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Concerted Control of Multiple Histone Promoter Factors during Cell Density Inhibition of Proliferation in Osteosarcoma Cells: Reciprocal Regulation of Cell Cycle-controlled and Bone-related Genes[1]

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ABSTRACT

Cell density-induced growth inhibition of osteosarcoma cells (ROS 17/2.8) results in the shutdown of proliferation-specific histone H4 and H2B genes and the concomitant up-regulation of several osteoblast-related genes. In several respects, this reciprocal regulatory relationship is analogous to the proliferation/differentiation transition stage during development of the bone cell phenotype in normal diploid osteoblasts. Here, we comprehensively analyzed the promoter binding activities interfacing with key regulatory elements in the cell cycle-dependent histone and bone-specific osteocalcin genes. Similarly, we examined factors interacting with a series of general transcription regulatory elements that are present in a broad spectrum of promoters. The results show that histone promoter binding activities HiNF-D, HiNF-P/H4TF-2, H4UA-1, and OCT-1, as well as AP-1 activity, are proliferation dependent. These factors decline coordinately during the cessation of proliferation in both ROS 17/2.8 bone tumor cells and normal diploid osteoblasts. Collective down-regulation of these *trans*-activating factors occurs in both cell types within the physiological context of constitutive regulation of ubiquitous transcription factors (Sp1, ATF, and CCAAT binding proteins). In addition, during growth inhibition of ROS 17/2.8 cells we observe a complex series of modifications in protein/DNA interactions of the osteocalcin gene. These modifications include both increased and decreased representation of promoter factor complexes occurring at steroid hormone response elements as well as tissue-specific basal promoter sequences. These results demonstrate cell growth regulation of the promoter factors binding to the proliferation-specific histone and tissue-specific osteocalcin genes during the cessation of proliferation.

INTRODUCTION

Osteosarcoma cells (ROS 17/2.8) continuously express a series of bone-related genes (1, 2) that in normal diploid cells or *in vivo* are expressed only postproliferatively (3, 4). The expression of genes characteristic of postproliferative stages of osteoblast differentiation (*e.g.*, OC,[2] AP, and OP) occurs concomitantly with the expression of cell growth-regulated genes required for ordered progression through the cell division cycle (*e.g.*, histones H4, H3, H2B, H2A, and H1) (3, 5). Comparison of gene regulatory mechanisms operative in ROS 17/2.8 cells with those functioning in normal diploid rat osteoblast allows an assessment of the extent to which relationships between proliferation and differentiation are compromised during neoplastic transformation.

Differentiation of ROBs derived from fetal calvarial bone occurs in three well-defined stages (6–11; reviewed in Refs. 3 and 12). First, actively proliferating ROB cells express cell growth-related genes and genes associated with extracellular matrix biosynthesis (*e.g.*, type I collagen, fibronectin, transforming growth factor β). Subsequently, at a critical transition point, an integrated series of signaling mechanisms mediates cessation of cell division, resulting in the down-regulation of proliferation-specific genes and the initiation of osteoblast phenotype gene expression (*e.g.*, AP). As a result of genes expressed during both the initial proliferative and the immediate postproliferative periods, the extracellular matrix is rendered competent for the ordered deposition of hydroxyapatite by mature osteoblasts. Extracellular matrix mineralization is initiated at a second transition point, coincident with the up-regulation of mature bone phenotypic genes, including OC and OP. This staged cascade of sequential gene expression is controlled to a significant extent at the transcriptional level.

The stringently orchestrated expression of diverse genes accompanies and is required for the normal development of bone tissue, but osteosarcoma cells do not produce a bone tissue-like organization in culture. However, in these cells increased expression of bone-related genes and shutdown of proliferation-specific genes are clearly evident following cell density-dependent growth inhibition (1, 4). Thus, it is important to assess gene regulatory mechanisms operative during the proliferation arrest of ROS 17/2.8 cells for comparison with those occurring at the analogous key transition stage during normal osteoblast development.

To address transcriptional mechanisms during osteoblast development our laboratory has focused on the regulation of histone and osteocalcin genes (Refs. 10 and 13; reviewed in Ref. 14). Transcription of vertebrate proliferation-specific histone genes (15–24) and the bone-specific osteocalcin gene (25–31) is controlled by multiple evolutionarily conserved *trans*-activating factors interacting at a series of well-defined promoter recognition sites. The regulation of several of these DNA binding activities has been studied in relation to modulations of transcription during progressive development of the osteoblast phenotype (4, 13, 25, 32). These studies provide insight into transcriptional mechanisms controlling the individual histone and osteocalcin genes and address aspects of the developmental regulation of key nuclear factors executing the selective expression of these and other target genes. In this study, we compare regulatory events occurring during the cessation of ROS 17/2.8 proliferation with those occurring during normal osteoblast differentiation to gain insight into the mechanisms mediating stringent growth control of gene expression. We observe reciprocal and cell density-dependent modifications in the expression of a series of proliferation-specific (*e.g.*, histone H4 and H2B) and tissue-specific (*e.g.*, OC, OP, and AP) genes during growth inhibition of osteosarcoma cells. We systematically examined regulation of the entire complement of promoter DNA-binding activities that have been characterized previously and shown to interact with histone H4 (17, 21–36) and osteocalcin (25–27, 29, 30, 37) promoters to evaluate potential mechanisms controlling these reciprocal gene regulatory events.

MATERIALS AND METHODS

Cell Culture. Rat osteosarcoma cells (ROS 17/2.8; Merck, Sharp and Dohme, West Point, PA) were grown in F12 media (Gibco, Grand Island, NY)

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[2] The abbreviations used are: OC, osteocalcin; AP, alkaline phosphatase; OP, osteopontin; ROB, normal diploid rat calvarial osteoblasts; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; VDRE, vitamin D response element; nt, nucleotide; SSC, standard saline citrate; SDS, sodium dodecyl sulfate.

and supplemented with 5% horse serum (ICN, CA). Cells (passage 18, passed by subcultivation every 8–10 days) were seeded at a density of 1×10^6 cells/150-mm dish. Osteocalcin samples were taken from the media every other day and analyzed by radioimmunoassays (38). At days 3, 5, 7, and 10 2–5 dishes were processed for mRNA (Fig. 1) and nuclear run-on analysis (4). Nuclear extracts were made at days 5 and 10 after the cells were plated.

ROB were isolated from 21-day-old rat calvaria by sequential enzyme digestions as described (6, 39). Cells were passaged at confluency, seeded at a density of 1×10^6 cells/150-mm dish, and maintained in minimal essential media (Gibco) supplemented with 10% fetal calf serum (HyClone, Logan, Utah), 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate while changing media every other day. Proliferating ROB cells were collected 2 days after plating, and differentiated cells were harvested at day 30.

