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Elisheva Smith
University of Massachusetts Medical School

Et al.

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Expression of Cell Cycle Regulatory Factors in Differentiating Osteoblasts: Postproliferative Up-Regulation of Cyclins B and E[1]

Elisheva Smith, Baruch Frenkel, Robert Schlegel, Antonio Giordano, Jane B. Lian, Janet L. Stein, and Gary S. Stein[2]

Department of Cell Biology and Cancer Center, University of Massachusetts Medical Center, Worcester, Massachusetts 01655 [E. S., B. F., J. B. L., J. L. S.]; Harvard School of Public Health, Boston, Massachusetts 02115 [R. S.]; and Jefferson Cancer Institute, Thomas Jefferson University, Philadelphia, Pennsylvania 19107 [A. G.]

ABSTRACT

The representation of cyclins and cyclin-dependent kinases (cdks) was analyzed during progressive development of the bone cell phenotype in cultures of normal diploid rat calvarial osteoblasts. Three developmental stages were examined: (*a*) proliferation; (*b*) monolayer confluency; and (*c*) mineralization of the bone extracellular matrix. We demonstrate that the presence of cyclins and cdks is not restricted to the proliferation period. Consistent with their role in cell cycle progression, cdc2 and cdk2 decrease postproliferatively. However, cdk4 and cyclins A, B, and D1 persist in confluent cells. Cyclin E is significantly up-regulated during the extracellular matrix mineralization developmental period. Examination of the cytoplasmic levels of these cell cycle regulatory proteins indicates a marked increase in cyclin B in the late differentiation stage. The elevation of nuclear cyclin E and cytoplasmic cyclin B is not observed in osteoblasts maintained under culture conditions that do not support differentiation. Furthermore, treatment with transforming growth factor β for 48 h during the proliferation period renders the cells incompetent for differentiation and abrogates the postproliferative up-regulation of cyclins B and E. Density-induced growth inhibition of ROS 17/2.8 osteosarcoma cells is not accompanied by up-regulation of nuclear cyclin E and cytoplasmic cyclin B when compared to the proliferation period. This observation is consistent with abrogation of both growth control and differentiation regulatory mechanisms in tumor cells. These results suggest that cell cycle regulatory proteins function not only during proliferation but may also play a role in normal diploid osteoblast differentiation.

INTRODUCTION

Interrelationships between proliferation and differentiation are mediated by cross talk between regulatory mechanisms controlling the cell cycle and initiation of gene expression required for phenotypic properties characteristic of specialized cells and tissues. During the past several years there have been significant advances in our understanding of both the regulated and regulatory events that control competency for proliferation and cell cycle progression. Particularly important have been the identification and characterization of the proliferation-related proteins, which include the cyclins, cdks,[3] and cdk inhibitors (reviewed in Refs. 1–5). As cells progress through the cell cycle, stage-specific modifications in the representation of cyclins occur. These cell cycle-regulated proteins function as regulatory subunits for constitutively expressed cdks, supporting activation of kinase activity for phosphorylation of regulatory molecules that contribute to control of passage through the cell cycle. In addition, cyclins and cdks participate in transcription factor complexes that interact with promoter elements of cell cycle regulated genes (6, 7).

Abbreviation of G_1 and acceleration of the G_1-S transition by overexpression of cyclins D1 and E directly implicate cyclins in cell cycle control (8–11). Other examples of functional involvement for cyclins in cell cycle regulation are provided by induction of the G_2-M transition when the cyclin B-cdc2 complex was microinjected into oocytes (12). Consistent with primary roles for cyclins in cell cycle control, aberrations in cellular levels of cyclins, particularly cyclins D1, D2, and E, have been observed in transformed and tumor cells where growth-regulatory mechanisms have been compromised (4).

Several lines of evidence implicate the cyclin-related proteins in control of competency for differentiation. Down-regulation of cell cycle-regulatory genes has been observed after induction of differentiation. HL60 promyelocytic leukemia cells exhibit decreased mRNA levels for the G_2-related proteins cyclin B1, cdc2, and cdc25 after phorbol ester induction of differentiation along the monocyte/macrophage lineage (13). Permissiveness for hexamethylene bisacetamide induction of differentiation in murine erythroleukemia cells is dependent on reduced expression of the G_1-related kinase cdk4 (14). Differentiation of 32D myeloid cells by granulocyte colony-stimulating factor is blocked by overexpression of the G_1 D2 or D3 but not D1 (15). Cyclin D1 impedes lymphocyte maturation in transgenic mice (16), and ectopic expression of cyclin D1 inhibits C2C12 myoblast differentiation in a dose-dependent manner (17).

