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Expression of Cell Growth and Bone Phenotypic Genes During the Cell Cycle of Normal Diploid Osteoblasts and Osteosarcoma Cells

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Abstract Establishing regulatory mechanisms that mediate proliferation of osteoblasts while restricting expression of genes associated with mature bone cell phenotypic properties to post-proliferative cells is fundamental to understanding skeletal development. To gain insight into relationships between growth control and the developmental expression of genes during osteoblast differentiation, we have examined expression of three classes of genes during the cell cycle of normal diploid rat calvarial-derived osteoblasts and rat osteosarcoma cells (ROS 17/2.8): cell cycle and growth-related genes (e.g., histone), genes that encode major structural proteins (e.g., actin and vimentin), and genes related to the biosynthesis, organization, and mineralization of the bone extracellular matrix (e.g., alkaline phosphatase, collagen I, osteocalcin, and osteopontin). In normal diploid osteoblasts as well as in osteosarcoma cells we found that histone genes, required for cell progression, are selectively expressed during S phase. All other genes studied were constitutively expressed both at the transcriptional and posttranscriptional levels. Alkaline phosphatase, an integral membrane protein in both osteoblasts and osteosarcoma cells, exhibited only minimal changes in activity during the osteoblast and osteosarcoma cell cycles. Our findings clearly indicate that despite the loss of normal proliferation-differentiation interrelationships in osteosarcoma cells, cell cycle regulation or constitutive expression of growth and phenotypic genes is maintained.

Key words: osteoblasts, osteosarcoma, osteocalcin, cell cycle, alkaline phosphatase

Fundamental to understanding skeletal development is establishing the regulatory mechanisms that mediate proliferation of osteoblasts while restricting expression of genes associated with mature bone cell phenotypic properties to post-proliferative cells. Previous studies from our laboratory have shown that calvarial derived normal diploid fetal rat osteoblasts progress through a three stage developmental sequence of gene expression that is functionally related to the establishment of bone tissue-like organization in culture. In each developmental stage a distinct set of genes is principally expressed. The first developmental stage involves active proliferation during which time expression of genes associated with cell cycle and cell growth control (e.g., histone) as well as genes associated with extracellular matrix biosynthesis (e.g., collagen I) dominate. These genes are down regulated as the cells progress into a post-proliferative period of extracellular matrix maturation. During this second developmental stage alkaline phosphatase expression is elevated and the extracellular matrix undergoes modifications in composition and organization which render it competent for the ordered deposition of mineral. In the final developmental period, alkaline phosphatase is down regulated and osteocalcin is expressed at maximal levels as extracellular matrix mineralization occurs [reviewed in Stein et al., 1990a,b; Owen et al., 1990; Pockwinse et al., 1992; Lian and Stein, 1992; Stein and Lian, 1993].

It is apparent that a physiologically responsive series of developmental regulatory signaling mechanisms are operative to modulate the interrelationships between expression of genes characteristic of each osteoblast developmental period. In striking contrast, loss of growth control in the rat osteosarcoma tumor cell line, ROS 17/2.8, is reflected by abrogation of developmental interrelationships. Expression of both cell growth and bone phenotypic genes occur concomitantly rather than sequentially. Addition-

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ally, despite expression of genes associated with development and maintenance of the bone extracellular matrix, a bone tissue-like organization does not develop in culture [reviewed in Stein et al., 1990a,b; Owen et al., 1990; Lian and Stein, 1992; Stein and Lian, 1993; Rodan and Noda, 1991].

To gain further insight into relationships between growth control and the developmental expression of genes during osteoblast differentiation, we have experimentally addressed expression of three classes of genes during the cell cycle of normal diploid osteoblasts and osteosarcoma cells: 1) growth-related genes, those associated with competency for proliferation and those expressed in a cell cycle dependent manner which facilitates progression through the cell cycle; 2) genes that encode proteins which support major structural properties of cells; and 3) genes related to the biosynthesis, organization, and mineralization of the bone extracellular matrix. Comparisons were made between control at the transcriptional and post-transcriptional levels. Our results demonstrate that bone phenotype related genes are constitutively expressed during the cell cycle. In both normal diploid osteoblasts and osteosarcoma cells transcriptional as well as post-transcriptional control appear to be operative.