Northern Blot Analysis. Total RNA was extracted from cell pellets as described previously (40), with a LiCl precipitation procedure. RNA samples were quantitated by absorbance at 260 nm and electrophoresed on a 6.6% formamide–1% agarose gel. The integrity of the RNA was assessed by ethidium bromide staining. RNA was transferred to a zeta-probe membrane (Bio-Rad, Richmond, CA) and hybridized with probes as indicated in the figure legend. The probes were labeled with [α-^{32}P]dCTP by using a Prime-It kit (Stratagene, La Jolla, CA). Prehybridizations and hybridizations were performed at 42°C in 50% formamide, 5× SSC (20 × SSC = 0.3 M sodium chloride, 0.3 M sodium citrate), 10× Denhardt's solution, 50 mM sodium phosphate (pH 6.5), 1% SDS, and 250 μg/ml salmon sperm DNA. For hybridizations 10^6 cpm/ml probe were added, and the salt concentration was adjusted by adding 20× SSC. Blots were washed at 60°C, three times in 20× SSC, 0.1% SDS and two times in 1× SSC, 0.1% SDS. The blots were exposed to X-AR (Eastman Kodak Company, Rochester, NY).

Probes for Protein/DNA Interactions. The probes used in this study were prepared from synthetic oligonucleotides (17, 26, 36, 48) which were quantitated and 5′ end-labeled using T4 polynucleotide kinase according to standard procedures (17, 41). The radiolabeled DNA fragment used to measure HiNF-D activity was plasmid derived and encompasses the proximal promoter region of the H4-F0108 histone gene (spanning nt −97 to −38, cap site at nt −30) (36).

Preparation of Nuclear Extracts. The preparation of nuclear extracts from rat osteosarcoma cells was performed as described previously (36, 42). Nuclear extracts from rat osteoblasts were prepared as follows. Collected cells were lysed in 5 ml lysis buffer/10^8 cells (10 mM KCl, 0.5% Nonidet P-40, 10 mM HEPES, pH 7.5), and 20 ml of solution A (0.32 M sucrose, 11% w/v; 0.01% Nonidet P-40; and 5 mM HEPES, pH 7.5) were added. This suspension was carefully layered over 10 ml solution B (2.1 M sucrose, 72% w/v; 5 mM HEPES, pH 7.5). The nuclei were recovered as a pellet after centrifugation (Beckman SW 27 rotor, 13,000 rpm, for 45 min, at 4°C). The nuclei were washed with solution C (10 mM KCl, 10 mM HEPES) and recovered by centrifugation (IEC, 1500 rpm, for 5 min, at 4°C). The pellet was resuspended in extraction buffer (420 mM KCl, 0.2 mM EDTA, 20 mM HEPES, pH 7.5) in a 1.5-ml microcentrifuge tube, using approximately 0.5 ml for 1×10^8 nuclei. The nuclei were extracted for 30 min by rotating at 4°C and removed by centrifugation at 14,000 rpm for 15 min at 4°C. During the extraction procedure a broad spectrum of fresh protease inhibitors was used (phenylmethylsulfonyl fluoride, leupeptin, pepstatin, trypsin inhibitor, tolylsulfonyl phenylalanyl chloromethyl ketone, at concentrations recommended by Boehringer Mannheim, Indianapolis, IN). Desalting of the nuclear extracts was performed by diluting with storage buffer without KCl (KN0 buffer: 20% glycerol; 0.2 mM EDTA; 0.01% Nonidet P-40; 25 mM HEPES, pH 7.5; and 1 mM dithiothreitol). The protein concentration was determined by Bradford analysis (Pierce).

Gel Retardation Assays. Gel retardation assays were performed as described previously (17, 43). Protein/DNA binding reactions were obtained by combining 10 μl of a protein mixture (in KN100 buffer, which is as KN0 buffer but contains 100 mM KCl) with 10 μl of a DNA mixture, including probe DNA (10–50 fmol), 1 mM dithiothreitol, and nonspecific competitor DNA. In most gel retardation assays 0.1 μg/μl poly (dI-dC)·(dI-dC) was used as a nonspecific competitor. The type and amount of nonspecific competitor DNA used for the detection of the sequence-specific DNA binding activities described in this study [*e.g.*, poly (dG-dC)·poly (dG-dC) and poly (dA-dT)·poly (dA-dT)] were optimized for the detection of each factor as indicated in the figure legends. Competition assays were performed with a 50- to 100-fold molar excess of specific competitors (described in the figures) by incubation with the DNA mixture prior to addition of the protein mixture. The samples were loaded on a 4% polyacrylamide gel with various acrylamide:bisacrylamide cross-linking ratios and buffer conditions as optimized for the different proteins; *i.e.*, interactions of HiNF-D, ATF, SP1, AP1, CCAAT, and OCT-1 were studied using [80:1] gels with LIS buffer (43); factors HiNF-M and HiNF-P were detected using [20:1] gels with TGE buffer (50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5) (41); protein/DNA interactions at the VDRE, OC box, and TATA region were analyzed using [40:1] gels with TBE buffer (89 mM Tris, 89 mM Borate, 2 mM EDTA, pH 8.4) (4).

RESULTS

Reciprocal Patterns of Gene Regulation during Cell Density-induced Growth Inhibition of ROS 17/2.8 Cells. Cultures of ROS 17/2.8 cells are subject to an initial period of active growth (day 0 to day 6 after plating) (Fig. 1*A*), during which a low level of OC secretion into the culture medium is detected (Fig. 1*B*) and high levels of H4 and H2B gene expression are observed (Fig. 1*C*). In the subsequent stage (day 7 to day 10), OC secretion reaches maximum levels (Fig. 1*B*), and the levels of H4 and H2B mRNA are down-regulated below the level of detection (Fig. 1*C*). Because of the tight linkage between histone mRNA levels, DNA replication, and progression through S phase in the cell division cycle (14, 44), these results indicate that under these culture conditions ROS 17/2.8 cells cease to proliferate and enter a cell growth-inhibited state. In contrast to the down-regulation of proliferation-specific histone genes, constitutive levels of type I collagen expression are observed, whereas the expression of OC, OP, and AP genes is up-regulated above basal levels initially observed in actively dividing ROS 17/2.8 cells (Fig. 1*C*). These results suggest that the levels of OC, OP, and AP gene expression are coupled in part to the cessation of cell proliferation.

