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Influence of DNA Replication Inhibition on Expression of Cell Growth and Tissue-Specific Genes in Osteoblasts and Osteosarcoma Cells

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Abstract Interrelationships between proliferation and expression of cell growth as well as bone cell-related genes were examined from two standpoints. First, the consequence of downregulating proliferation by DNA synthesis inhibition on expression of a cell cycle-regulated histone gene and genes associated with development of the bone cell phenotype (type I collagen, alkaline phosphatase, osteopontin, and osteocalcin) was investigated. Second, the requirement for stringent growth control to support functional relationships between expression of proliferation and differentiation-related genes was explored. Parameters of cell growth and osteoblast-related gene expression in primary cultures of normal diploid osteoblasts, that initially express proliferation-dependent genes and subsequently postproliferative genes associated with mature bone cell phenotypic properties, were compared to those operative in ROS 17/2.8 osteosarcoma cells that concomitantly express cell growth and mature osteoblast phenotypic genes. Our findings indicate that in both normal diploid osteoblasts and osteosarcoma cells, expression of the cell cycle regulated histone genes is tightly coupled with DNA synthesis and controlled predominantly at a posttranscriptional level. Inhibition of proliferation by blocking DNA synthesis with hydroxyurea upregulates a subset of developmentally expressed genes that postproliferatively support progressive establishment of mature osteoblast phenotypic properties (e.g., alkaline phosphatase, type I collagen, and osteopontin). However, the osteocalcin gene, which is expressed during the final stage of osteoblast differentiation when extracellular matrix mineralization occurs, is not upregulated. Variations in the extent to which inhibition of proliferation in normal diploid osteoblasts and in ROS 17/2.8 osteosarcoma cells selectively affects transcription and cellular levels of mRNA transcripts from bone cell-related genes (e.g., osteocalcin) may reflect modifications in proliferation/differentiation interrelationships when stringent growth control is abrogated.

Key words: proliferation/differentiation, transcription, osteoblasts, bone cell-related genes, DNA synthesis inhibition

Interrelationships between the expression of cell growth and tissue-specific genes characterize progressive development of the osteoblast phenotype supporting both the establishment and maintenance of structural and functional properties of bone tissue both in vivo and in vitro. Initially, proliferating osteoblasts express genes associated with cell cycle and cell growth control (e.g., histone and c-fos) together with genes encoding extracellular matrix proteins (type I collagen, fibronectin) and modulators of extracellular matrix biosynthesis (e.g., TGFβ). Then postproliferatively, there is an upregulation in the expression of genes that support the maturation and organization of the bone extracellular matrix (e.g., alkaline phosphatase). Subsequently, genes that facilitate extracellular mineralization (osteopontin and osteocalcin) are initiated [reviewed in Stein et al., 1990a,b; Owen et al., 1990; Pockwinse et al., 1992; Lian and Stein, 1992; Stein and Lian, 1993]. In contrast, osteosarcoma cells such as the ROS 17/2.8 cell line have abrogated stringent growth regulatory mechanisms and concomitantly express genes transcribed in early stage proliferating osteoblasts together with genes expressed only in postproliferative osteocytes in a mineralizing bone extracellular matrix [reviewed in Stein et al., 1990a,b; Owen et al., 1990; Lian and Stein, 1992; Stein and Lian, 1993; Rodan and Noda, 1991].

A fundamental question is determining whether osteosarcoma cells have modifications

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in the control mechanisms that mediate restriction of cell growth regulated genes to proliferating normal diploid osteoblasts. We therefore investigated the extent to which expression of the cell cycle regulated histone genes remains coupled to DNA synthesis in the ROS 17/2.8 osteosarcoma cell line. Results presented here indicate that in normal diploid osteoblasts as well as in osteosarcoma cells, inhibition of DNA synthesis is paralleled by downregulation of histone gene expression through a posttranscriptional mechanism that selectively destabilizes histone messenger RNA. Equally important are consequences of abrogated growth control on signalling mechanisms associated with the induction of postproliferative genes during osteoblast differentiation. Our findings support modifications in the extent to which downregulation of proliferative activity by DNA synthesis inhibition upregulates expression of postproliferative genes in osteosarcoma cells compared to normal diploid osteoblasts.