MATERIALS AND METHODS

Cell Isolation and Culture

To obtain normal osteoblasts, calvariae from fetal rats of 21 days gestation were isolated and subjected to sequential digestions of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase A (Boehringer-Mannheim, Indianapolis, IN) with 0.25% trypsin (Gibco, Grand Island, NY). The cells of the first two digests were discarded, and those released from the third digestion were plated in minimal essential medium (MEM; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum. After 5 days in culture osteoblasts were trypsinized and replated at a density of 2.6×10^5 cells per 100 mm plate. ROS 17/2.8 osteosarcoma cells were provided to us by Dr. Sevgi and Dr. Gideon Rodan (Merck Sharp and Dohm, West Point, PA). These cells were cultured in DMEM (Gibco, Grand Island, NY) supplemented with calcium and 5% fetal calf serum as described by Majeska et al. [1989].

Cell Synchronization

A thymidine/aphidicolin double block was used to synchronize both normal diploid osteoblasts and osteosarcoma cells at the G1/S boundary [Stein et al., 1994; Fedarko et al., 1990]. Briefly, to actively proliferating cells, 24–36 h after plating, thymidine was added to a final concentration of 2 mM. After 16–18 h cells were released from the first block by washing the monolayers once with serum free medium prior to addition of normal growth medium containing 24 μM deoxycytidine. Following a 9 h release period, a second block was performed in 5 μg/ml aphidicolin (obtained from the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute). After 16 h the blocking medium was aspirated, cells were washed, and fresh medium was added to each dish. This point is designated the 0 time point in all experiments.

Cell synchronies were monitored by examining several parameters. First, cell counts were performed during each experiment to assure a doubling of the cells. Second, ^{3}H-thymidine (Dupont, NEN, Wilmington, DE) incorporation was determined every hour during the time course. ^{3}H-thymidine was added to the culture medium at a final concentration of 5 μCi/ml and the cells were incubated at 37°C for 30 min. DNA was precipitated with 5% TCA and cells were suspended in 10% SDS. Radioactivity was then determined by liquid scintillation counting. Third, measurements of histone mRNA levels, an S-phase marker, were obtained for each experiment.

RNA Isolation, Northern Blot Hybridization, and Analysis

To isolate total cellular RNA cells were scraped, centrifuged at 800*g* for 5 min at 4°C, and quick frozen in liquid nitrogen. For each experiment samples obtained at all time points were thawed and processed together. RNA was extracted according to Chomczynski and Sacchi [1987], gel electrophoresed, and transferred to Zetaprobe membrane (Bio-Rad Laboratories, Hercules, CA) for northern blot analyses. Blots were hybridized with random primed (Prime It kit; Stratagene, La Jolla, CA) ^{32}P-labeled (Dupont, NEN) cDNA probes to H4 histone, H2B histone, collagen I, alkaline phosphatase, osteopontin, osteocalcin, actin, vimentin, and 18S ribosomal subunit, washed, and exposed to film

for varying lengths of time. Signals were quantitated by laser densitometry and expressed relative to levels of 18S ribosomal subunit.

Protein Isolation, Alkaline Phosphatase Assay

Cells were harvested for alkaline phosphatase assay by scraping in culture media (to prevent loss of mitotic cells which may be loosened if cells were rinsed in the culture dish), centrifuging, and rinsing twice with 0.01 M Tris-saline. After centrifugation at 800*g* for 5 min, the cell pellet was resuspended in sterile water and frozen at −70°C. Upon thawing cells were treated with 0.1% triton X-100, vortexed and put on ice for 2 h. Alkaline phosphatase activity was determined by the amount of *p*-nitrophenol phosphate conversion to *p*-nitrophenol during a 30 min incubation (Alkaline phosphatase colorimetric kit, Sigma, St. Louis, MO). Protein concentrations were determined by the BCA* Protein Assay (Pierce, Rockford, IL).

Nuclear Run on Analysis

Nuclei were isolated at indicated time points by homogenizing (dounce) in solution A (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40), frozen in liquid nitrogen, and stored at −70°C. Transcription reactions were performed in the presence of ^{32}P-UTP (Dupont NEN) according to Greenberg and Bender [1991]. Radiolabeled RNA, isolated using phenol: chloroform extractions [Greenberg and Bender, 1991], was hybridized to blots containing H4 histone, H2B histone, collagen I, AP, OP, OC, actin, vimentin, and 18S ribosomal subunit cDNAs. Blots were washed to a final stringency of 0.2× SSC/0.1× SDS at 55°C and exposed for autoradiography. Signals were quantitated by laser densitometry and expressed relative to the intensity of binding to 18S ribosomal DNA.

