented in Figure 2B,C are from Northern blot analysis of total cellular RNA isolated from osteoblasts throughout the developmental-differentiation sequence. The data indicate that when cultured in the presence of β-glycerol, under conditions that support expression of the mature bone cell phenotype, both HMG 14 and HMG 17 are expressed in proliferating osteoblasts. Post-proliferatively, there is a rapid decline in cellular levels of HMG 14 and HMG 17 mRNA and at the onset of extracellular mineralization, these two classes of HMG gene transcripts are only detectable at basal levels. Under these conditions, the expression of HMG 14 and HMG 17 parallels that of histone H4.

We addressed whether expression of HMG 14 and HMG 17 during the initial period of the osteoblast developmental sequence is solely related to the proliferative process and/or to establishment of competency for post-proliferative gene expression necessary for structural and functional properties of fully differentiated osteocytic cells. As shown in Figure 2D, normal diploid osteoblasts cultured in the absence of ascorbic acid and β-glycerol phosphate undergo proliferation for the same period of time and express cell cycle and cell growth-related genes to the same extent as osteoblasts cultured in the presence of ascorbic acid and β-glycerol phosphate. This occurs despite the impairment in expression of post-proliferative mature osteoblast phenotypic properties. While HMG 17 expression, as reflected by mRNA levels, remains confined to the proliferative period, in the culture conditions that are not conducive to extracellular matrix mineralization there is a striking modification in the expression of HMG 14 (Fig. 2E,F). Here, only low levels of HMG 14 gene expression are observed during proliferation. Post-proliferatively, during the period of osteoblast differentiation, when genes associated with extracellular matrix maturation and organization are maximal, expression of HMG 14 levels are significantly elevated. It therefore appears that HMG 17 expression is associated with proliferation independent of the commitment to completion of osteoblast differentiation and may reflect a relationship with the proliferative process in a general biological context. By contrast, HMG 14 expression may be more dependent upon post-proliferative properties of cells undergoing differentiation.

HMG 14 and HMG 17 Gene Expression During Promyelocytic Leukemia Cell Differentiation

To further pursue the relationships between HMG expression and proliferation within the context of cells undergoing differentiation, we examined HMG 14 and HMG 17 mRNA levels in HL-60 promyelocytic leukemia cells following monocytic differentiation by treatment with phorbol ester [Huberman and Callahan, 1979; Rovera et al., 1979]. As shown in Figure 3, high levels of both HMG 14 and HMG 17 expression are transiently detected in proliferating HL-60 cells, followed by a decline to undetectable levels. HMG 14 remains down-regulated post-proliferatively but with expression of the monocytic phenotype there is a reinitiation of HMG 17 expression.

DISCUSSION

Our results indicate that two HMG genes are selectively expressed during progressive development of the osteoblast phenotype. Expression of HMG 14 and HMG 17 mRNA is at least in part dependent on culture conditions that are conducive to post-proliferative gene expression associated with extracellular matrix mineralization in mature osteocyte-like cells. Such developmental modifications in HMG expression is consistent