Coordinate Regulation of H4 Gene Promoter Factors HiNF-D, HiNF-P/H4TF-2, and H4UA-1 during Cessation of ROS Proliferation. The down-regulation of histone H4 and up-regulation of OC gene expression during density-induced growth inhibition of osteosarcoma cells is mediated in part by changes in gene transcription rates as measured by nuclear run-on analysis (4). Multiple promoter factors involved in the control of histone and osteocalcin genes have been extensively studied. Transcriptional control of histone H4 gene expression during the onset of differentiation is reflected by selective modifications in interactions of *trans*-activating factors with both proximal and distal *cis*-acting elements (13, 17, 33, 35, 45). However, the regulation of H4 promoter factors has not been assessed at the cessation of cell proliferation, irrespective of mechanisms mediating the initial onset of differentiation. To gain insight into regulation of both H4 gene transcription as well as the corresponding control of H4 promoter factors, we analyzed the entire complement of H4 promoter DNA binding activities when ROS 17/2.8 cells become cell growth inhibited. We used a comprehensive panel of promoter segments (Fig. 2), representing all protein/DNA recognition sites established for a prototypical H4 gene in gel mobility shift assays. We monitored binding activities interacting with each DNA probe using nuclear protein preparations of actively proliferating (day 5) and density-inhibited (day 10) ROS 17/2.8 cells (Figs. 3 to 5). Our results show that factors HiNF-D (Fig. 3*A*), HiNF-P/H4TF-2 (Fig. 3*B*), and H4UA-1 (Fig. 3*C*) are not detected in cell density-inhibited ROS 17/2.8 cells. In comparison, HiNF-M (Fig. 3*B*) is present in ROS 17/2.8 cells both early and late during the culturing period. These results indicate that the binding activities of HiNF-D and HiNF-P/H4TF-2 are selectively down-regulated, whereas HiNF-M binding activity is constitutive.

The interactions of HiNF-D, HiNF-P/H4TF-2, and HiNF-M occurring at the cell cycle regulatory region of this gene (designated H4 Site II) have been studied in detail previously by, *i.e.*, methylation inter-

ference and mutational analyses (17, 36). Unambiguous assignment of these factors in this study is based on competition analyses using specific wild-type and mutant competitors (see Fig. 3, *right*) as well as exact comigration with previously characterized protein/DNA complexes. However, distinct probes spanning H4 Site II sequences will also interact less reproducibly with uncharacterized and mostly nonspecific factors (*e.g.*, see Fig. 3, *A* and *B*). For example, a band migrating more slowly than the HiNF-M complex is observed in both proliferating and confluent cells (Fig. 3*B*, *band NS*). This and other nondesignated bands represent nonspecific DNA binding proteins, because in each case no competition is observed with specific oligonucleotides. Similarly, unidentified complexes were observed with most of the other factor-specific probes used in this study, and analogous explanations apply in each case.

Two complexes migrating faster than the HiNF-P/H4 Site II interaction occur with proteins from confluent cells (Fig. 3*B*). We have previously shown that there is at least one posttranslationally modified electrophoretic variant of HiNF-M (36). Whereas HiNF-M may be a phosphorylated heteromeric factor, to date only a single electrophoretic form of the HiNF-P/H4TF-2 complex is known (17, 34). Competition characteristics of these two high-mobility complexes are indistinguishable from those of HiNF-M (data not shown), suggesting a relationship with HiNF-M. The physiological significance of the abundance of these factors in confluent cells, when HiNF-M activity persists, remains to be established.

Binding Activities of Ubiquitous Transcription Factors Remain Constitutive during ROS 17/2.8 Cell Density Inhibition. Members of transcription factor families with broad promoter binding specificity, including Sp1-, ATF-, and CCAAT box-related factors (46), have been shown to interact with the promoters of several distinct histone gene subtypes (*e.g.*, H4, H3, and H1) using several *in vitro* (35, 36, 47, 48) and *in vivo* (49, 50) approaches. The nuclear abundance of promoter factors binding to Sp1, ATF, and CCAAT elements is similar in proliferating and density-inhibited ROS 17/2.8 cells (Fig. 4). For example, Sp1 (Fig. 4*A*), ATF-84 (Fig. 4*B*), and CCAAT box protein HiNF-B (Fig. 4*C*) remain constitutive in both proliferating and confluent ROS 17/2.8 cells. The constitutive presence of these factors is consistent with their postulated roles in regulating general ("housekeeping") and tissue-specific genes (46). In addition, these results provide qualitative and quantitative internal standards for the general integrity and extractability of nuclear factors in our protein preparations, apart from normalization based on protein content.

