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Accelerated Publications

Downregulation of Cell Growth and Cell Cycle Regulated Genes during Chick Osteoblast Differentiation with the Reciprocal Expression of Histone Gene Variants†

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Received March 6, 1989; Revised Manuscript Received April 26, 1989

ABSTRACT: Expression of cell cycle (core and H1 histone) and cell growth (c-myc and c-fos) regulated genes was examined in primary cultures of chick calvarial osteoblasts during a developmental sequence associated with the progressive maturation of the osteoblast in a bonelike mineralized extracellular matrix. We have identified a transition point early in the developmental sequence which occurs when proliferation ceases and expression of genes related to the differentiated phenotype of osteoblasts is initiated. During this transition period, cellular levels of RNA transcripts from core and H1 histone genes and the c-myc and c-fos protooncogenes decrease in a parallel and coordinate manner. The decline in histone gene transcription that accompanies the loss of accumulated histone mRNA indicates that the downregulation of histone gene expression is at least, in part, transcriptionally mediated. In contrast, persistence of c-myc and c-fos transcription following completion of the proliferation period, when the mRNAs are no longer present at detectable levels, suggests that the initial downregulation of protooncogene expression is controlled at the level of messenger RNA stability. Thus, two types of signaling mechanisms are operative in the downregulation of cell proliferation genes during osteoblast differentiation—one that impinges on regulatory sequences that influence the interactions of transcription factors with cis-acting promoter elements and a second that modulates messenger RNA turnover. Of significance, downregulation of the cell cycle regulated histone genes is accompanied by a reciprocal increase in the expression of a structurally distinct subset of the histone genes that are not coupled with DNA replication during the period of expression of osteoblast phenotype markers. A similar increase in expression of these histone genes that encode high molecular weight, poly(A+) transcripts was also observed during the shutdown of proliferation and onset of differentiation in promyelocytic leukemia cells. Thus, expression of this subset of histone genes serves as a marker for the onset of differentiation. Involvement of these genes may relate to modifications of the structural and transcriptional properties of chromatin that occur at the initiation of tissue-specific gene expression.

Development of the osteoblast phenotype is associated with a complex and interdependent sequence of events reflected by modifications at the cellular, biochemical, and molecular levels. Recently, the development of osteoblast culture systems of normal diploid cells isolated from embryonic chick (Aubin et al., 1982; Gerstenfeld et al., 1987) and fetal rat (Bellows et al., 1986; Ecarot-Charrier et al., 1983) calvaria has provided an experimental system to address control of events related to the onset and progressive maturation and differentiation of proliferating osteoblasts into fully mature, nonproliferating osteocytic cells in a tissuelike mineralized matrix (Stein et al., 1988a,b). A temporal expression of proteins that are phenotypically unique to the osteoblast occurs in these cultured cells. The biological relevance of this in vitro developmental sequence leading to a bonelike matrix is supported by a similar developmental sequence during fetal formation of the calvarium in the intact animal (Yoon et al., 1987).

In situ, several osteoblast populations that include proliferating preosteoblasts and nondividing osteoblast cells at various developmental and maturational stages are identified by morphological criteria and tissue organization (Nijweide et al., 1986). We have identified in vitro (Stein et al., 1988b) a key transition point in the ordered developmental sequence associated with osteoblast differentiation that occurs early in the process when cell proliferation ceases and expression of genes associated with extracellular matrix maturation and mineralization is initiated. An understanding of molecular mechanisms operative in the downregulation of cell growth

† These studies were supported by grants from the National Institutes of Health (GM32010, GM32381, AR35166, AR39122, and HD22400), the National Science Foundation (DCB88-96116), and the March of Dimes Birth Defects Foundation (1-813).
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0006-2960/89/0428-5318$01.50/0

genes can provide insight into control of events associated with osteoblast differentiation. In this study we addressed the relationship between proliferation and the sequence of events associated with the progressive development of the bone cell phenotype.