Taken together, these and other studies are consistent with a dual role for cyclin-related proteins in supporting cell cycle progression in proliferating cells while preventing expression of genes associated with postproliferative phenotypic properties of differentiated cells. Evidence for involvement of cyclin-related proteins in control of differentiation is further provided by the selective expression of cdk5 in terminally differentiated neurons (18). These findings implicate cyclin-related proteins in control of gene expression required for initiation and maintenance of phenotypic properties characteristic of specialized cells and tissues.

Osteoblast differentiation is a multistep developmental process initially involving expression of cell growth and cell cycle regulatory genes to support proliferation and genes associated with extracellular matrix biosynthesis (reviewed in Ref. 19). Then, postproliferatively, there is a sequential expression of genes required for the organization and mineralization of a bone extracellular matrix. Down-regulation of proliferation at a key developmental transition point is necessary for transcription of genes functionally related to expression of the mature osteoblast phenotype. In contrast to the sequential and mutually exclusive expression of cell growth and tissue-specific genes in normal diploid osteoblasts, osteosarcoma cells coexpress genes involved with growth control and regulation of bone phenotypic genes (20).

In this paper we report modifications in both the representation and subcellular distribution of cell cycle regulatory proteins during sequential stages of osteoblast differentiation. Increased nuclear levels of cyclin E and cytoplasmic levels of cyclin B are observed postproliferatively during differentiation of normal diploid osteoblasts. These alterations do not occur during growth arrest, which is uncoupled from expression of skeletal phenotypic genes, or in osteosarcoma cells.

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[2] To whom requests for reprints should be addressed, at Department of Cell Biology and Cancer Center, University of Massachusetts Medical Center, Worcester, MA 01655-0106. Phone: (508) 856-5625; Fax: (508) 856-6800.

[3] The abbreviations used are: cdk, cyclin-dependent kinase; TGF-β, transforming growth factor β; OC, osteocalcin.

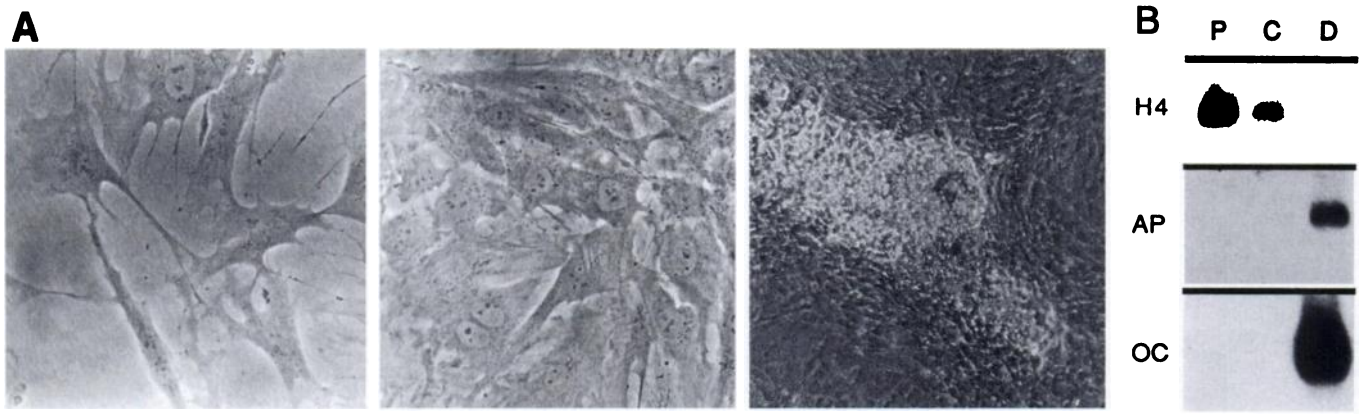

Fig. 1. Cultures of normal diploid rat calvarial osteoblasts were maintained under conditions that support differentiation, as described in "Materials and Methods." *A*, three developmental stages [proliferation (*left*; × 250); monolayer confluency (*middle*; × 250); and differentiation (*right*; × 75)] are shown by light microscopy. Note the postconfluent nodule formation in the differentiation stage, where mineralization of the extracellular matrix occurs. *B*, mRNA was isolated at the three developmental stages shown in *A*, and Northern analysis was performed with 15 μg of RNA. Blots were probed with either a histone H4 cDNA as a marker for proliferation or alkaline phosphatase (*AP*) and OC cDNAs as differentiation markers. *P*, proliferation; *C*, confluency; *D*, differentiation.