MATERIALS AND METHODS

Cell Culture

Rat osteoblasts from fetal rat calvaria were isolated and maintained in culture as described previously [Owen et al., 1990; Aronow et al., 1990]. Cells were grown in minimal essential medium (MEM; Gibco) supplemented with 10% fetal calf serum (FCS). Second or third passaged cells were used in the studies reported in this paper. ROS 17/2.8 cells, provided by Dr. Sevgi and Dr. Gideon Rodan, were cultured in F12 supplemented with L-glutamine (Sigma), penicillin/streptomycin (Sigma), 5% horse and 2% fetal calf serum. Cells were plated in 100 mm and six-well dishes at a density of $4.5 \cdot 10^4$ cells/cm^2. Experiments were performed 1 or 2 days after plating when the cells were actively proliferating.

Hydroxyurea Treatment

In initial experiments, hydroxyurea (HU; Sigma) toxicity was examined using increasing concentrations and lengths of treatment. Exposure to 1 mM HU resulted in a 97% inhibition of DNA synthesis without affecting cell viability. This concentration was used in subsequent experiments. Cells were treated with 1 mM HU for 0, 15, 45, 90, and 180 min by adding HU from a 50-mM stock solution directly to the medium of the growing cells. After completion of treatment the cells were rinsed with ice-cold phosphate-buffered saline (PBS) and harvested by scraping with a rubber policeman.

DNA Synthesis

The rate of DNA synthesis was determined by pulse labeling with [^{3}H]-Thymidine (Dupont, NEN) for 30 min with 5 μCi/ml. DNA was precipitated with 5% TCA and cells were lysed with 10% SDS. Radioactivity was then determined by liquid scintillation counting. Measurements for each timepoint were determined in triplicate and plotted at the midpoint in the 30-min labeling period.

RNA Isolation and Analysis

Total cellular RNA was isolated from frozen cell pellets stored at −70°C [Chirgwin et al., 1979]. The intactness of the RNA was monitored by electrophoretic fractionation in denaturing 6.6% (v/v) formaldehyde, 1% (w/v) agarose gels and ethidium bromide staining. Five or 10 μg of the RNA preparations was transferred to Zetaprobe membranes (Bio-Rad).

Blots were probed with random primed ^{32}P-labeled cDNA probes (Stratagene, Prime It kit). Hybridization was performed at 43°C in 5× SSC containing 50% formamide, 1% SDS, 50 mM $NaPO_4$, 10× Denhardt's solution and 100 μg/ml salmon sperm DNA. Blots were washed twice for 30 min in 1× SSC/0.1% SDS at 55°C (for hybridization with H3, H4, H2B histone gene probes) or at 60°C (for hybridization with AP, OP, OC, COL I, LS2 probes). The blots were exposed to preflashed XAR-5 X-ray film (Kodak) using a Cronex Lightning Plus intensifying screen at −75°C. Autoradiograms were quantitated densitometrically using the gel analysis program on the Ultra-Violet Products (UVP) gel documentation system. All mRNA levels were normalized to the amount of 18S ribosomal RNA determined by hybridization with a LS2 ribosomal gene probe.

Nuclear Run-on and In Vitro Transcription

Nuclei were isolated from proliferating ROB and ROS cells and stored at −70°C in 50 mM Tris pH 8.3, 40% glycerol, 5 mM $MgCl_2$ and 0.1 mM EDTA. Isolation involved Dounce homogenization in a Triton lysis buffer (10 mM Tris, pH 7.4, 2 mM $MgCl_2$, 10 mM NaCl, 2 mM $CaCl_2$, and 0.6% Triton X-100), followed by incubation in a NP-40 buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40). Transcription

reactions using between 5×10^5 and 5×10^6 nuclei were carried out for 30 min in the presence of 100 μCi [^{32}P]-UTP (Dupont NEN), 2 mM ATP, 0.5 mM GTP, and 0.5 mM CTP. Radiolabeled RNA was isolated using phenol: chloroform extractions [Greenberg and Bender, 1991] or by a guanidinium-cesium–chloride gradient method (McCabe et al., unpublished results).

Equivalent amounts of radiolabeled RNA were hybridized to 2 μg of plasmid DNA containing the genes of interest, which were immobilized onto Zetaprobe membranes (Schleicher and Schuell, Keene, NH). Autoradiograms were quantitated by scanning laser densitometry (LKB 2400 Gelscan XL). Data were expressed in arbitrary densitometry units and normalized to the amount of radiolabelled 28S ribosomal RNA bound to the blot.