Our studies were carried out with primary cultures of chick osteoblasts which over a 30-day culture period develop a mineralized extracellular matrix and a tissuelike organization characteristic of embryonic bone (Ecarot-Charrier et al., 1988; Bhargave et al., 1988; Aubin et al., 1982). During the first 12 days in culture, the cells actively proliferate and synthesize and assemble an extracellular matrix composed of type I collagen fibrils; no type III is present, characteristic of an osteoblast matrix (Gerstenfeld et al., 1987, 1988; Stein et al., 1988b). This is followed by a period of matrix maturation and specialization during which time cells become multilayered and greater than 80% of the cells in the culture are alkaline phosphatase positive. Proteins are synthesized that are functionally related to the ordered deposition and organization of hydroxyapatite within the extracellular matrix comprised of orthogonally organized bundles of collagen fibrils (Gerstenfeld et al., 1988). Two experimental approaches to defining expression of the osteoblast-differentiated phenotype in relation to proliferation are presented. First, we examined expression of cell cycle and cell growth genes during the osteoblast developmental sequence. Second, we addressed molecular mechanisms operative in the control of cell cycle and cell growth regulated genes during the proliferative period of osteoblast development and the subsequent downregulation of these genes that occurs with the onset of events involved with cellular specialization associated with the appearance of osteoblast markers, alkaline phosphatase, osteocalcin, phosphoprotein, and mineralization of the osteoblast's extracellular matrix.

Materials and Methods

Osteoblast Cell Cultures. Osteoblasts were isolated from calvaria of 16-day embryonic chicks by digestion of the tissue with a mixture of trypsin/collagenase [2.5 mg of trypsin (1:250 GIBCO Co.) and 2.0 mg of bacterial collagenase per milliliter] as described by Gernstenfeld et al. (1988). Cells were plated in MEM supplemented with 10% fetal bovine serum for 3 weeks, a period that enriches the growth and differentiation of cells that will express osteoblast phenotype properties. Cells were then subcultivated into BGJ_b media contain 10% fetal bovine serum, 15 mg/mL ascorbate, and 10 mM β-glycerol phosphate, which promotes expression of osteoblast markers. Greater than 95% of the cells were osteocalcin positive by immunofluorescence antibody staining on day 30 (Gernstenfeld et al., 1987), indicating that the entire culture had progressed to a stage where the cells are expressing this marker of the mature osteoblast phenotype (Lian et al., 1989). During primary and secondary culture, the cells were replenished with fresh media every 3 days.

RNA Isolation and Analysis. Total cellular RNA was isolated as previously described (Rowe et al., 1978), and where indicated, poly(A+) RNA was selected by oligo(dT)-cellulose chromatography (Maniatis et al., 1982). It should be noted that the oligo(dT) selection procedure enriches for poly(A+) RNA but does not totally exclude all poly(A-) RNA. Northern blot analysis was carried out following electrophoretic fractionation in a 1.5% agarose-6% formaldehyde gel followed by diffusion transfer to a Zeta probe nylon membrane with 20× SSC (1× SSC = 15 mM sodium citrate, 150 mM sodium chloride, pH 7.25). The membrane was prewashed in 0.5% SDS and 0.1× SSC for 1 h at 65 °C and then prehybridized for 2 h in 50 mM sodium phosphate (pH 6.5), 0.45% SDS, 42% formaldehyde, 9% dextran sulfate, and 500 mg/mL *Escherichia coli* DNA. After prehybridization, ^{32}P-labeled gene probes were added to a final concentration of 10^6 cpm/mL, and hybridization was carried out for 6-18 h at 48 °C. The membrane was then washed in a series of increasing stringency steps, the final which was 0.1% SDS and 0.1× SSC, 65 °C for 30 min, and was followed by autoradiography.

Transcription Analysis. Isolated nuclei were transcribed in the presence of ^{32}P-labeled UTP as reported (Greenberg & Ziff, 1984). Radiolabeled RNA transcripts were hybridized to slot blots of cloned H4 histone and ribosomal genes and to c-myc and c-fos cDNAs. Each filter containing the immobilized DNAs was hybridized with 6×10^6 cpm of nuclear RNA transcripts from 4.0×10^6 nuclei as determined by hemocytometry after nuclei isolation.