MATERIALS AND METHODS

Cell Culture. Primary osteoblasts were isolated from fetal rat calvariae and maintained in MEM supplemented with 10% FCS (21). Ascorbic acid (50 μg/ml) and β-glycerophosphate (10 mM) supplementation of the medium was initiated at confluence. For secondary cultures, cells were trypsinized before reaching confluency (day 5) and replated at a density of 9×10^5 cells/100-mm plate. Unless otherwise stated, secondary cultures were fed as above. The first two feedings contained 10^{-7} M dexamethasone to enhance expression of the bone cell phenotype. At principal developmental stages, cells were harvested for protein and RNA isolation. To obtain nondifferentiating osteoblasts, secondary cultures were maintained in MEM supplemented with 10% FCS but with no additions of dexamethasone, ascorbic acid, and β-glycerophosphate. For TGF-β treatment, 100 pM TGF-β was added to primary cultures during the proliferation period for 48 h. The cultures were returned to differentiation medium and collected at later times with untreated controls. Rat osteosarcoma cells (ROS 17/2.8; Merck, Sharp and Dohme, West Point, PA) were grown in F-12 medium supplemented with 5% horse serum. Cells (passages 16–18) were plated at a density of 7×10^5/100-mm dish. At three different time points (proliferation, confluence, and a late time point of density-induced growth inhibition), cells were harvested for protein and RNA isolation. Medium from all cultures was collected for OC analysis by RIA (22). Unless otherwise stated, all cell samples for protein and mRNA analyses were collected 24 h after medium change.

Western Analysis. Cells were scraped in ice-cold PBS, centrifuged, and resuspended in ice-cold 1% citric acid for cell fractionation as described by Bailly *et al.* (23). Briefly, the resuspended cells were homogenized (35 strokes) in a tight fitting Dounce homogenizer, sedimented at 300 × *g*, and washed once in 0.1% citric acid and then in a 10 mM Tris-HCl buffer (pH 7.4) containing 5 mM $MgCl_2$. The citric acid-extracted cytoplasmic proteins were precipitated in 90% methanol (overnight at −20°c). The nuclear and cytoplasmic fractions were solubilized in a 60 mM Tris-HCl buffer (pH 6.8) containing 2% SDS. Before each Western analysis, protein concentration was determined (Pierce Micro BCA Protein Assay kit 23235), and equivalent amounts were subjected to SDS PAGE (12%) in the absence of reducing agents, followed by electroblotting to nitrocellulose membrane (Hoefer Scientific Instrument, CA). Immunodetection was performed with the enhanced chemiluminescence kit (Amersham). Antibodies to cdc2, cdk2, and cdk4, and cyclins B1, D1, and E (sc-54, sc-163, sc-260, sc-245, sc-246, and sc-247, respectively) were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Rabbit polyclonal antibodies to human cyclins A and