RESULTS

Cell Cycle-Regulated Histone mRNA Levels Are Functionally Coupled to DNA Synthesis in Osteoblasts and Osteosarcoma Cells

Our initial experimental approach was to address the extent to which expression of the cell cycle regulated histone genes is coupled to DNA replication in normal diploid rat osteoblasts where stringent growth control is operative and in ROS 17/2.8 osteosarcoma cells. We compared total cellular levels of histone mRNA following inhibition of DNA synthesis by treatment of exponentially growing cells with hydroxyurea. As shown in Figure 1, treatment of ROS and ROB cells with 1 mM hydroxyurea resulted in a rapid decrease in DNA synthesis within 15 min, declining to 3% of control levels after 3 h. In the same experiments cellular H3, H4, and H2B mRNA levels were determined by northern blot analysis using radiolabeled histone gene probes. Representative blots for H2B histone mRNA and 18S ribosomal RNA (internal control for intactness of RNA preparations and quantitation) are shown in Figure 2. The amount of each histone mRNA was quantitated densitometrically and normalized to the amount of 18S ribosomal RNA.

As shown in Figure 2, upon DNA synthesis inhibition, the cellular level of each histone mRNA species was decreased 70–80% in relation to control values. Although initially histone mRNA levels show a gradual decline, a parallel downregulation was observed in the rate of DNA synthesis. It is evident from these results that the kinetics of decline in histone mRNA levels following inhibition of DNA synthesis is similar in both ROB and ROS cells, and that the relationship between cellular histone mRNA levels and DNA synthesis is maintained.

Downregulation of Histone Gene Expression Following DNA Synthesis Inhibition in Osteoblasts and Osteosarcoma Cells Is Postproliferatively Mediated

To investigate the extent to which transcriptional regulation contributes to the coupling of histone gene expression with DNA synthesis in

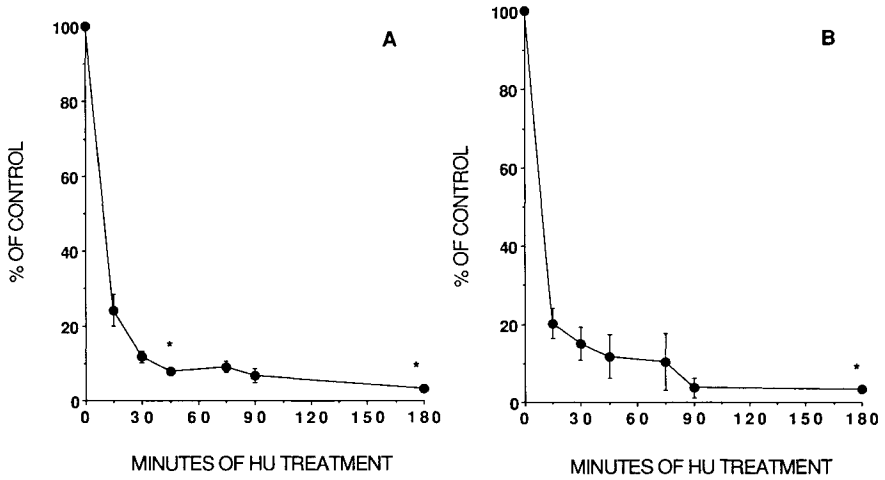

Fig. 1. Inhibition of DNA synthesis after HU treatment in proliferating ROB (A) and ROS (B) cells. Cells were treated with 1 mM HU for indicated times and the rate of DNA synthesis was monitored by incorporation of [^{3}H]-thymidine as described in Materials and Methods. Time points were plotted halfway the 30-min labeling time. Brackets indicate the standard error of three (A) or four (B) independent experiments. At some timepoints brackets fell within the symbol (*).