Cloned Gene Probes. Recombinant plasmids containing chicken histone genes that were used include pHU 4.8E (H1, H2A, and H2B) and pHU 2.6H (H3 and H4) (Sierra et al., 1982; Engel & Dodgson, 1981; Bruschi & Wells, 1981). Human histone gene clones that were used as hybridization probes include pFF 435B (H2A and H2B) (Plumb et al., 1983) and pFN C16A (H1) (Rickles et al., 1982).

Results and Discussion

Expression of Cell Cycle and Cell Growth Regulated Genes. Expression of histone genes is restricted to the S phase of the cell cycle and functionally coupled with DNA replication as reflected by a rapid and selective decrease in histone protein synthesis and a decline in histone mRNA levels when DNA synthesis is inhibited (Carozzi et al., 1984; Baumbach et al., 1984; DeLisle et al., 1983; Heintz et al., 1983; Lichtler et al., 1980). The tight coupling of histone gene expression with DNA replication is consistent with the involvement of these basic (enriched in arginine, lysine, and histidine residues) chromosomal polypeptides with the packaging of newly replicated DNA into chromatin.

Cellular levels of mRNAs transcribed from the cell cycle regulated core (H2A, H2B, H3, and H4) and H1 histone genes were determined throughout the osteoblast developmental/differentiation sequences. As shown in Figure 1, cellular levels of core and H1 histone mRNAs are elevated in a coordinate parallel manner during the initial 12 days in culture when the osteoblasts are actively proliferating. Maximal levels of histone mRNA accumulation and rates of DNA synthesis are observed between days 9 and 15. By day 20, when cell proliferation has declined to less than 10% of the level at day 12 (when proliferative activity is maximal), a similar decline in histone mRNA levels occurs. Also shown in Figure 1 are cellular levels of mRNAs transcribed from the c-myc and c-fos protooncogenes, which encode cell growth regulated nuclear proteins. c-myc and c-fos mRNA levels are elevated during the proliferative period and then decrease to below detectable levels by day 18.

To address the level(s) at which expression of histone genes and the c-myc and c-fos protooncogenes is regulated during the osteoblast developmental sequence, cellular mRNA content and rates of transcription were compared at two points during the proliferative period (days 4 and 8) and following the completion of proliferation (day 18). The rationale was that transcriptionally mediated control would be reflected by parallel modifications of mRNA levels and rates of transcription. Continued transcription in the absence of accumulated mRNAs would be indicative of control at the level of mRNA stability. mRNA levels were determined by slot blot analysis and transcription rates were assayed by nuclear

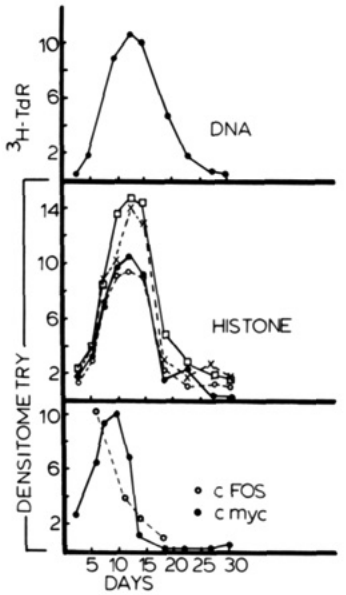

FIGURE 1: Comparison of osteoblast proliferation and cell cycle (core and H1 histone) and cell growth (c-myc and c-fos) regulated gene expression in chick calvarial osteoblast cultures. The rate of DNA synthesis was determined by pulse labeling (60 min) with [^{3}H]thymidine followed by determination of TCA-precipitable radioactivity. Cellular levels of mRNA for histone [H2A and H2B (●), H3 (×), H4 (□), and H1 (○)] and cellular protooncogenes [c-myc (●) and c-fos (○)] were determined by Northern blot analysis using radiolabeled (^{32}P) gene-specific hybridization probes. Autoradiograms were quantitated by densitometry.