RESULTS

Osteoblasts and Osteosarcoma Cells Are Effectively Synchronized by Thymidine/Aphidicolin Blockade

Normal calvarial-derived rat osteoblasts (ROB cells) and transformed rat osteosarcoma (ROS) cells were synchronized at the G1/S phase boundary by first blocking exponentially growing cells with thymidine, releasing cells for 9 h, and then blocking with aphidicolin (see Materials and Methods). Cell synchrony was verified by measuring DNA synthesis every hour after the cells were released from the aphidicolin block. In both ROS and ROB cells levels of ^{3}H-thymidine incorporation peaked 4 h after release from the block (Fig. 1) indicating that the cells were synchronously entering S phase. Cell counts and mitotic index demonstrate that by 12 h cells have doubled (data not shown).

RNA Levels of Developmentally Regulated and Structural Genes Are Not Cell Cycle Regulated in Normal Osteoblasts

Northern blot analyses were performed on RNA isolated from synchronized normal rat osteoblasts at 0, 2, 4, 6, 9, and 12 h following release from the final aphidicolin block to determine if cell cycle progression affects expression

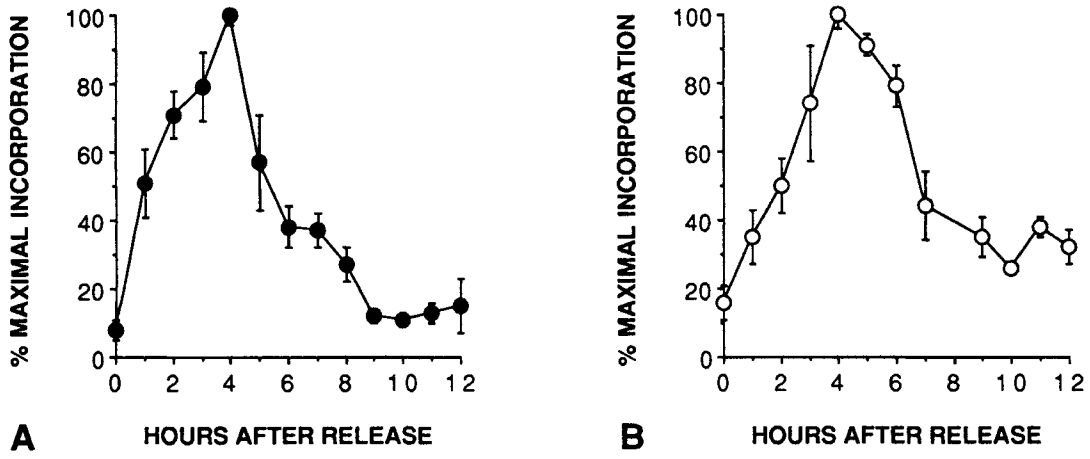

Fig. 1. DNA synthesis in synchronized normal rat osteoblasts (**A**) and osteosarcoma (**B**) cells. Cells were pulse labeled with [^{3}H]-thymidine for 30 min prior to scintillation counting of TCA precipitates. Values are expressed as a percentage of maximum radiolabel incorporation.

of cell cycle (histone), developmentally regulated [alkaline phosphatase (AP), osteopontin (OP), osteocalcin (OC), and collagen I], and/or structural (vimentin and actin) genes. Four hours after the final release histone H4 and histone H2B mRNA levels increased 5-fold (Fig. 2). As expected, this increase occurs during DNA synthesis (Fig. 1). In contrast to histone mRNA levels, no significant change in the steady state mRNA levels of any of the other genes studied could be seen during S, G2, and mitotic phases of the cell cycle (Fig. 3). Expression of bone-specific genes such as alkaline phosphatase was low. In fact, osteocalcin mRNA, a marker of osteoblast differentiation, was not detectable.

Alkaline Phosphatase Activity Is Not Cell Cycle Regulated in Normal Rat Osteoblasts

Because cell cycle progression could affect gene expression at the translational or post-translational levels, we measured the activity of alkaline phosphatase, a general integral membrane enzyme, during the cell cycle. Levels of total cellular AP activity were determined at 0, 2, 4, 6, 9, and 12 h after releasing the osteoblasts from the final aphidicolin block. The results, shown in Figure 4, demonstrate that levels of alkaline phosphatase activity are low in proliferating cells and are relatively constant during the cell cycle of normal rat osteoblasts. Three separate experiments confirmed this finding.