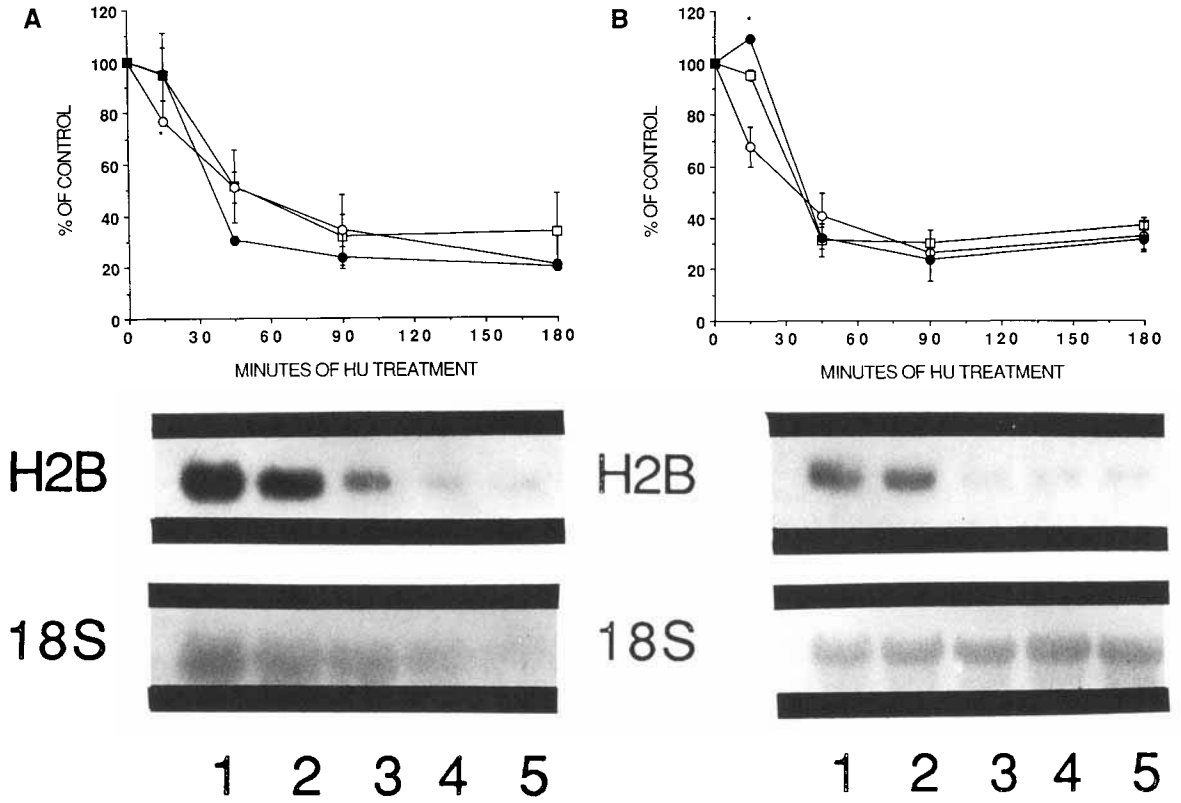

Fig. 2. Kinetics of histone mRNA levels after DNA synthesis inhibition by HU in proliferating ROB (**A**) and ROS (**B**) cells. At indicated times, following 1 mM HU treatment, total cellular RNA was isolated and H3 (●), H4 (○), and H2B (□) mRNAs were analyzed by Northern blotting as described in Materials and Methods. Histone mRNA levels of the HU treated cells are presented relative to the mRNA levels of the untreated cells. Brackets indicate the standard error of three (A) or four (B) independent experiments. At some timepoints brackets fell within the symbols (*). A representative autoradiogram is presented for both cell types, showing H2B and 18S RNA levels after exposure to 1 mM HU; **lane 1,** 0 min; **lane 2,** 15 min; **lane 3,** 45 min; **lane 4,** 90 min; and **lane 5,** 3 h.

ROB and ROS cells, nuclei were isolated from parallel cultures in which histone mRNA levels and rates of DNA synthesis were analyzed following DNA synthesis inhibition for determining rates of transcription by nuclear run-on. For both ROS and ROB cells two different run-on protocols were used, which differ in the method of isolating newly transcribed RNA. Equal amounts of incorporated radioactivity were included in each hybridization reaction and the resulting autoradiograph signals were quantitated densitometrically and normalized to the 28S ribosomal RNA signal. Both methods yielded similar results which are summarized in and reflected by the representative autoradiogram shown in Figure 3. It is evident that no significant decrease occurs in transcription of the H3, H4, and H2B histone genes following inhibition of DNA replication. Therefore, the mechanism by which histone gene expression is coupled with DNA synthesis is retained in osteosarcoma cells. In both normal diploid osteoblasts and osteosarcoma cells downregulation of histone gene expression that accompanies inhibition of DNA synthesis by hydroxyurea is controlled primarily at the posttranscriptional level.