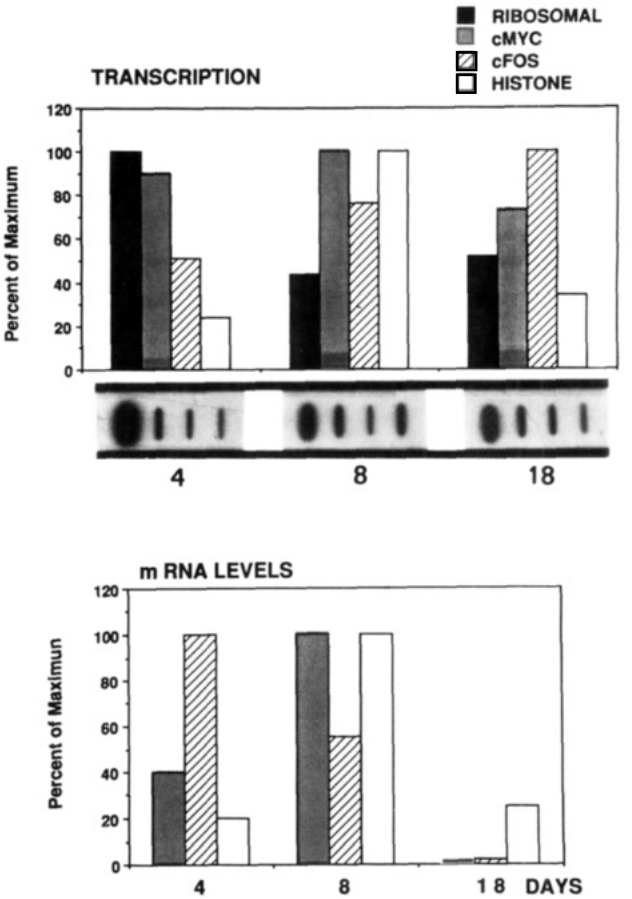

FIGURE 2: Expression of histone genes and protooncogenes during the developmental sequence associated with chick osteoblast differentiation. Rates of transcription were determined by nuclear runoff assays. Nuclei isolated at days 4, 8, and 18 of the culture period were transcribed in vitro in the presence of [^{32}P]UTP, and the radiolabeled RNAs were hybridized to nylon filter immobilized, cloned DNAs encoding H4 histone, c-myc, c-fos, and 18S ribosomal RNA. Autoradiograms are shown below the data from densitometry, which are plotted as percent of maximum level. For comparison, steady-state cellular levels of the H4 histone and the c-myc and c-fos protooncogene mRNAs are expressed as a percent of maximal levels in the lower panel. Total cellular RNA was isolated at days 4, 8, and 18 of the culture period, fractionated electrophoretically, and following transfer to a nylon membrane hybridized with ^{32}P-labeled probes for H4 histone, c-myc, and c-fos.

runoff transcription with the nuclear transcripts hybridized to cloned histone, c-myc, or c-fos DNA probes. As shown in Figure 2, changes observed in the rates of histone gene transcription parallel cellular mRNA levels, indicating that the downregulation of expression during the onset of osteoblast differentiation is transcriptionally mediated. In contrast, c-myc and c-fos transcription remains at elevated levels throughout the first 18 days of the in vitro osteoblast developmental sequence, while mRNA levels decline following the completion of proliferation, indicating that the downregulation is initially mediated by mRNA destabilization.

Taken together, these results are consistent with multiple mechanisms operative in downregulation of cell cycle and cell growth regulated genes at the transition point in the osteoblast developmental sequence when proliferative activity is completed and the extracellular matrix begins to undergo modifications related to mineralization. In the case of the histone genes where there is a stringent requirement for histone proteins to package newly replicated DNA into chromatin, the downregulation of expression at the transcriptional level appears to reflect a commitment of the osteoblast to the nonproliferative state. This shutdown of proliferation appears to signal increased expression of osteoblast markers [alkaline phosphatase, osteocalcin (Gernstenfeld et al., 1987)] that promotes further maturation of an extracellular matrix which supports the ordered deposition and organization of mineral. A similar developmental sequence of proliferation followed by expression of osteoblast phenotype markers occurs in cultured calvarial rat osteoblast (Stein et al., 1988b; Aronow et al., 1989). Since type I collagen genes are actively expressed during the proliferation period (Gernstenfeld et al., 1988; Stein et al., 1988b), the regulation of cell cycle and cell growth genes may be functionally related to the production and deposition and possibly the initial steps in the organization of the bone cell extracellular matrix.