Cell Cycle Changes in Transcription of Developmentally Regulated Genes Are Minimal or Absent

To directly determine if the observed constitutive expression of genes examined is a result of transcriptional control, run on analyses were performed using nuclei isolated from normal diploid osteoblasts at 0, 2, 4, 6, and 9 h following release from the aphidicolin block. Equal amounts of incorporated radioactivity were hybridized to blots containing cDNAs to H4, H3, H2B, OC, AP, collagen I (Col I), vimentin (Vim), actin, and OP. Representative blots from a nuclear run on assay are shown in Figure 5D. Results from three separate experiments expressed relative to signal intensity of ribosomal RNA were averaged and graphed in Figure 5A–C. Histone transcription increased (1.5–2-fold) at 2 h (Fig. 5A), just prior to the peak in histone mRNA levels (Fig. 2). This level of increase has been previously reported by our laboratory and others [Baumbach et al., 1987, 1984; Holthuis et al., 1990; Plumb et al., 1983a,b; Osley, 1991] for the histone genes. Although signals were low, a gradual increase in AP, OC, and OP transcription was detected during the time course. A small increase was also seen in actin and collagen I transcription which peaked 2 h after release of the cells from the aphidicolin block. In contrast, vimentin transcription appeared constitutive throughout the cell cycle.

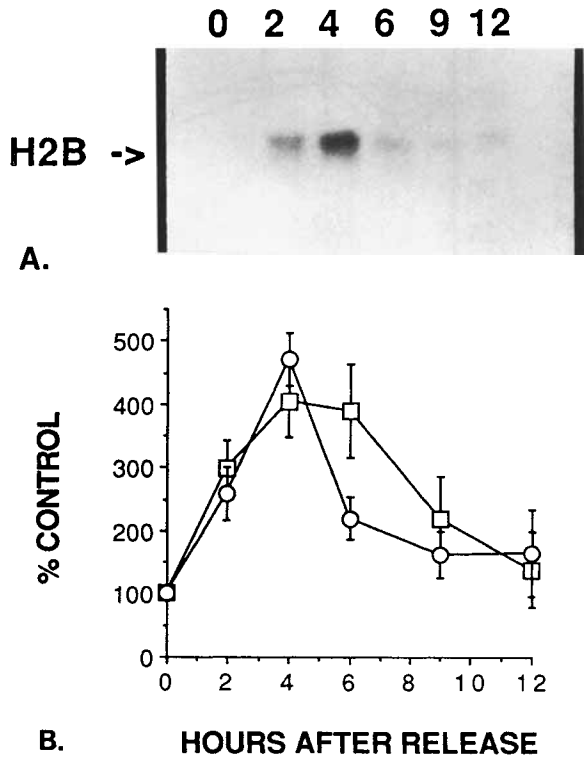

Fig. 2. Histone mRNA levels in synchronized normal diploid osteoblasts. **A:** Representative Northern blot hybridized to histone H2B cDNA. **B:** Histone H4 (open square) and histone H2B (open circle) mRNA levels following release from aphidicolin block. Levels normalized to 18S ribosomal RNA abundance and expressed relative to 0 h values. Values expressed ± SE, n = 3–4.

Abrogated Growth Control in Osteosarcoma Cells Does Not Affect Cell Cycle Expression of Developmental and Structural Genes at the Post-Transcriptional Level

ROS osteosarcoma cells are known to override the developmental sequence of gene expression; that is, they can express genes during proliferation that are normally expressed only postproliferatively in association with differentiation. To determine if cells with abrogated growth control also exhibit aberrant expression of these genes during the cell cycle, northern blot analyses were performed on RNA isolated at various time-

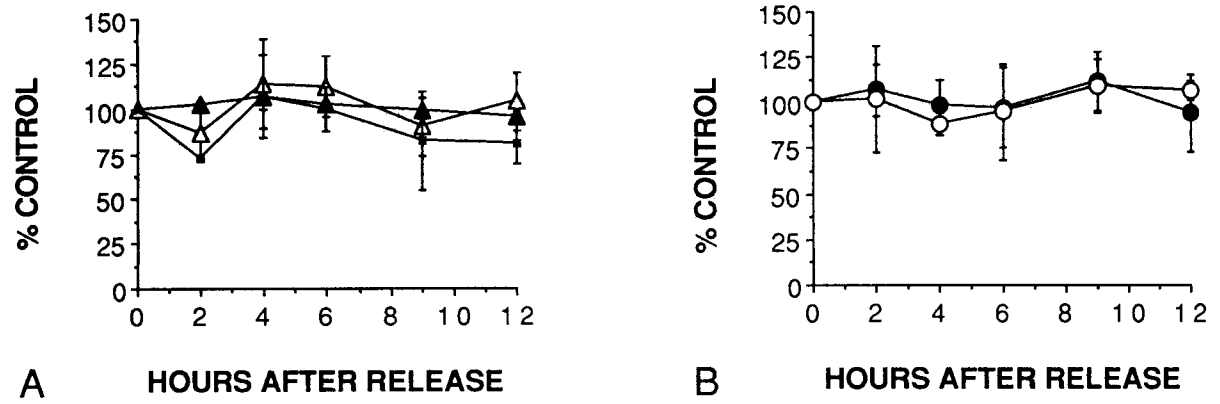

Fig. 3. Northern blot analyses of bone specific (A) and structural (B) genes in synchronized normal diploid osteoblasts. RNA levels were normalized to 18S ribosomal RNA abundance and expressed relative to 0 h values. **A:** Alkaline phosphatase (closed triangle), osteopontin (star), and collagen I (open triangle) mRNA levels. **B:** Vimentin (closed circle) and actin (open circle) mRNA levels. Values expressed as a percentage of control levels (0 h) ± SE, n = 3–4.