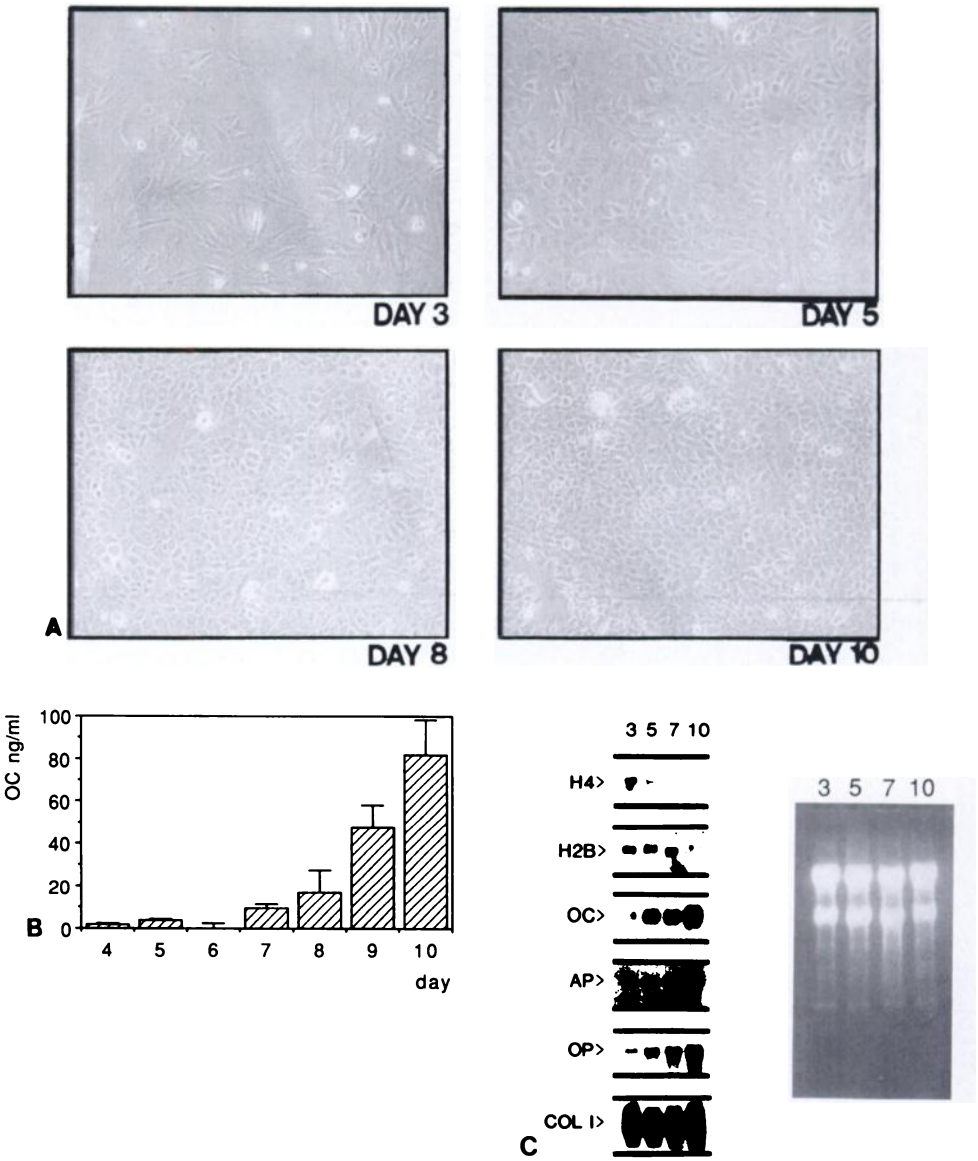


Fig. 1. *A*, rat osteosarcoma cells (ROS 17/2.8) cultured *in vitro* during density-induced cell growth inhibition. The photomicrographs were taken at days 3, 5, 8, and 10 after cell plating. *B*, osteocalcin production per day as determined by radioimmunoassays. The graph shows the increase in osteocalcin secretion into the culturing media when ROS 17/2.8 cells reach confluency. Each point represents the average of 5 samples, which were taken from the media of 5 different plates. *C*, *left*, Northern blot analysis of total cellular RNA from ROS 17/2.8 cells during the cessation of proliferation. Blots were analyzed for the representation of mRNAs encoded by cell cycle-dependent and tissue-specific genes. The probes used span mRNA coding sequences of H4, H2B, OC, AP, OP, or type I collagen (coll) and were prepared as reported previously (40, 60). The figure shows that the abundance of cell cycle-dependent histone H4 and H2B mRNAs decreases during density inhibition of rat osteosarcoma cells, whereas the levels for osteocalcin, alkaline phosphatase, and osteopontin increase. On the *right*, the integrity of mRNA extracted from rat osteosarcoma cells is shown in an ethidium bromide-stained gel. Each lane contains 10 μg of total RNA, extracted at, respectively, days 3, 5, 7, and 10.

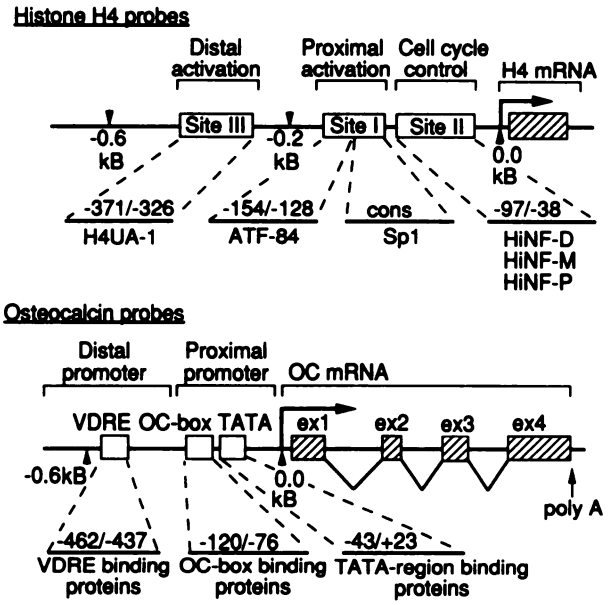


Fig. 2. The probe DNA fragments used in this study are shown relative to the modular organization of a prototypical cell cycle-regulated histone H4 gene promoter (*top*) and the bone-specific OC gene promoter (*bottom*). The nucleotide positions are indicated for each probe, except the Sp1 oligonucleotide, which conforms to an Sp1 consensus element. The histone H4 promoter contains a series of proximal and distal transcriptional domains (H4 Sites I to III) (*boxes*). These domains are bipartite and multipartite, and each interacts with several promoter factors as indicated (*e.g.*, HiNF-D, Sp1, and H4UA-1; reviewed in Refs. 14 and 17). The osteocalcin promoter contains steroid hormone response elements (*e.g.*, VDRE) and basal tissue-specific elements (OC box and TATA box). Whereas the OC gene represents a single gene in the rat genome (28), the H4 gene is present in multiple copies (44), each with a different organization of promoter elements. The distribution of protein/DNA interactions indicated in the diagram represents that of the human H4-FO108 gene, which has been most extensively studied (14, 17). Each of the evolutionarily conserved promoter elements depicted here has homologous counterparts in the promoters of several other vertebrate H4 genes, as well as other histone gene subtypes (17, 44).

Down-Regulation of the Binding Activities of Histone H2B Gene Transcription Factor OCT-1 and the FOS/JUN-related Oncoprotein AP-1. The octamer transcription factor OCT-1 was identified initially as a critical histone H2B gene *trans*-activating protein (51). OCT-1 belongs to a class of homeo- and POU/homeo-domain proteins that recognize the octameric sequence 5′-dATTTGCAT (52) and perform important gene-regulatory roles during development (53). The binding activity of OCT-1 is cell growth regulated in several cell types (53, 54, 55), but regulation of OCT-1 has not been studied in bone cells. Our data show that OCT-1 binding activity is detectable in proliferating ROS 17/2.8 cells but is absent in ROS 17/2.8 cells that are density inhibited (Fig. 5*A*). Together with results presented in Figs. 1*C* and 3, these findings establish that down-regulation of H4 and H2B gene expression in ROS 17/2.8 cells (Fig. 1*C*) corresponds with the down-regulation of the key cognate factors HiNF-D and HiNF-P/H4TF-2 for the H4 gene (Fig. 3, *A* and *B*) and OCT-1 for the H2B gene (Fig. 5*A*).