Inhibition of Proliferation Selectively Upregulates Expression of Bone Specific Genes

To determine whether genes expressed during bone cell differentiation and in mature osteocytes are modified by DNA synthesis inhibition, we investigated the effect of hydroxyurea treatment on the expression of the type I collagen, alkaline phosphatase, osteopontin, and osteocalcin genes in proliferating ROB and ROS cells. As shown in Figure 4, inhibition of DNA synthesis resulted in a slight increase in type I collagen, alkaline phosphatase, and osteopontin mRNA in ROB as well as ROS cells. Maximal levels of upregulation are 1.4- and 1.7-fold for collagen I, 1.3- and 1.7-fold for AP and 1.5- and 1.4-fold for

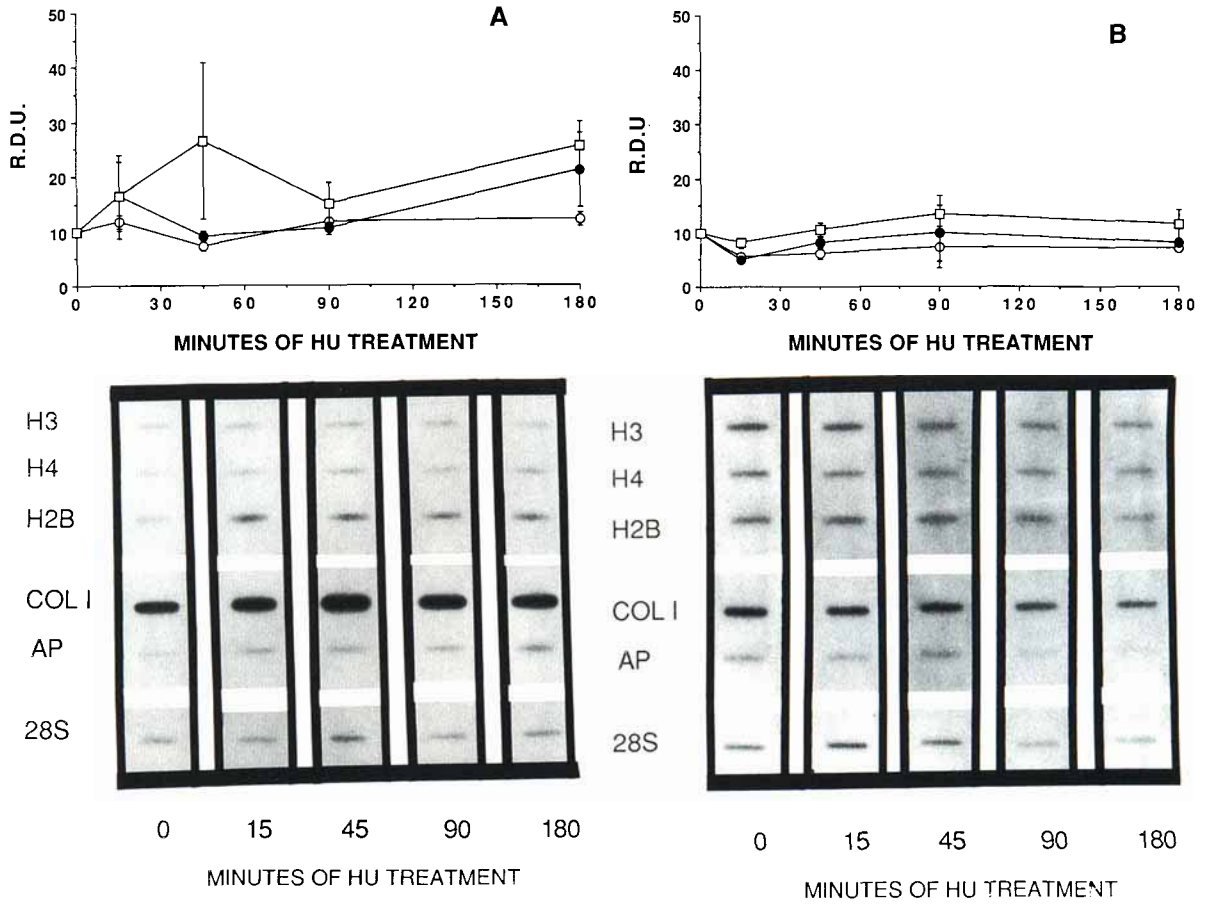

Fig. 3. Effect of DNA synthesis inhibition by HU on histone gene transcription rates in ROB **(A)** and ROS **(B)**. Cells were exposed to 1 mM HU and at indicated times nuclei were isolated and H3, H4, and H2B gene transcriptions were assayed by nuclear run-on transcription reactions as described in Materials and Methods. The graphs show the average of two independent experiments. Relative densitometry units (RDU) were normalized to the 28S ribosomal gene signal. For both cell types a representative autoradiogram is shown. 28S ribosomal signals were exposed for 30 min (A) and 1 h (B). Other gene signals were exposed for 48 h (A) and 72 h (B). Symbols are the same as Figure 2.

OP in ROB and ROS cells respectively. In general, the upregulation is more pronounced in the ROS cells, which show a concomitant expression of these genes during proliferation. No correlation between the length of treatment and extent of increased expression was observed. In contrast, hydroxyurea treatment did not affect the level of osteocalcin mRNA expression in ROS cells, and did not induce osteocalcin (data not shown) in proliferating ROB cells, which do not express the gene during the initial period of the osteoblast developmental sequence.