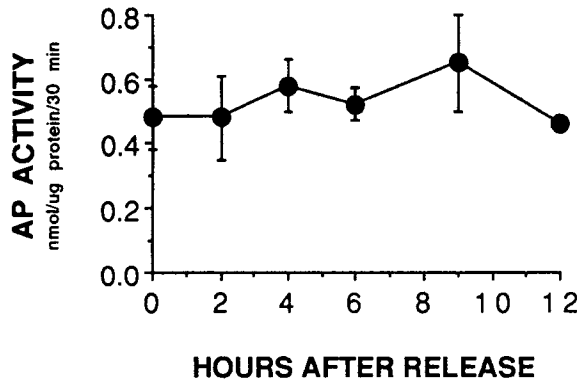

Fig. 4. Total cellular alkaline phosphatase activity in synchronized normal diploid osteoblasts. Values expressed ± SE, n = 4–6 plates per point.

points after releasing ROS cells from the aphidicolin block. As expected, histone mRNA levels (Fig. 6) increased 12-fold during the period of DNA synthesis (Fig. 1). AP and OP mRNA levels were constitutively expressed during the cell cycle, while OC mRNA levels appeared to double 4 h after releasing the cells from the aphidicolin block (Fig. 7). In addition, these developmental markers were expressed to a greater extent in the osteosarcoma cells compared to osteoblasts. Collagen I, vimentin, and actin mRNA levels did not change significantly during the cell cycle (Fig. 7).

Alkaline Phosphatase Activity Is Not Cell Cycle Regulated in Osteosarcoma Cells

To determine if levels of alkaline phosphatase activity are affected by cell cycle progression in osteosarcoma cells, total cellular AP activity was measured in ROS cells 0, 2, 4, 6, 9, and 12 h after release from the aphidicolin block. As shown in Figure 8, no cell cycle variation in the activity of alkaline phosphatase was found in the osteosarcoma cell line. However, the levels of this activity were nearly ten times those found in proliferating normal diploid osteoblasts. This finding further supports the abrogated control of developmentally expressed enzyme activity in a transformed cell line.

Transcription of Developmentally Expressed Genes in Osteosarcoma Cells Is Not Altered During the Cell Cycle

Run on analyses were performed on nuclei isolated at 0, 2, 4, 6, 9, and 12 h after releasing cells from the aphidicolin block (Figure 9). Although some changes were noted in individual experiments, no major changes in the transcription of the developmentally expressed genes studied were apparent over the course of the cell cycle. These findings were similar to those results found in ROB cells.

DISCUSSION

To gain insight into relationships between growth control and the developmental expression of genes during osteoblast differentiation, we have experimentally addressed expression of three classes of genes during the cell cycle of normal diploid osteoblasts and osteosarcoma cells: those associated with growth, cell structure, and the mature bone cell phenotype. By treating and releasing osteoblasts and osteosarcoma cells from a thymidine block and then an aphidicolin block, we were able to obtain a popu-