The AP-1 oncoprotein family represents a heterogeneous class of transcription factors that are intimately associated with cell growth-related intracellular signaling mechanisms (56, 57). AP-1 activity is thought to be involved in the positive regulation of several but not all histone genes (20) and is implicated in the cell growth-related suppression of basal level osteocalcin gene expression during osteoblast development (3, 26). The dual role of AP-1 proteins in the potentially negative or positive influence of gene transcription is consistent with the versatile role of AP-1 in developmental regulation (56, 57). Our results show that AP-1 binding activity is down-regulated in growth-inhibited ROS 17/2.8 cells (Fig. 5*B*). Hence, cell growth regulation of AP-1 binding activity correlates with both the up-regulation of the basal level of OC gene expression and the down-regulation of histone

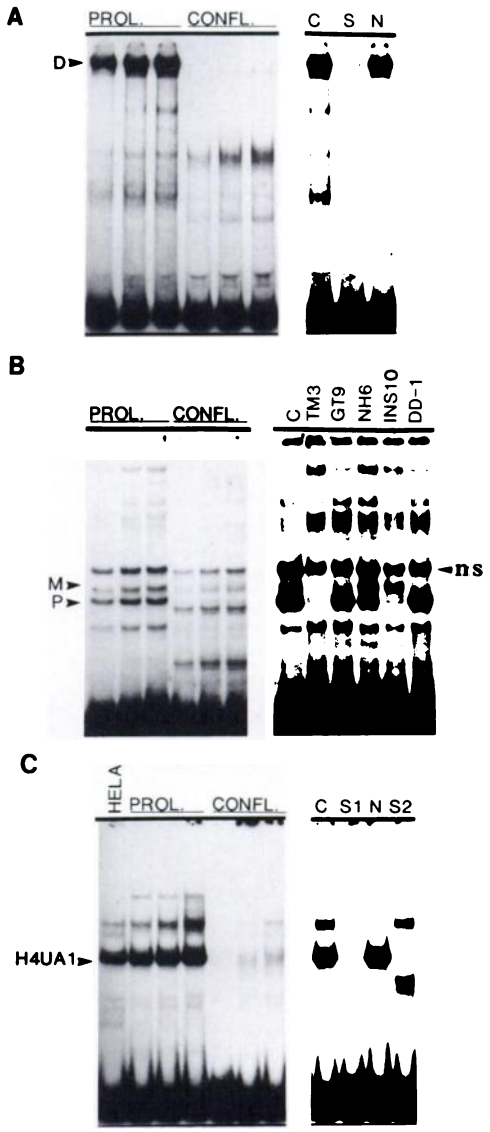


Fig. 3. Protein/DNA interactions at Sites II and III of the H4 gene promoter during growth inhibition of ROS 17/2.8 cells. In *A*, HiNF-D activity is detectable in proliferating rat osteosarcoma cells (*first 3 lanes from the left*, 2.5, 3.8, and 5 μg nuclear protein, respectively) but not in density-inhibited ROS cells (*middle 3 lanes, left to right*, 2.5, 3.8, and 5 μg, respectively). Competition analysis was performed in the presence of 5 μg protein from proliferating ROS 17/2.8 cells with a 100-fold molar excess of either a specific oligonucleotide spanning the HiNF-D binding site (designated TM3; *Lane S*) that competes or a HiNF-D mutant oligonucleotide (SUB-11; *Lane N*) that does not compete (17). The probe spans H4-Site II (nt −97 to −38) of the H4 gene promoter (*EcoRI/HindIII* fragment of pFP202 (41). *B*, HiNF-M binding activity is detectable in proliferating as well as in density-inhibited rat osteosarcoma cells, whereas the level of HiNF-P decreases to below the limit of detection during growth inhibition. Nuclear extracts from both proliferating (*PROL.*) and confluent (*CONFL.*) ROS 17/2.8 cells were used in the assay. The amount of nuclear protein used in each binding reaction was, respectively, 5, 7.5, and 10 μg for each preparation. Competition analysis is shown in *5 right-hand lanes* using oligonucleotides (described in detail in Ref. 17) that compete for both HiNF-P and HiNF-M (*TM3*) or specifically compete for either HiNF-P (*NH6*) or HiNF-M (*DD-1*) and specifically mutated DNA fragments that do not compete for HiNF-P (*GT9*) or HiNF-M (*INS10*). *ns*, nonspecific complex. The probe fragment encompasses Site II of the histone H4-FO108 gene promoter and spans nt −97 to −38; binding reactions were performed in the presence of 1 μg salmon sperm DNA. In *C*, H4UA-1 binding activity decreases during osteosarcoma cell growth inhibition. Shown is a gel retardation assay monitoring the abundance of H4UA-1 binding activity interacting with Site III of the H4 gene promoter using nuclear extracts from proliferating (*PROL.*) and density-inhibited (*CONFL.*) ROS 17/2.8 cells. The amounts of protein loaded for each extract were, respectively, 2.5, 3.8, and 5 μg. *First lane on the left*, pattern obtained using 5 μg of protein from HeLa S3 cells. Competition analysis was performed with 5 μg protein from proliferating ROS 17/2.8 cells in the absence of competitor DNA (C) or in the presence of specific competitor (*S1*, H4 Site III, spanning nt −369 to −323; *S2*, proximal part of H4 Site III (nt −349 to −323) or nonspecific competitor (*N*; distal part of H4 Site III (nt −369 to −349). The probe for H4 Site III encompasses nt −326 to −371 and was used in the presence of 2 μg poly (dA-dT)·poly (dA-dT) DNA and 1 μg poly (dI-dC)·poly (dI-dC) as described previously (33).