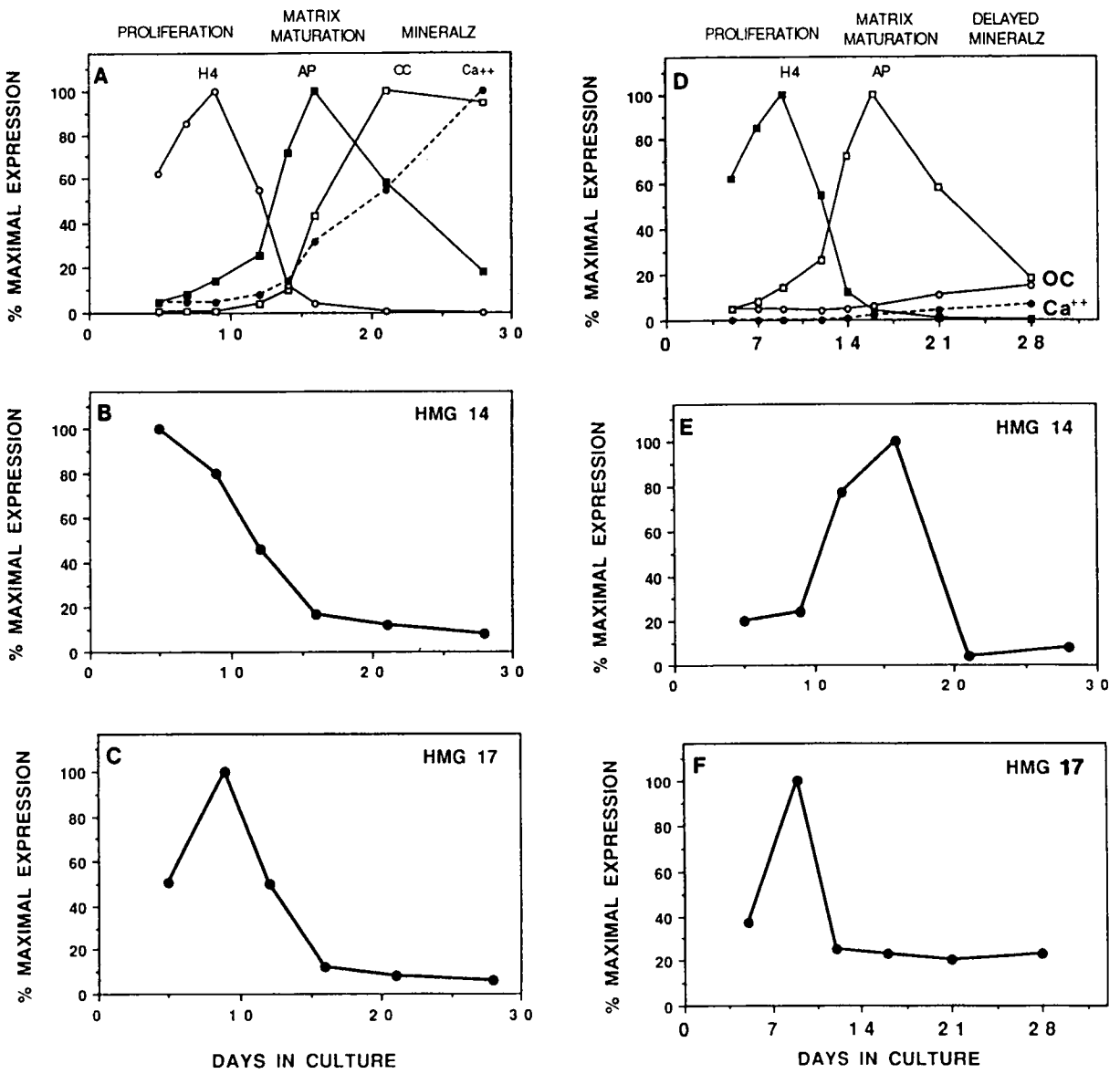

Fig. 2. Expression of HMG 14 and HMG 17 during osteoblast differentiation. Cells were maintained under growth conditions that support development of the mature osteoblast phenotype, an osteocytic cell in a mineralized extracellular matrix (A–C) or delayed and reduced levels of extracellular matrix mineralization (D–F). The upper panels, A, D: Cellular levels of H4 histone, type I collagen, alkaline phosphatase, osteopontin, and osteocalcin mRNAs, as well as calcium in the cell layers throughout the 35-day development of bone tissue-like organization in primary cultures of normal diploid osteoblasts. Cellular contents of HMG 14 and HMG 17 mRNAs during osteoblast phenotype development are shown in the middle and lower panels, respectively. All values are presented as percentage maximal levels. Note the three periods of osteoblast differentiation that are reflected by expression of cell growth and bone related genes. The proliferation period is reflected by H4 histone mRNA levels, and extracellular matrix biosynthesis occurs in proliferating osteoblasts as shown by type I collagen gene expression. The down-regulation of proliferation is accompanied by alkaline phosphatase gene expression as the maturation and organization of the bone extracellular matrix occurs. The period of extracellular matrix mineralization is reflected by expression of osteocalcin and osteopontin. At this time, accumulation of calcium is evident.

with involvement in transcriptional events that regulate bone cell differentiation.

There are multiple lines of evidence consistent with a role for HMG proteins in modulating the chromatin structure of active genes thereby suggesting a functional relationship between HMG expression and transcriptional regulation [reviewed in Einck and Bustin, 1985; Bustin et al., 1990].

Focusing on gene expression during osteoblast phenotype development, we observed that changes in HMG 14 and HMG 17 mRNA levels