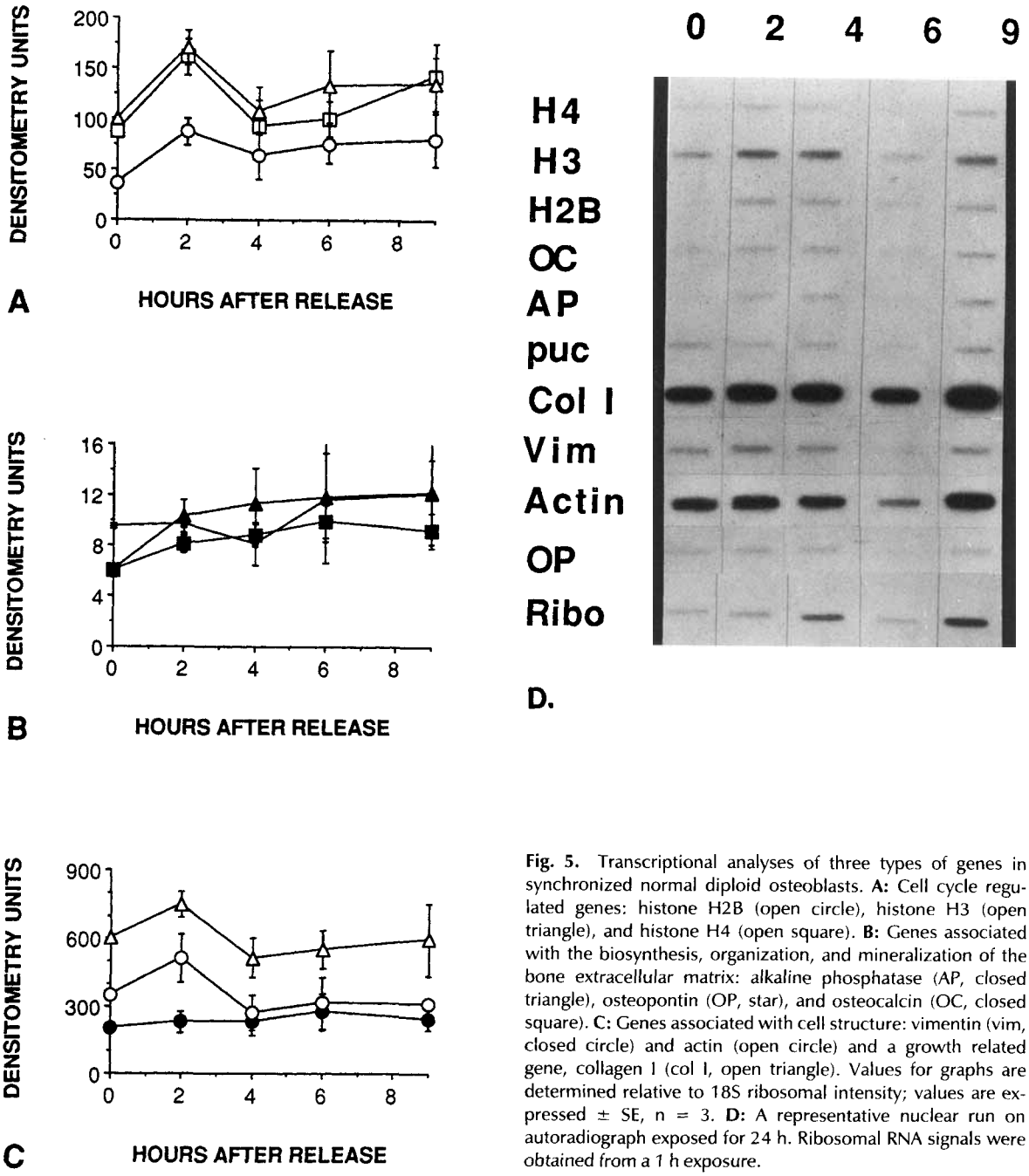

Fig. 5. Transcriptional analyses of three types of genes in synchronized normal diploid osteoblasts. **A:** Cell cycle regulated genes: histone H2B (open circle), histone H3 (open triangle), and histone H4 (open square). **B:** Genes associated with the biosynthesis, organization, and mineralization of the bone extracellular matrix: alkaline phosphatase (AP, closed triangle), osteopontin (OP, star), and osteocalcin (OC, closed square). **C:** Genes associated with cell structure: vimentin (vim, closed circle) and actin (open circle) and a growth related gene, collagen I (col I, open triangle). Values for graphs are determined relative to 18S ribosomal intensity; values are expressed ± SE, n = 3. **D:** A representative nuclear run on autoradiograph exposed for 24 h. Ribosomal RNA signals were obtained from a 1 h exposure.

lation of cells which progressed synchronously through the cell cycle. This allowed us to determine the expression of genes with respect to cell cycle traverse. We found that histone genes, which are required for cell cycle progression, are selectively expressed during S phase as has been reported by our lab and others [Baumbach et al., 1987, 1984; Holthuis et al., 1990; Plumb et al., 1983a,b; Osley, 1991]. In contrast, alkaline phosphatase, collagen I, osteocalcin, and osteopontin are constitutively transcribed at low levels and mRNA transcripts are minimally detectable with the exception of type I collagen. This result is consistent with the absence of a requirement for expression of these genes in proliferating osteoblasts. Because both transcription and cellular retention of mRNAs are reduced in parallel during proliferation in normal diploid osteoblasts, stringent control mechanisms are operative to selectively down regulate transcription and destabilize mRNAs expressed by these post-proliferative genes.