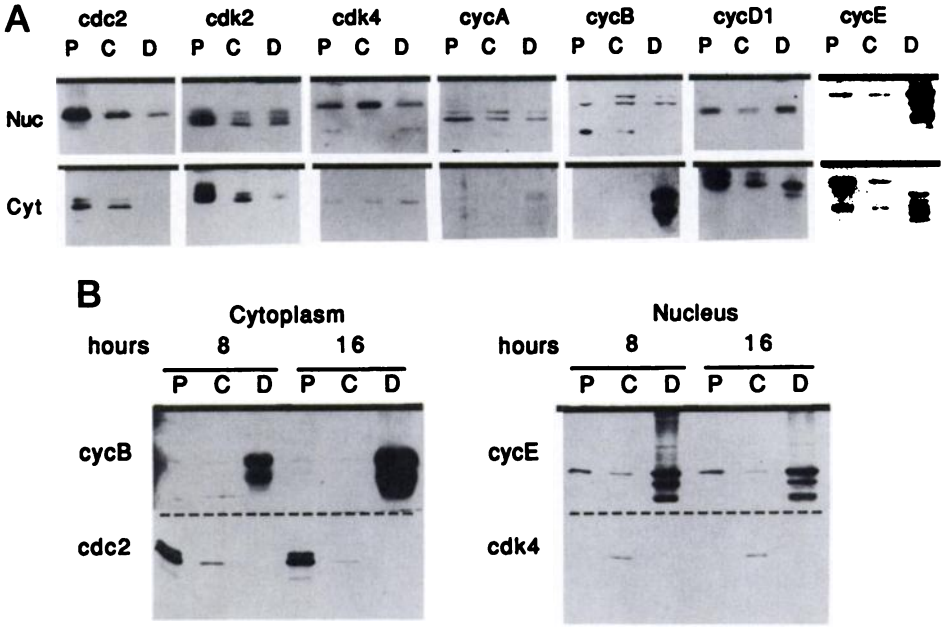

Fig. 2. *A*, normal diploid osteoblasts shown in Fig. 1*A* were harvested 24 h after medium change, at the three developmental stages, followed by isolation of the nuclear (*Nuc*) and cytoplasmic (*Cyt*) fractions. Twenty μg of either nuclear proteins or the methanol precipitate of the cytoplasmic fractions were subjected to Western analysis using antibodies to the kinases cdc2, cdk2, and cdk4, and the cyclins (*cyc*) A, B, D1, and E. *P*, proliferation; *C*, confluency; *D*, differentiation. *B*, cells as in *A* were harvested at the indicated time points after feeding; analyses of representative proteins are shown.

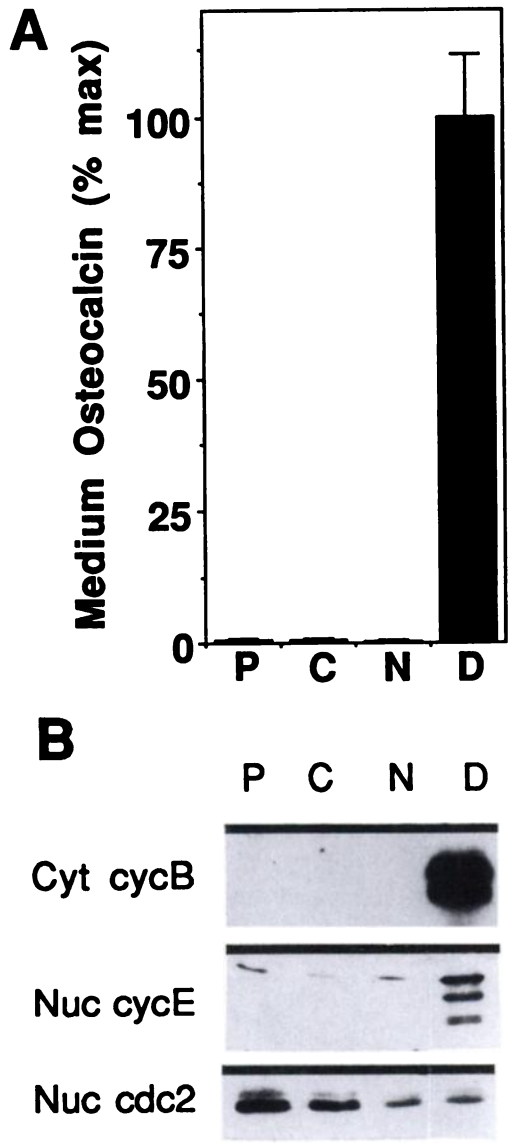

Fig. 3. Normal diploid osteoblasts isolated as in Figs. 1 and 2 were maintained under nondifferentiating culture conditions as described in "Materials and Methods." *A*, samples of 24-h conditioned medium were collected during proliferation (*P*), at confluency (*C*), and at a late postconfluent stage (*N*; nondifferentiated) for OC RIA. The OC level measured in 24-h conditioned medium of differentiating osteoblasts (*D*) maintained as in Fig. 1 is also shown. *Columns*, mean values from 3 independent determinations; *bars*, SD. The OC level in differentiated cultures is defined as 100%. *B*, Western analysis is shown for cytoplasmic cyclin B (*Cyt cycB*), and nuclear (*Nuc*) cyclin E and cdc2. Results from differentiated osteoblasts (*D*) are shown for comparison.

B1 were prepared in the laboratory of Dr. R. Schlegel. Results of the Western analysis were reproduced in at least 3 independent osteoblast differentiation courses.