Transcriptionally mediated downregulation of histone gene expression as a marker for bone cell differentiation is supported by histone mRNA destabilization as the predominant downregulation mechanism. This does not occur in growth-arrested osteoblasts which remain poised to reinitiate proliferation, but rather, histone gene transcription continues. A transcriptionally mediated shutdown in the expression of cell cycle regulated histone genes at the onset of osteoblast differentiation as shown here and during monocyte differentiation (Stein et al., 1988a) is further supported by the selective loss of occupancy of a rate-limiting proximal transcription regulatory element designated site II by a promoter binding factor, HiNF-D: site II–HiNF-D interactions persist in cells retaining the potential to reinitiate proliferation.

Expression of Non Cell Growth Regulated Histone Genes. Histone genes are represented as a multigene family consisting of multiple copies of the core and H1 histone coding sequences, with each gene an independent transcription unit and each unique with respect to both the mRNA coding and the mRNA flanking regulatory sequences, despite a high level of amino acid sequence conservation of the histone polypeptides (Lichtler et al., 1980, 1982). While greater than 90% of the human and

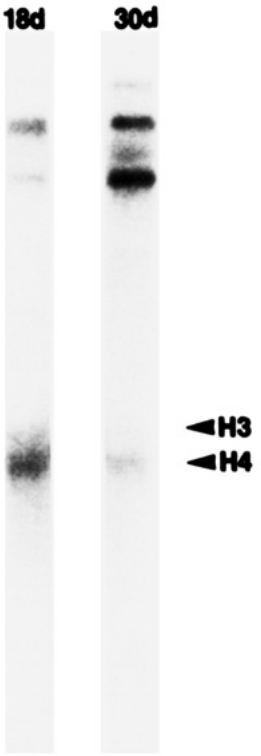

FIGURE 3: Northern blot analysis of RNA isolated from chick osteoblasts at days 18 and 30. RNAs were fractionated electrophoretically in 1.5% agarose–6% formaldehyde gels, transferred to nitrocellulose, and hybridized with a segment of cloned chicken genomic DNA containing an H3 and H4 histone mRNA coding sequence (PHU 2.6). The bands designated H4 and H3 are 425 and 500 nucleotides, respectively, representing transcripts from cell cycle regulated histone genes. The higher molecular weight bands are histone gene transcripts that are present at increased levels during the decline in proliferative activity of the cultures. Equal amounts of RNA from the 18- and 30-day cultures were analyzed; thus, the signals reflect the relative representation of cell cycle (lower molecular weight bands) and non cell growth regulated (upper bands) histone gene transcripts. The diffuseness of the lower molecular weight H3 and H4 bands represents a series of RNA transcripts from the multigene family of the cell cycle regulated histone genes.