Genes which are involved in cell cyto-architecture such as actin and vimentin were also constitutively expressed during the cell cycle. However, we cannot dismiss the possibility that cell cycle changes occur at the translational or post-translational levels. Several studies suggest that cell cycle regulation of vimentin protein occurs by differentially distributing the protein within the cell at different times during the cell cycle and by phosphorylation during mitosis [Skalli et al., 1992]. Cell cycle changes in actin expression can be seen at the level of monomer polymerization [Jackson and Bellett, 1989]. Thus, it is likely that these cell structural proteins are regulated, at least in part, post-translationally during the cell cycle.

Abrogation of developmental interrelationships in osteosarcoma cells, reflected by co-expression of genes which are developmentally regulated in normal diploid osteoblasts, was the basis for determining the extent to which cell cycle control of these genes is retained. No significant cell cycle changes were apparent in mRNA levels or levels of transcription of a broad spectrum of both cell growth and tissue-related genes that we studied. However, alkaline phosphatase mRNA levels were 5–10-fold higher in ROS compared to ROB cells (data not shown) and OC expression, observed in ROS cells, was absent in ROB cells. These findings correspond with previous studies that demonstrate that ROS cells exhibit expression of genes during proliferation which in normal osteoblasts are stringently controlled and expressed at significant levels only post-proliferatively during late developmental stages [reviewed in Stein et al., 1990a,b; Owen et al., 1990; Lian and Stein, 1992; Stein and Lian, 1993; Rodan and Noda, 1991]. It is interesting that despite the loss of developmental regulation, ROS cells still maintain control of cell cycle and cell growth-dependent gene expres-

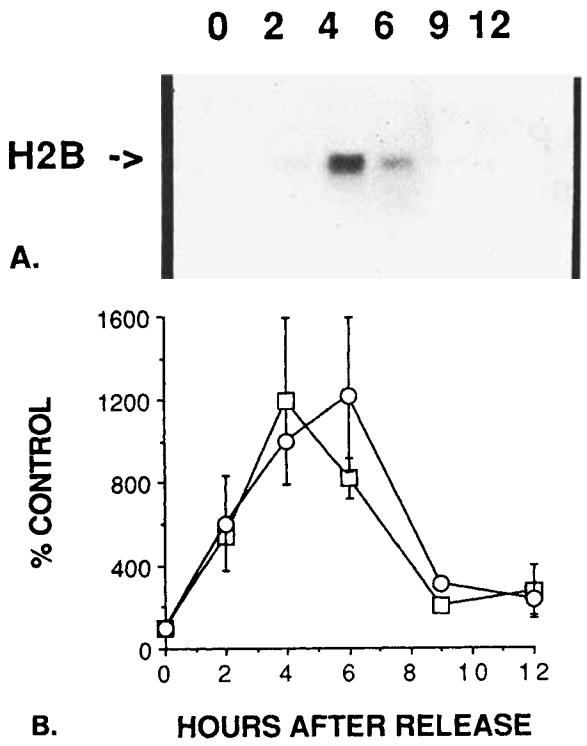

Fig. 6. Histone mRNA levels in synchronized osteosarcoma cells. **A:** Representative Northern blot hybridized to histone H2B cDNA. **B:** Histone H4 (open square) and histone H2B (open circle) mRNA levels following release from aphidicolin block. Levels normalized to 18S ribosomal RNA abundance and expressed relative to 0 h values. Values expressed ± SE, n = 3–4.