Northern Analysis. Cells were scraped in guanidinium solution (24), and RNA was analyzed as described (25). Samples were electrophoresed in 1% agarose gels and blotted overnight onto nylon membrane (Zetaprobe Blotting Membrane, Bio-Rad). Prehybridization (12 h at 42°C) and hybridization (overnight at 42°C) were performed in 50% formamide-4.7× SSPE [1× SSPE is 0.18 M NaCl-10 mM NaH_2PO_4 (pH 7.4)-1 mM EDTA], 0.5% SDS, 5× Denhardt's solution (1× Denhardt's solution = 0.2(w/v) each of Ficoll, polyvinylpyrolidone, and BSA), and 200 μg/ml salmon-sperm DNA. Membranes were washed in a 2× SSC solution (1× SSC is 0.15 M NaCl-15 mM sodium citrate) containing 0.1% SDS at 50°C for 25 min, followed by a more stringent wash (1× SSC-0.1% SDS) when necessary. cDNAs for histone H4 (26), alkaline phosphatase (27), and OC (28) were used.

RESULTS AND DISCUSSION

Nuclear Cyclin E and Cytoplasmic Cyclin B Are Elevated in Postproliferative Differentiating Osteoblasts. To address involvement of cell cycle regulatory proteins in osteoblast differentiation, we determined the nuclear and cytoplasmic representation of 4 cyclins and 3 cdks in cultured rat calvarial osteoblasts during 3 developmental periods (proliferation, monolayer confluency, and extracellular matrix mineralization). Fig. 1*A* presents micrographs of cells in these three developmental periods. Fig. 1*B* shows Northern analysis of histone, alkaline phosphatase, and OC gene transcripts during osteoblast differentiation, indicating that there is a reciprocal relationship between proliferation and postproliferative expression of bone cell phenotypic properties. The high levels of OC expression in postproliferative mature osteoblasts provide evidence for differentiation of the osteoblast cultures (see Fig. 1 and Ref. 19).

Western analysis of cyclins and cdks is shown in Fig. 2. The nuclear levels of cdc2 and cdk2 kinases decline postproliferatively. A parallel relationship is therefore observed between proliferation and expression of two kinases that are primary mediators of cell cycle progression. However, low but detectable levels of these kinases are present throughout the osteoblast developmental sequence including in differentiated osteoblasts. Nuclear levels of cdk4 exhibit a modest increase at confluency. In postproliferative differentiated cells, cdk4 declines to levels below those observed during active proliferation. Significant nuclear levels of cyclins A, B, D1, and E are retained in differentiated osteoblasts. Particularly striking is the increase in nuclear abundance of cyclin E in the differentiated osteoblasts (Fig. 2*A*). The multiple cyclin E-immunoreactive bands (Figs. 2 and 4*B*) may represent products of cyclin E-related genes or alternative splicing of cyclin E mRNA (10, 29).

Fig. 2*A* also shows cytoplasmic levels of cell cycle regulatory factors during the osteoblast developmental sequence. As observed in the nuclear fractions, both cdc2 and cdk2 but not cdk4 are significantly reduced in differentiating cells. A dramatic increase is observed in cytoplasmic cyclin B during differentiation. This increase was documented in three independent experiments using two different cyclin B antibodies. The findings confirm the correlation between cyclin B expression and the extent of osteoblast differentiation. It is noteworthy that the increase in cyclin E observed in differentiated cells is specific to the nuclear fraction, whereas cytoplasmic cyclin E is reduced. Taken together, these results indicate that in contrast to the well documented role for cyclins and cdks in governing proliferation, nuclear cyclin E and cytoplasmic cyclin B are up-regulated during osteoblast differentiation. Whereas Fig. 2*A* presents data from samples harvested 24 h after medium change, Fig. 2*B* demonstrates similar increase in cytoplasmic cyclin B and nuclear cyclin E in differentiating osteoblasts in samples collected 8 and 16 h after feeding. The expression patterns of cytoplasmic cdc2 and nuclear cdk4 analyzed using the same blots (Fig. 2*B*), as well as those of all the other components shown in Fig. 2*A*, were also essentially unaffected by the time period between feeding and collection (not shown). Thus, the developmental changes in cellular levels of cyclin-related proteins are independent of the serum response that occurs as a result of medium changes during the developmental time course in cultured osteoblasts.