Figure 5 shows the relative transcription rates of collagen I and alkaline phosphatase genes after HU treatment. The osteopontin and osteocalcin signals did not exceed background levels and are therefore not plotted. In both ROB and ROS cells no obvious increase in the rate of alkaline phosphatase transcription is observed. However, there appears to be a 2-fold increase in the rate of collagen I transcription in ROB cells following inhibition of DNA synthesis. Interestingly, despite the increase in collagen I mRNA levels associated with inhibition of DNA synthesis in ROS cells (Fig. 4) this was not associated with a change in transcriptional activity of the type I collagen gene.

DISCUSSION

We have experimentally addressed the interrelationships between proliferation and genes expressed during progressive development of the bone cell phenotype from two standpoints. First, we evaluated the consequences of downregulated proliferation by DNA synthesis inhibition on expression of genes associated with cell growth and osteoblast differentiation. Second, by evaluating the effects of proliferation inhibi-

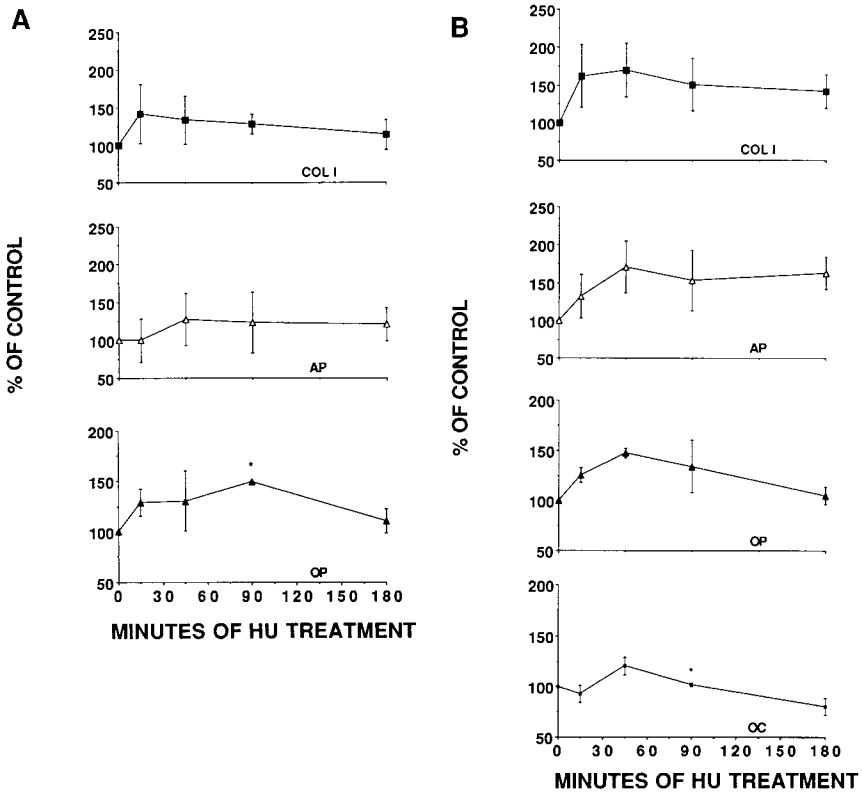

Fig. 4. Effect of DNA synthesis inhibition on mRNA levels of bone-specific genes in proliferating ROB (**A**) and ROS (**B**) cells. Treatments and conditions are similar as described in Figure 2. Collagen I (COL I), alkaline phosphatase (AP), osteopontin (OP) and osteocalcin (OC) mRNAs were analyzed by Northern blotting as described in Materials and Methods. The mRNA levels of the HU treated cells are presented relative to the mRNA levels of the untreated cells. No osteocalcin mRNA was detected in the ROB cells and was therefore not plotted. Brackets indicate the standard error of three (A) or four (B) independent experiments. At some timepoints, brackets fell within the symbols (*).