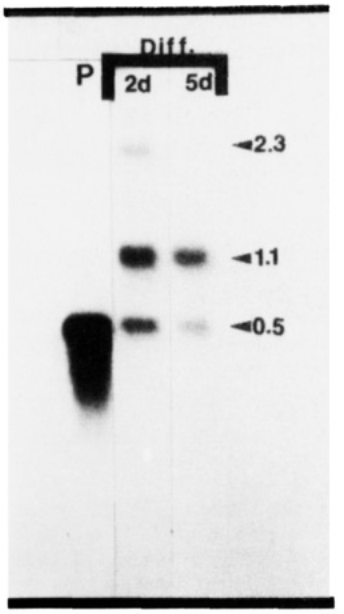

FIGURE 4: Northern blot analysis of poly(A+) RNAs from HL60 promyelocytic leukemia cells following phorbol ester induced differentiation into monocytes. Poly(A+) RNA from actively proliferating HL60 cells (P, the untreated control) and from cells at 2 and 5 days following treatment with TPA (12-*O*-tetradecanoylphorbol 13-acetate) were fractionated electrophoretically in 1.5% agarose–6% formaldehyde gels. Following transfer to a nylon membrane, the blots were hybridized with a 340-nucleotide, ^{32}P-labeled probe from the cloned protein coding region of a DNA replication-independent H2B histone gene (Collart et al., 1989). The two high molecular weight histone H2B mRNA species that are present (2320 and 1100 nucleotides) are detected at significant levels only following TPA-induced differentiation. The 500-nucleotide band is a poly(A−) H2B histone mRNA that remains with the poly(A+) mRNA during oligo(dT)–cellulose fractionation and is detectable due to conservation in the nucleotide sequences encoding the cell cycle regulated and non cell growth dependent H2B histone proteins. When this Northern blot analysis was carried out with a ^{32}P-labeled probe from the 3′ nontranslated region of the replication-independent H2B histone gene, only the 2320-nucleotide histone H2B species is detected. Therefore, it appears that the various high molecular weight H2B histone mRNAs originate from at least two distinct H2B histone genes. It should be noted that when these RNA preparations are fractionated in a higher resolution gel, the 1100-nucleotide band can be resolved into two distinct species.

murine histone genes are expressed coordinately and tightly coupled with DNA replication (Rickles et al., 1982; Baumbach et al., 1984; DeLisle et al., 1983; Heintz et al., 1983; Lichtler et al., 1980, 1982; Lau & Nathans 1985), other histone coding sequences are nontranscribed pseudogenes (Marashi et al., 1984) and genes encoding histone polypeptides which have been reported to be expressed independent of proliferation (Wells & Kedes, 1985; Bird et al., 1985a,b; Wu & Bonner, 1981). Structurally, the cell cycle regulated histone genes encode 400–700-bp mRNAs lacking introns with nontranslated leader and trailer sequences generally less than 50 base pairs. In contrast, most of the non cell cycle regulated histone mRNAs that have thus far been examined are poly(A+), with an extensive trailer, and some have introns as well as extensive leader sequences. Most of these non cell cycle histones mRNAs are greater than 1000 base pairs (Ernest et al., 1987; Engel et al., 1982; Wells & Kedes, 1985; Hatch & Bonner, 1988; Bird et al., 1985a,b; Collart et al., 1989). To date, expression of these non cell cycle regulated histone genes has been observed at similar levels in both proliferating and nonproliferating cells with the exception of $H1^0$, which increases in the absence of proliferation (Marsh & Fitzgerald, 1973).

When total cellular RNAs from osteoblasts, at the end of the proliferative period (day 18) and later in the developmental sequence when proliferation is no longer occurring and mineralization is ongoing (day 30), were assayed by Northern blot analysis, a reciprocal relationship was observed between the representation of transcripts from the cell cycle and the appearance of non cell cycle regulated histone genes (Figure 3). As expected, the levels of cell cycle regulated H4 and H3 histone gene transcripts reflected proliferative activity and on day 30 decreased to less than 10% of the levels seen at day 12 (Figures 1 and 3). But, as proliferation declined, there was a progressive increase in transcripts from the non cell cycle regulated histone genes with a 10-fold increase at day 30 compared to day 18. A similar reciprocal relationship between expression of the cell cycle regulated and non cell growth regulated histone genes was observed during phorbol ester induced differentiation of promyelocytic leukemia HL60 cells into monocytes where H2B histone gene expression was examined (Figure 4). Thus, the enhanced expression of this histone gene subclass at the onset of differentiation appears to be correlated with the shutdown of proliferation and development of tissue-specific structure and function in general and may serve as a marker for the onset of differentiation.

For those histone proteins that are synthesized independent on ongoing DNA synthesis, there are only subtle differences in the amino acid sequences of proteins (Wu & Bonner, 1982).

Thus, it appears that the properties of the proteins do not directly mediate chromatin-associated modifications that accompany differentiation. However, differences in the sequences that regulate transcription of these genes and differences in the mRNAs that influence stability may be rate-limiting determinants for availability of the non cell cycle regulating histone proteins to support the transition point in the osteoblast developmental sequence when proliferation is completed and expression of bone cell phenotype markers is initiated. Within this context, the possibility can be considered that the transient expression of core histone genes with the onset of differentiation may be related to a requirement for reorganization of chromatin structure and nucleosome organization in the absence of DNA synthesis to support changes in gene expression associated with the development of cell- or tissue-specific function (differentiation), i.e., in the case of osteoblasts matrix maturation and mineralization and with HL60 cells specialization related to phagocytic activity.

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