Postproliferative Elevation of Cyclins B and E in Osteoblasts Is Dependent on Differentiation. To address further the involvement of cell cycle-regulatory factors in osteoblast differentiation, we examined cyclins and cdks in osteoblast cultures under conditions that do not support differentiation. It was necessary to establish that changes observed in cellular levels of cyclin-related proteins in differentiating osteoblasts do not reflect a prolonged postproliferative

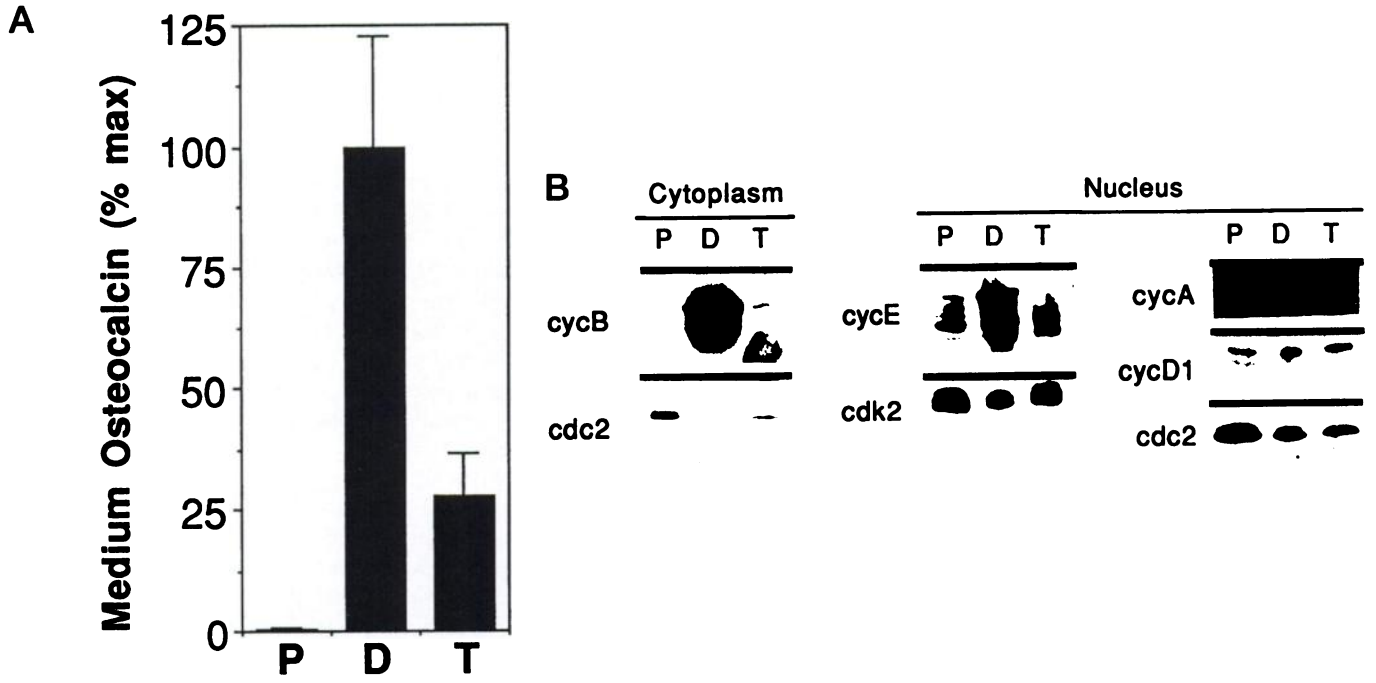

Fig. 4. Primary cultures of rat calvarial osteoblasts were maintained under differentiation conditions as described in "Materials and Methods." At the proliferating stage, one-half of the plates were treated for 48 h with 100 pM TGF-β to inhibit differentiation. *A*, 24-h accumulation of OC in the medium was determined by RIA of the proliferating (*P*), differentiating (*D*), and TGF-β-treated (*T*) cells. *Columns*, mean values from 3 independent determinations; *bars*, SD. The values from differentiated cells are defined as 100%. *B*, Western analysis was performed as described in Fig. 2 for cytoplasmic cyclin B and cdc2, as well as nuclear cyclins A, D1, and E, and the kinases cdc2 and cdk2.