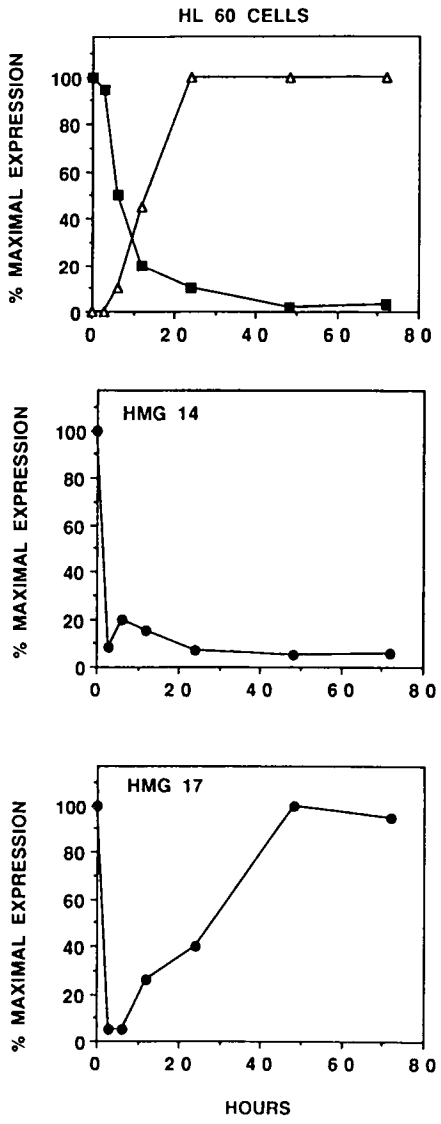

Fig. 3. Expression of HMG 14 and HMG 17 during HL-60 cell differentiation. The upper panel shows cellular levels of H2B histone mRNA reflecting the extent of proliferation following phorbol ester induction of monocytic differentiation (■). Differentiation of the cells following phorbol ester induction was monitored by reduction of nitro blue tetrazolium (△). Cellular levels of HMG 14 and HMG 17 mRNAs are shown in the middle and lower panels, respectively. All values are expressed as percentage maximal levels.

correlate with a reciprocal relationship between transcription of cell growth and tissue-specific genes [Owen et al., 1990a]. Histone gene transcription is down-regulated post-proliferatively, while osteocalcin gene transcription is induced [Owen et al., 1990b; Bortell et al., 1992]. These developmental modifications in transcription are associated with changes in chromatin structure and nucleosome organization in principal gene regulatory elements. For histone genes that are cell cycle regulated, changes in chromatin structure during the cell cycle are reflected by DNAse I hypersensitivity [Chrysogelos et al., 1985], S1 nuclease sensitivity [Chrysogelos et al., 1989] and restriction endonuclease accessibility [Moreno et al., 1986]. Cell cycle-dependent modifications in nucleosome organization and spacing have also been established [Bortell et al., 1992]. For the osteocalcin gene, changes in nucleosome hypersensitivity at primary basal and steroid hormone enhancer elements parallel the developmental expression and steroid hormone-responsive transcriptional upregulation [Montecino et al., 1992]. These modifications in chromatin structure are accompanied by alterations in nucleosome organization [Bortell et al., 1992].

Our observation of modifications in HMG expression during phorbol ester-induced monocytic differentiation are consistent with previous reports of proliferation and differentiation associated-changes in HMG 14 and HMG 17 mRNA levels during differentiation of HL-60 cells induced by dimethyl sulfate, retinoic acid, $1,25(OH)_2D_3$ and TPA [Crippa et al., 1990]. Here, variations in the extent to which HMG 14 and HMG 17 are expressed as the different phenotypes of these pluripotent promyelocytic leukemia cells develop may reflect subtleties in chromatin structure and organization requisite for transcriptional modifications that mediate unique requirements of the monocyte, macrophage, granulocyte, and neutrophil. The changes in the relative levels of HMG expression are indicated in HMG17/HMG14 mRNA ratios shown in Table 1 and Figure 4 for osteoblasts and HL60 cells. Changes in HMG17/14 mRNA ratios have previously been reported during both cell growth and following the onset of differentiation [Crippa et al., 1990, 1991].

The most significant change is the dramatic reinitiation of HMG 17 synthesis during differentiation of HL-60 cells into macrophages which was observed both here and in a completely independent previous set of experiments [Crippa et al., 1990]. The gradual increase in HMG 17 mRNA levels is related to cellular differentiation rather than proliferation, since the levels of histone mRNA did not change during this period. These results are consistent with previous studies which indicated that the down-regulation of HMG 14/HMG 17 mRNAs during myogenesis is related to differentiation, rather than to proliferation [Pash et al., 1990]. We wish to

TABLE I. HMG17/HMG14 Ratios in Differentiating Rat Osteoblasts and HL60 Promyelocytic Leukemia Cells*

	Rat osteoblasts		HL60 cells	
Days in culture	Extracellular matrix mineralization conditions	Delayed and reduced extracellular matrix mineralization conditions	Hours in culture +TPA	Macrophage phenotype development
0	—	0.4	—	—
1	0.52	—	1	1.05
4	—	1.8	4	0.71
8	1.25	4.16	8	0.23
12	1.00	0.37	12	2.16
16	0.86	0.26	24	5.00
21	0.40	5.00	48	20.00
28	0.50	3.50	72	9.50

*Ratios were determined by laser densitometry (in %) from Northern blots of cellular mRNAs hybridized with human HMG14 and HMG17 probes.

point out however, that the levels of HMG 14/HMG 17 mRNAs do fluctuate during the cell cycle [Bustin et al., 1987]. Thus the expression of these mRNAs may be controlled by both cell-cycle and differentiation-related events.