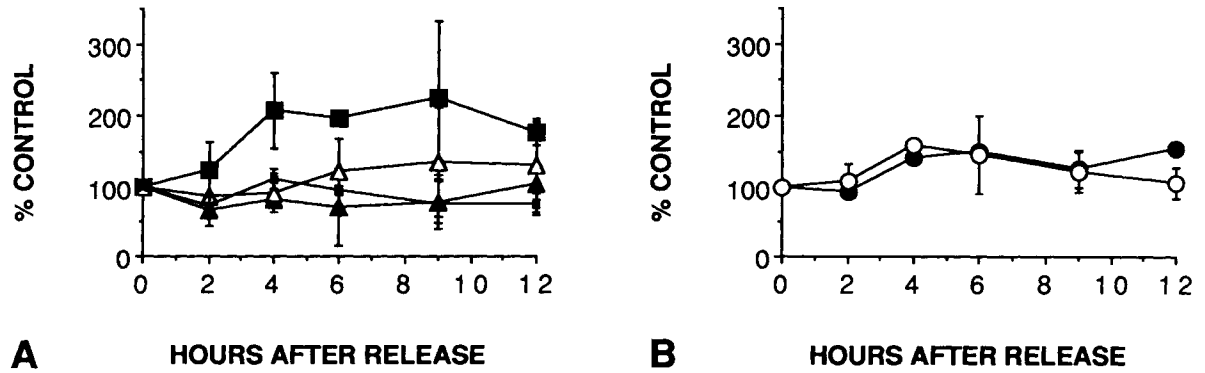

Fig. 7. Northern blot analyses of bone specific (**A**) and structural (**B**) genes in synchronized osteosarcoma cells. RNA levels were normalized to 18S ribosomal RNA abundance and expressed relative to 0 h values. A: Alkaline phosphatase (closed triangle), osteopontin (star), collagen I (open triangle), and osteocalcin (closed square) mRNA levels. B: Vimentin (closed circle) and actin (open circle) mRNA levels. Values expressed as a percentage of control levels (0 h) ± SE, n = 3.

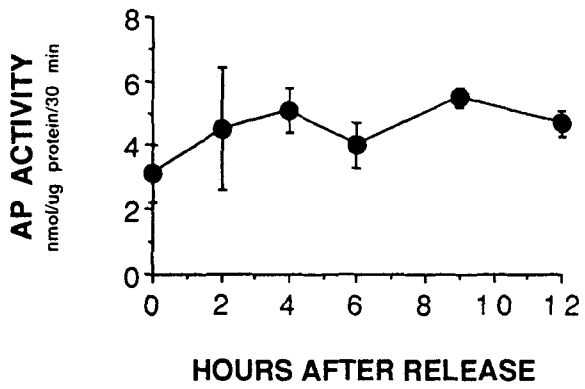

Fig. 8. Total cellular alkaline phosphatase activity in synchronized osteosarcoma cells. Values expressed ± SE, n = 4–6.

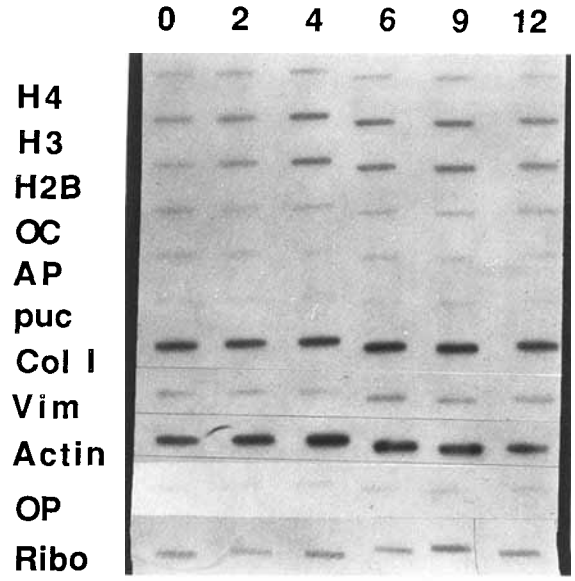

Fig. 9. Nuclear run on analysis in synchronized osteosarcoma cells; a representative autoradiograph. Transcription of three types of genes was examined: cell cycle regulated genes: histone H2B, histone H3, histone H4; genes associated with the biosynthesis, organization, and mineralization of the bone extracellular matrix: alkaline phosphatase (AP), osteopontin (OP), collagen I (col I), and osteocalcin (OC); and genes associated with cell structure: vimentin (vim) and actin. All signals were obtained from a 24 h autoradiograph exposure except for the ribosomal RNA signal which received a 1 h exposure.

sion in a manner equivalent to that observed in normal diploid osteoblasts.

Because alkaline phosphatase is an integral membrane protein in both normal diploid osteoblasts and in osteosarcoma cells, we examined alkaline phosphatase activity during the cell cycle to determine if changes occur when the cell membrane is "remodeled" as mitotic division occurs. We observed only minimal modifications in alkaline phosphatase activity during the cell cycle consistent with the absence of cell cycle variations in transcription and mRNA levels. Fedarko et al. [1990] have reported increased alkaline phosphatase activity during S-phase which declines during mitosis. These apparent variations may reflect species differences (rat vs. human), or differences in origin of the cultured bone cells (calvaria vs. trabecular bone).

The regulatory mechanisms that restrict cell cycle and growth related genes to the proliferation period of osteoblast differentiation and the signaling mechanisms that support upregulation of bone phenotypic genes post-proliferatively have yet to be defined. However, our findings clearly indicate that regardless of whether proliferation-differentiation interrelationships are operative as in normal diploid osteoblasts or abrogated as in osteosarcoma cells, cell cycle regulation or constitutive expression of growth and phenotypic genes is maintained.

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