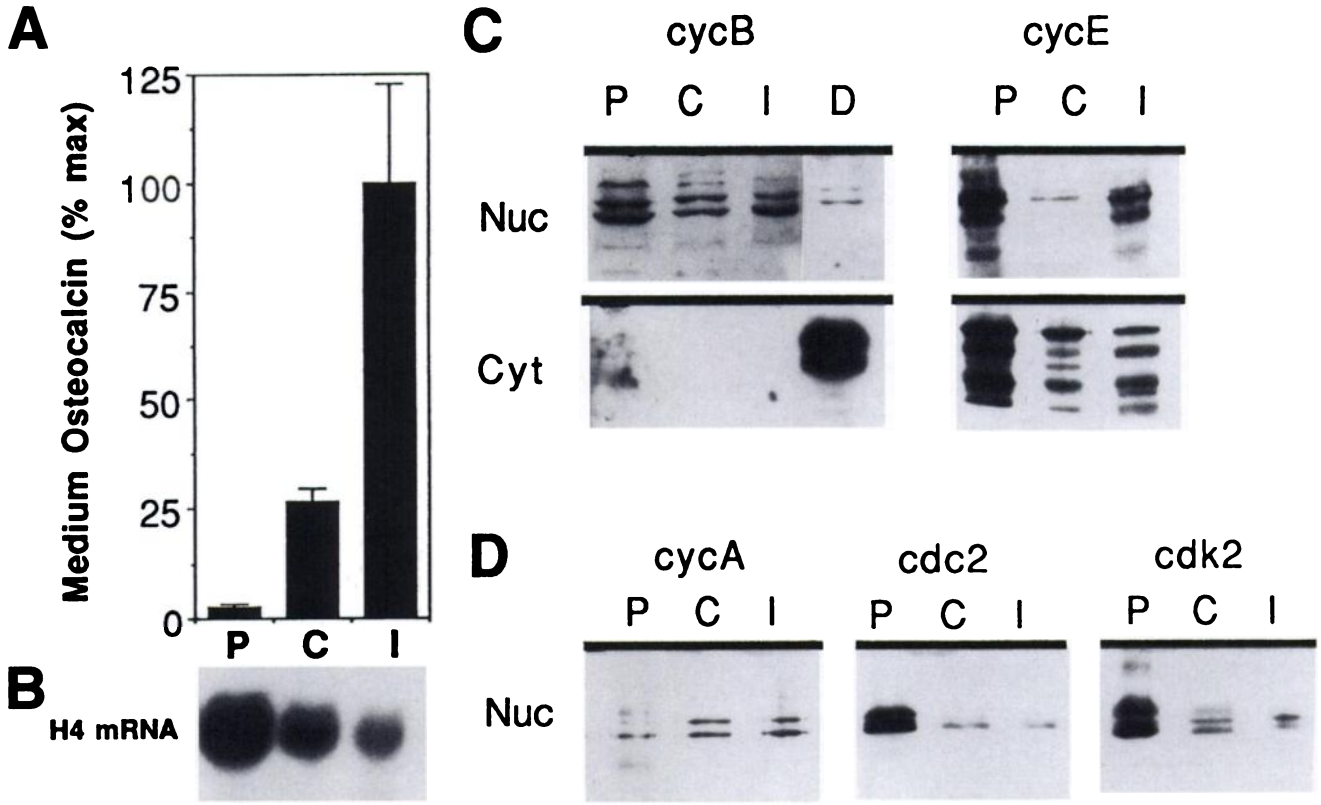

Fig. 5. ROS 17/2.8 osteosarcoma cells were maintained as described in "Materials and Methods" and harvested when actively proliferating (*P*), at confluency (*C*), and after density-mediated growth inhibition (*I*). *A*, 24-h OC accumulation in the culture medium was determined at the three stages by RIA. *B*, Northern analysis of histone H4 mRNA was performed as in Fig. 1*A*. *C*, cyclins B (*cycB*) and E (*cycE*), exhibiting specific developmental regulation in normal diploid osteoblasts (Fig. 2), were analyzed in the nuclear and cytoplasmic fractions of ROS 17/2.8 cells. Cyclin B of normal diploid differentiated osteoblasts (*D*) was analyzed as a reagent control. *D*, Western analysis of ROS 17/2.8 nuclear cyclin A, cdc2, and cdk2, showing a pattern similar to that observed in normal diploid cells.

period. Osteoblasts were isolated and cultured under conditions that preclude differentiation as described in "Materials and Methods" (absence of dexamethasone, ascorbic acid, and β-glycerophosphate). Fig. 3*A* indicates that under culture conditions that support proliferation and subsequent growth arrest but not expression of mature osteoblast phenotypic properties, the expression of OC, a late developmental marker, is completely blocked. As shown in Fig. 3*B*, nuclear cyclin E and cytoplasmic cyclin B are not elevated in the nondifferentiating cells, further indicating linkage of these two cyclins (Fig. 2) with osteoblast differentiation. Data for differentiating osteoblasts are shown for comparison. The patterns of appearance of cdc2 (Fig. 3), cdk2, cdk4, and cyclins A and D1 (not shown) in nondifferentiating cells were generally similar to those of differentiating cells.