The functional consequences of the variations in the HMG 14/HMG 17 mRNA ratios are not presently understood. In humans, the gene coding for HMG 17 is located on chromosome #1, while that coding for HMG 14 is located on chromosome #21. Thus it is conceivable that the levels of HMG mRNA are related to an overall elevation in the transcriptional level of distinct transcriptional domains. Indeed, previous studies failed to detect significant fluctuations in the HMG 14 to HMG 17 protein ratios during development [Bustin et al., 1992]. On the other hand, we have recently noted that myoblast differentiation requires stringent regulation of HMG 14 expression [Pash et al., submitted]. Thus, it is possible that the variation in HMG 14:HMG 17 mRNA ratios reflect subtle temporary changes in protein levels. Although the nucleosomal binding regions of HMG 14 and HMG 17 differ [Bustin et al., 1990], it remains to be seen whether these differences have functional implications.

The specific contribution of HMG 17 and HMG 14 to structural organization of chromatin remains to be established. However, developmental modifications in HMG expression during osteoblast growth and differentiation, together with modifications in the expression of HMG proteins during muscle [Begum et al., 1990; Pash et al., 1990], avian red blood cell [Crippa et al., 1991], monocyte [Crippa et al., 1990; Boix,

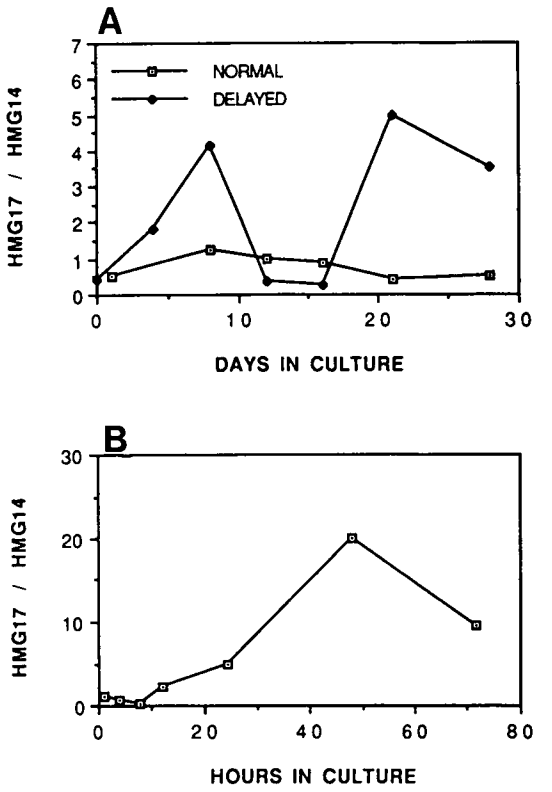

Fig. 4. HMG17/HMG14 ratios in differentiating rat osteoblasts and HL60 promyelocytic leukemia cells. Note the peak in cultured rat osteoblasts during the proliferative period, when maintained in culture medium promoting extracellular matrix mineralization. When the cells were maintained in the medium (absence of β-glycerol phosphate) not supporting mineralization of extracellular matrix, the HMG17/HMG14 ratios give two prominent peaks, one in the proliferative phase and the other in post-proliferative phase. In the HL60 cells, differentiating into macrophages in the presence of TPA, the HMG17/HMG14 ratios are maximal in the postproliferative period. **A**: Osteoblasts. **B**: HL-60 cells.

1991], and granulocyte [Crippa et al., 1990; Boix, 1991] differentiation, are consistent with broad-based biological relevance to the relationship of HMG proteins with developmental transcriptional control. These proteins, in association with histones, may serve to define competency for binding of sequence-specific transactivation factor complexes which contribute to (establish) activity of promoter elements. Further understanding of the manner in which HMG proteins modulate chromatin structure can additionally provide insight into mechanisms by which the three-dimensional organization of promoter sequences facilitate the interrelationships of independent positive and negative regulatory elements that determine the level of transcription in response to an interdependent series of physiological signaling mechanisms.

ACKNOWLEDGMENTS

These studies were supported by grants AR35166, AR33920, AR39588, GM32010 from the National Institutes of Health. The authors are most appreciative of editorial assistance from Ms. Patricia Jamieson in the preparation of this manuscript.

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