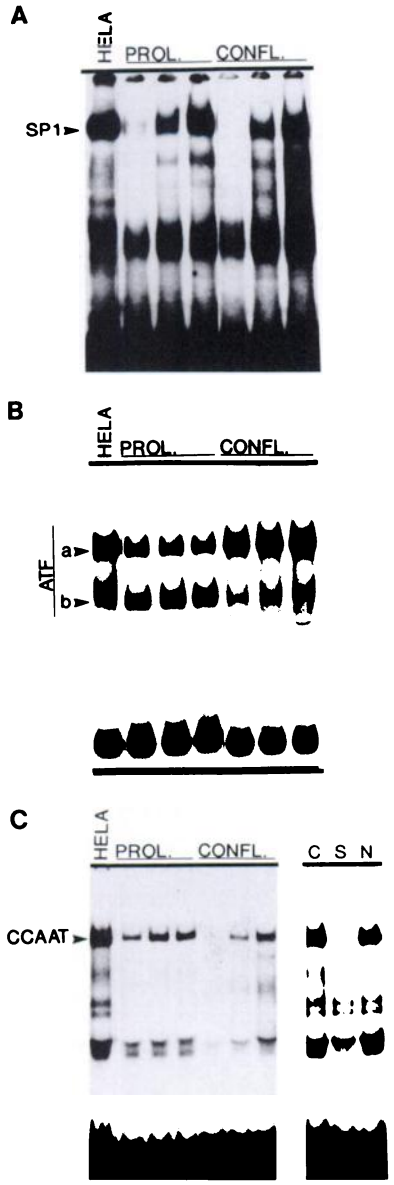


Fig. 4. Analysis of general transcription factor binding activities in nuclear extracts derived from proliferating and density-inhibited rat osteosarcoma cells. *A*, Sp1 binding activity was assayed in nuclear extracts from proliferating (*PROL.*) and growth-inhibited (*CONFL.*) ROS 17/2.8 cells. The *first lane on the left* shows Sp1 binding activity present in HeLa nuclear extracts (6 μg); *Lanes 2–4 from the left*, 1.9, 2.9, and 5.8 μg of nuclear protein, respectively, from proliferating cells; *Lanes 5–7 from the left*, 2, 4, and 6 μg of nuclear protein, respectively, from growth-inhibited cells. The probe is a consensus Sp1 binding site oligonucleotide (36) similar to the sequence present in the proximal portion of H4-Site I (Fig. 2). In *B*, total ATF binding activity (*a* and *b*) was monitored during density inhibition of rat osteosarcoma cells. The *first lane from the left* contains 2.5 μg protein from HeLa cells; *Lanes 2–4 from the left*, 2.3, 4.5, and 6.8 μg protein, respectively, from proliferating ROS 17/2.8 cells; *Lanes 5–7 from the left*, 3, 4.8, and 6.3 μg of protein, respectively, from density-inhibited osteosarcoma cells. The probe spans the ATF binding site present in the distal portion of H4-Site I (nt −154 to −128; DS-I oligonucleotide) in the H4-FO108 gene promoter. The biological importance of differences in relative band intensities of complexes *a* and *b* is not clear. For example, this ratio varied from preparation to preparation with proliferating cells, and we could not discern a consistent pattern. In *C*, protein/DNA interactions at the CCAAT-box element of the H3 histone gene promoter was analyzed during growth inhibition of osteosarcoma cells. *Lane HELA*, 7.5 μg protein from HeLa nuclei; *Lanes 2–4 from the left*, 3.9, 7.7, and 11.5 μg protein, respectively, from proliferating ROS 17/2.8 cells; *Lanes 5–7 from the left*, 2.5, 3.8, and 5 μg protein, respectively, from density-inhibited ROS. The CCAAT sequence used as probe is derived from the histone H3-ST519 gene promoter (nt −139 to −110) (48). Competition analysis was performed with 5 μg protein from HeLa nuclear extracts in the presence of no competitor (*Lane C*), the unlabeled CCAAT element oligonucleotide as specific competitor (*Lane S*), or the Sp1 fragment as nonspecific competitor (*Lane N*).

gene expression (Fig. 1). Moreover, the decrease in overall AP-1 activity occurs in concert with the decline in H4 promoter factors HiNF-D, HiNF-P/H4TF-2, and H4UA-1 (Fig. 3*C*) as well as OCT-1 binding activity (Fig. 5*A*). The down-regulation of this series of proliferation-related transcription factors during cell growth inhibition of ROS 17/2.8 cells may reflect global modifications of gene-regulatory mechanisms.

Protein/DNA Interactions in the OC Promoter during Up-Regulation of OC Gene Expression from Basal Level. Regulation of osteocalcin transcription is mediated by several distinct transcriptional mechanisms, including the involvement of factors regulating tissue-specific basal levels of transcription and steroid hormone responsiveness (*e.g.*, the synergistic and/or antagonistic interplay of vitamin D and glucocorticoid) (3, 5). In addition, a postulated model for transcriptional repression may be operative whereby AP-1 protein interacts with specific sites in the OC promoter and suppresses the osteoblast phenotype-specific expression of the OC gene (26). Vitamin D and dexamethasone-inducible protein/DNA interactions have been extensively studied during osteoblast development at three key *cis*-acting elements in the OC promoter (see Fig. 2): respectively, the VDRE, the OC box, and the most proximal promoter region containing the TATA box region (4, 25–27, 32, 37). However, to date regulation of OC promoter element occupancy in the absence of exogenous, pharmacological doses of steroid hormones has not been studied

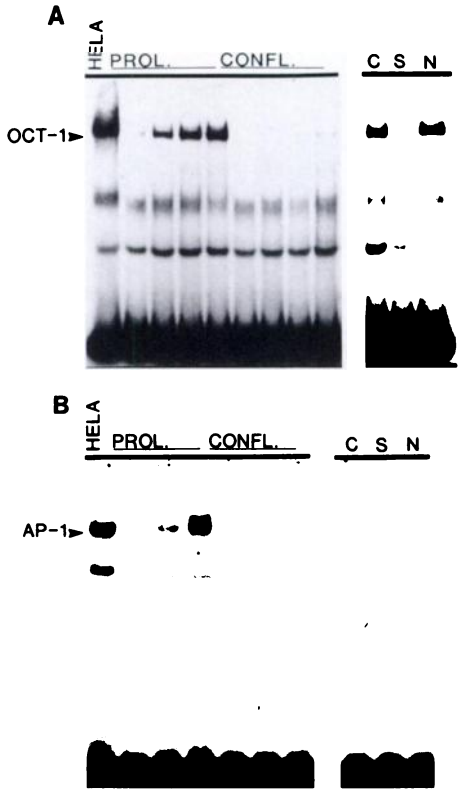


Fig. 5. *A*, OCT-1 binding activity decreases during density inhibition of rat osteosarcoma cells. *Lane HELA*, 1 μg HeLa nuclear protein. The amounts of protein loaded from proliferating ROS (*Lanes 2–5 from the left*) and from density-inhibited ROS (*Lanes 6–9 from the left*) cells were, respectively, 0.4, 0.8, 1.1, and 1.5 μg in each set. The probe used a synthetic oligonucleotide spanning an OCT-1 element of a human H2B gene (51), and 2 μg of poly (dI-dC)·poly (dI-dC) were present in the binding reaction. Competition analysis was performed with the unlabeled OCT-1 DNA fragment (*Lane S*), with the Sp1 oligonucleotide (*Lane N*), or in the absence of competitor DNA fragments (*Lane C*) using 1 μg HeLa nuclear protein. *B*, AP-1 binding activity is present in proliferating (*PROL.*) but not in confluent (*CONFL.*) ROS cells. For both nuclear extract preparations, binding reactions contained, respectively, 2.5, 3.8, and 5 μg protein. Competition analysis was performed with 5 μg protein from confluent ROS 17/2.8 cells using the following DNA fragments, respectively: *Lane 3 from the left*, no competitor DNA added (*C*); *Lane 4 from the left*, AP-1 as specific competitor (*S*) and lane 5 with SP1 as nonspecific competitor (*N*). The probe is a synthetic AP-1 consensus oligonucleotide (26), and 2 μg of poly (dI-dC)·poly (dI-dC) were present in the binding reaction. The protein/DNA complexes were separated on a 4% (40:1) polyacrylamide gel using TGE buffer.