TGF-β Impedes Osteoblast Differentiation and Up-Regulation of Cytoplasmic Cyclin B and Nuclear Cyclin E. TGF-β inhibits osteoblast differentiation *in vitro* (30–32). TGF-β treatment during osteoblast proliferation results in loss of competency for differentiation at later postconfluent stages. If changes in nuclear cyclin E and cytoplasmic cyclin B are functionally linked to osteoblast differentiation, decreased levels can be anticipated in cells treated with TGF-β. Primary cultures of rat calvarial derived osteoblasts exposed to TGF-β during the period of active growth exhibited a block in differentiation, as indicated by reduced bone nodule formation (not shown) and OC biosynthesis (Fig. 4*A*). The inhibitory effect of TGF-β is associated with abrogation of the high levels of nuclear cyclin E and cytoplasmic cyclin B, which is related to osteoblast differentiation as shown in Fig. 4*B*. Down-regulation of cytoplasmic cdc2 and nuclear cdk2 is also prevented by TGF-β inhibition of differentiation. Nuclear levels of cyclin A, cyclin D1, and cdc2 were not altered in the TGFβ-treated cells as compared to differentiated cells.

Growth Inhibition of ROS 17/2.8 Osteosarcoma Cells Is Not Accompanied by High Levels of Cytoplasmic Cyclin B or Nuclear Cyclin E. ROS 17/2.8 osteosarcoma cells exhibit increased levels of OC and reduced levels of histone H4 mRNA when growth inhibited at high density (Fig. 5, *A* and *B*). However, the elevated OC levels are not accompanied by the nodule formation or matrix mineralization characteristic of differentiated normal rat diploid osteoblasts. The growth-inhibited cells do not exhibit an increase in cytoplasmic cyclin B (Fig. 5*C*, compare *Lanes I* and *D*), indicating the specificity of up-regulation observed in association with cessation of proliferation during differentiation in normal diploid cells. When compared to confluent cells, the growth-inhibited osteosarcoma cells exhibit higher levels of nuclear cyclin E. In contrast to the pronounced increase in nuclear cyclin E during differentiation of normal diploid osteoblasts, cyclin E levels are similar during proliferation and growth inhibition. Similar patterns of cyclin A, cdc2, and cdk2 expression are observed in osteosarcoma cells and normal diploid osteoblasts in relation to the extent of proliferative activity (compare Fig. 5*D* to Fig. 2). Hence, up-regulation of cytoplasmic cyclin B and nuclear cyclin E is associated with the expression of postproliferative differentiation markers (OC) when coupled to the developmental process (nodule formation) that occurs in normal diploid osteoblast differentiation.

Conclusions. During osteoblast differentiation, two cyclins (B and E) are up-regulated postproliferatively when expression of tissue-specific phenotypic properties are established. This activity complements the well documented expression of these cyclins in proliferating cells (1–5). Validation of the biological relevance of this observation is provided by the absence of cyclin B and cyclin E up-regulation when growth inhibition is not accompanied by differentiation under multiple independent conditions.

Although it has been well documented that cyclins regulate phosphorylation of proteins functionally involved with growth control, contributions to differentiation remain to be defined. During differentiation, cyclins and cdks may function by phosphorylation of substrates either similar to or distinct from those that play a role in cell cycle regulation. Particularly important will be identification of the catalytic subunits with which cyclins B and E interact during osteoblast differentiation. These may be known or novel cdks (33–35). Alterations in the representation of cdk inhibitors (36, 37) may also be important to channel kinase activities in postproliferative osteoblasts to differentiation-related processes. The cascade of regulatory events that accompany and are functionally related to late stages of postproliferative osteoblast differentiation are unquestionably accompanied by and functionally coupled with phosphorylation-dependent regulatory mechanisms. Potential involvement of cyclins that exhibit increased representation in mature osteoblasts in control of bone cell structure and function is being addressed experimentally.

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