tion on developmentally expressed genes in both normal diploid osteoblasts and in osteosarcoma cells, we addressed the role of stringent growth control operative only in normal diploid osteoblasts on functional relationships between proliferation and differentiation.

Our findings indicate that expression of the cell cycle regulated histone genes remains tightly coupled with DNA replication and is principally controlled posttranscriptionally in normal diploid osteoblasts and in osteosarcoma cells. These findings are consistent with studies carried out over the last decade that demonstrate the existence of a temporal and functional coupling of cell cycle-regulated histone gene expression and DNA replication. The coordination between histone protein synthesis and DNA synthesis results from modulating cellular levels of histone mRNA [Sive et al., 1984; Stein et al., 1989; Bird et al., 1985]. When cells exit S phase, histone protein synthesis, cellular histone mRNA levels and DNA synthesis decline coordinately [DeLisle et al., 1983; Heintz et al., 1983; Baumbach et al., 1987; Morris et al., 1991]. However, in proliferating cells, the cell cycle regulated histone genes are transcribed throughout the cell cycle exhibiting only a 2- to 4-fold enhanced level of transcription during S phase [Baumbach et al., 1987; Ramsey-Ewing et al., in press] indicating involvement of both transcriptional and post-transcriptional control in confining histone protein synthesis to S phase.

Our findings that cellular levels of mRNA transcripts from cell cycle regulated histone genes are tightly coupled with DNA replication in osteoblasts and osteosarcoma cells following inhibition of DNA synthesis is in agreement with studies carried out with a broad spectrum of cell types which have demonstrated that inhibition of DNA synthesis by hydroxyurea, aphidicolin or cytosine arabinoside [Sittman et al., 1983; Gallwitz and Mueller, 1969; Sourlingas et al., 1991; Sariban et al., 1985; Baumbach et al., 1984] results in the rapid and selective decline

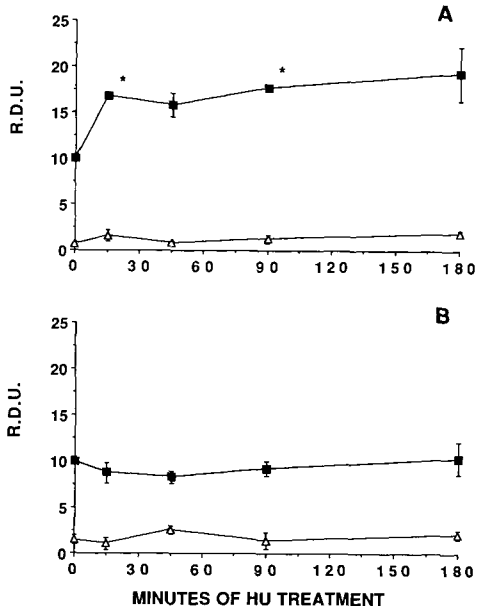

Fig. 5. Relative transcription rates of bone specific genes after DNA synthesis inhibition in proliferating ROB (A) and ROS (B) cells. Treatments and conditions are similar, as described in Figure 3. Osteopontin and osteocalcin signals did not exceed background levels and were therefore not plotted. The graphs show the average of two independent experiments. Relative densitometry units (RDU) for collagen I (■) and alkaline phosphatase (△) were normalized to the 28S ribosomal gene signal. At some timepoints, brackets fell within the symbols (*).

in histone mRNA levels from the cytoplasm [Baumbach et al., 1984, 1987; Sittman et al., 1983; Sariban et al., 1985; Zambetti et al., 1987].