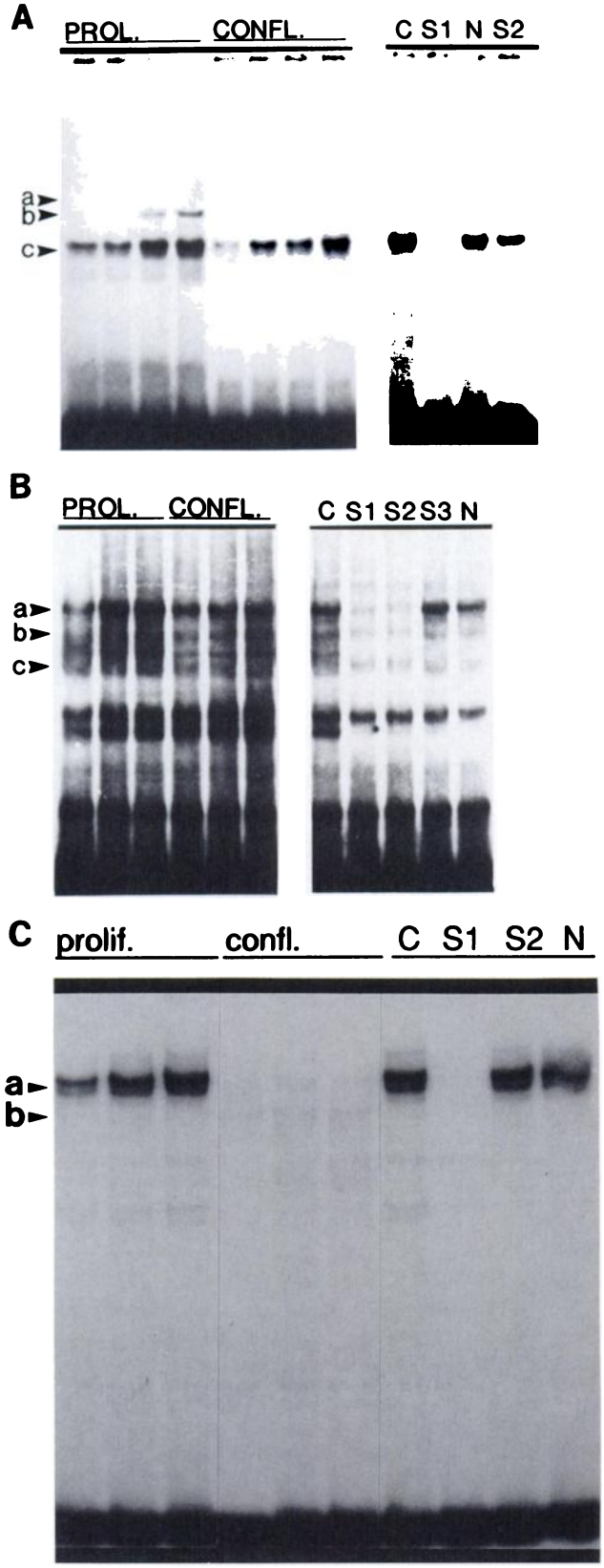


Fig. 6. Protein/DNA interactions in the osteocalcin gene promoter during growth inhibition of rat osteosarcoma cells. *A*, nuclear proteins interacting with the rat vitamin D-responsive element (nt −462 to −437) are observed in both proliferating (*PROL.*) and density-inhibited (*CONFL.*) ROS cells (for each set, respectively, 1.25, 2.5, 3.8, and 5 μg protein were used). Competition assays were performed with 5 μg of nuclear extract derived from proliferating ROS cells using a panel of oligonucleotides (*S1*, unlabeled rat osteocalcin VDRE; *N*, Sp1; *S2*, human osteocalcin VDRE). In *B*, the protein/DNA interactions at the osteocalcin box are similar in proliferating (*PROL.*) and confluent (*CONFL.*) ROS cells. Increasing amounts of nuclear protein were used in each set: 3.2, 4.8, and 6.4 μg, respectively. Competition analysis was performed with 5 μg of confluent ROS nuclear extract (*Lanes 7–11 from the left*) using DNA fragments spanning nt −120 to −76 (*S1*), nt −120 to −89 (*S2*), nt −99 to −76 (*S3*), and a CCAAT box containing oligonucleotide (*N*). The probe spans nt −120 to −76 of the rat osteocalcin promoter. The final salt concentration in the binding reaction was 90 mM KCl. In *C*, factors binding to the probe spanning the TATA region (nt −43 to +23) are observed in nuclear extracts prepared from proliferating (*prolif.*) and density-inhibited (*confl.*) ROS cells. Increasing amounts of nuclear proteins were used in each set: 2.5, 3.8, and 5 μg, respectively. Competition analysis was performed with 5 μg protein from proliferating ROS 17/2.8 cells using DNA fragments spanning nt −43 to −16 (*S1*), nt −26 to +10 (*S2*), or an SP1 consensus oligonucleotide (*N*). Based on the conditions used here (*e.g.*, modifications in nuclear extract procedures) we do not detect glucocorticoid receptor complexes that have been observed recently in related studies (63).

comprehensively in direct comparison with the down-regulation of proliferation-specific transcription factors.