While the effect of DNA synthesis inhibition on histone mRNA levels is well documented, our understanding of effects on histone gene transcription rates are limited and appear to be dependent on the cell type [DeLisle et al., 1983; Baumbach et al., 1987; Morris et al., 1991; Sittman et al., 1983; Zambetti et al., 1987; Harris et al., 1991]. Baumbach et al. [1984] demonstrated that hydroxyurea treatment of HeLa cells minimally affected histone gene transcription, which remained at approximately 80% of control levels. DeLisle et al. [1983] describe a 25% decrease in the rate of H3 histone gene transcription in 3T6 fibroblasts following cytosine arabinoside exposure. These results are similar to findings of other laboratories that have reported that following DNA synthesis inhibition cellular histone mRNA levels are predominantly downregulated at a posttranscriptional level [DeLisle et al., 1983; Baumbach et al., 1987; Morris et al., 1991; Zambetti et al., 1990; Harris et al., 1991]. In contrast, treatment of S49 lymphoma cells with either cytosine arabinoside or hydroxyurea resulted in an 80% reduction of histone gene transcription [Sittman et al., 1983]. In the present study we found that in ROB or ROS cells inhibition of DNA synthesis did not significantly reduce histone gene transcription rates. It appears that despite the abrogation of growth control in osteosarcoma cells, the fidelity of mechanisms that support the coupling of histone gene expression to DNA replication may be retained. In a more general context, variations in cell phenotype rather than transformation may be more relevant in determining the extent to which transcription is involved in controlling the relationship between histone gene expression and DNA replication.

A compelling question is the effect of downregulation of proliferation on the expression of bone specific genes. We therefore inhibited proliferative activity in ROB and ROS cells by blocking DNA synthesis with hydroxyurea. An induction of collagen I, alkaline phosphatase and osteopontin mRNA levels was observed following hydroxyurea treatment. The absence of a correlation between the length of treatment and extent of upregulation suggests that DNA synthesis inhibition itself, and not the extent of the inhibitory effect, is functionally relevant. The upregulation of alkaline phosphatase and osteopontin mRNA levels is consistent with our previous findings in normal diploid osteoblasts [Owen et al., 1990]. While the magnitude of the upregulation of alkaline phosphatase and osteopontin mRNA levels is less than previously reported, this may reflect subtle differences in primary osteoblast cultures compared to second- or third-passaged cells [Shalhoub et al., 1992]. Increased expression of differentiation-associated genes with premature downregulation of proliferation has also been described in other studies [Stepp et al., 1986; Sumpoi et al., 1990; Dedhar 1989; Arbiser et al., 1991; Cox and Park, 1989]. While Majeska et al. [1985] did not observe increased alkaline phosphatase activity in ROS 17/2.8 cells following hydroxyurea treatment, this may in part reflect cytotoxic effects of long-term exposure (3 days) to the DNA synthesis inhibitor [Coyle and Strauss, 1970; Pedrali-Noy et al., 1980].

The extent to which the upregulation of bone cell-related genes is transcriptionally mediated remains to be determined. Observed increases in some of the mRNA levels studied may be too small to detect corresponding increases at the

transcriptional level by nuclear run-on analysis. However, we were able to detect an increase in collagen I transcription following DNA synthesis inhibition in ROB cells that was not evident in osteosarcoma cells suggesting a proliferation dependent effect on transcription of bone tissue related genes that is associated with stringent growth control in normal diploid osteoblasts.

The inability of DNA synthesis inhibition to induce osteocalcin gene expression in ROB cells is consistent with the concept that there is at least a second set of genes expressed postproliferatively in mature osteoblasts. This expression is not directly coupled to the downregulation of proliferation [Stein et al., 1990a; Lian and Stein, 1992; Stein and Lian, 1993]. Gene regulatory mechanisms operative at this late transition point (onset of extracellular mineralization) during osteoblast differentiation may be retained in osteosarcoma cells despite the concomitant expression of proliferation-related and postproliferative genes, since inhibition of proliferation in ROS cells does not affect osteocalcin mRNA levels or transcription.

It is becoming increasingly evident that molecular mechanisms mediating proliferation/differentiation interrelationships that control the sequential expression of cell growth and bone cell-related genes during osteoblast differentiation are at least in part modified in osteosarcoma cells. These alterations in gene regulatory mechanisms are reflected at the transcriptional and at a series of posttranscriptional levels. Further understanding of growth regulatory mechanisms that are compromised with loss of stringent growth control in osteosarcoma cells may provide valuable insight into signaling mechanisms that support progressive development of the bone cell phenotype.

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