To correlate the regulation of protein/promoter interactions at the differentiation-specific OC promoter with cell growth-controlled binding activities, we assessed the formation of protein/DNA interactions at the VDRE, OC box, and the TATA box regions during cell growth inhibition of osteosarcoma cells (Fig. 6). The interaction patterns at the VDRE (Fig. 6*A*) of proliferating and density-inhibited ROS 17/2.8 cells are similar but not identical. At least two of the protein/DNA complexes at the VDRE observed under the conditions examined here are sequence-specific (Fig. 6*A*) and reflect the binding of a distinct basal promoter factor (VDRE complex C) as well as one interaction involving the vitamin D receptor (VDRE complex A) (4, 27). We observed a third complex (VDRE complex B), but characterization of this complex is incomplete at present. VDRE complex A competes with both the rat (Fig. 6*A*, *Lane S1*) and human (Fig. 6*A*, *Lane S2*) osteocalcin VDREs, because the vitamin D receptor is capable of binding to steroid hormone half-sites present in both DNA fragments as observed previously (27, 32). VDRE complex C competes only with the rat osteocalcin VDRE, suggesting that this factor recognizes sequences that are present in the rat OC VDRE fragment but not in the human OC VDRE fragment.

The detection of the rat vitamin D receptor by gel shift assays (*i.e.*, complex A in Fig. 6*A*) is related to activation of this steroid hormone-binding protein by endogenous vitamin D present at low levels in the culture medium. However, because the nuclear protein preparations were derived from uninduced osteosarcoma cells (*i.e.*, not treated with additional vitamin D), the signal on the autoradiogram that represents the vitamin D receptor complex (complex A) is weak. The similarities in protein/DNA interactions at the VDRE of dividing and nondividing ROS 17/2.8 cells suggests that the up-regulation of OC transcription from minimal basal levels when ROS 17/2.8 reach confluency does not involve dramatic changes in the levels of these DNA binding activities interacting with the VDRE. However, we cannot exclude the possibility that subtle differences in the representation of the complexes detected here may have physiological significance.

Several interactions occur at the OC box (Fig. 6*B*), and one of these (OC box complex A) involves a recently characterized protein related to the steroid hormone receptor family. OC box complex A is presumably related to two other complexes (OC box complexes B and C) by phosphorylation-dependent protein/protein associations (58). The constitutive presence of proteins mediating complexes A, B, and C at the OC box during ROS 17/2.8 cell density inhibition indicates that the basal level of OC transcription is not induced by dramatic changes in the nuclear abundance of these DNA-binding activities. The results obtained for factors interacting with the VDRE and OC box (Fig. 6*B*), in direct comparison with those presented in Figs. 3 and 5, are in contrast to the modifications in protein/DNA interactions observed for cell growth-controlled histone genes.

The TATA region interacts with a novel and pronounced DNA-binding activity (TATA region complex A) that is clearly present in proliferating cells, but its abundance is severely reduced in nonpro-

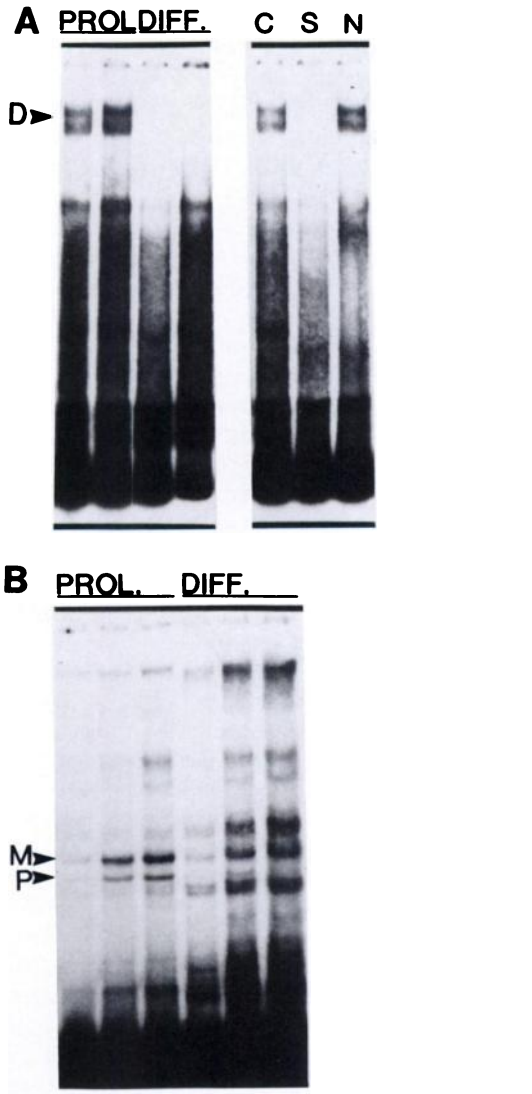


Fig. 7. *A*, protein/DNA interactions to Site II of the H4 histone gene promoter during differentiation of normal rat osteoblasts. The autoradiogram shows a gel shift assay indicating that HiNF-D binding activity is detectable in nuclear extracts from proliferating rat osteoblasts (*Lanes 1 and 2 from the left*, 2 and 4 μg, respectively) but not in differentiated rat osteoblasts (*Lanes 3 and 4 from the left*, 1.9 and 3.8 μg, respectively). Competition analysis is shown in the *last 3 lanes on the right*. Reactions were performed in the absence of competitor (*C*, control) or the presence of a 100-fold molar excess of specific (*S*, TM3) or nonspecific (*N*, DD-1) competitor DNA fragment (17). Binding reactions were performed using the nt −130 to −38 promoter fragment of the H4-F0108 gene cloned in pFP-1 (43) in the presence of 2 μg of poly (dG-dC)·poly (dG-dC). *B*, regulation of HiNF-P and HiNF-M binding activity during rat osteoblasts differentiation (*PROL.*, 2.6, 3.9, and 5.2 μg; *DIFF.*, 2.8, 4.1, and 5.5 μg). Reactions were performed using a fragment spanning nt −97 to −38 of the histone H4-FO108 gene promoter in the presence of 1 μg salmon sperm DNA.

liferating ROS 17/2.8 cells. In contrast, a second activity (TATA region complex B) is present primarily in confluent cells. We also observed several weaker interactions (not indicated) mediated by factors that are constitutively present in both proliferating and nonproliferating ROS 17/2.8 cells, similar to the observations for the OC box and VDRE. These minor complexes were not further characterized. Competition analysis using oligonucleotides spanning specific portions of the TATA box region shows that a DNA fragment spanning nucleotides −26 to +10 (Fig. 6*B*, *Lane S2*) does not compete for TATA region complexes A and B. However, an oligonucleotide spanning nucleotides −43 and −16 (Fig. 6*B*, *Lane S1*) competes for both complexes. The presence of the TATA box consensus sequence in this fragment and the strong detection of TATA region complex A in proliferating cells suggest a repressive function for the cognate protein. Because complexes A and B have similar competition behaviors, it is possible that complex A is converted into complex B at confluency. Regardless of the last conclusion, the down-regulation of the factor mediating complex A establishes that at least one protein/DNA

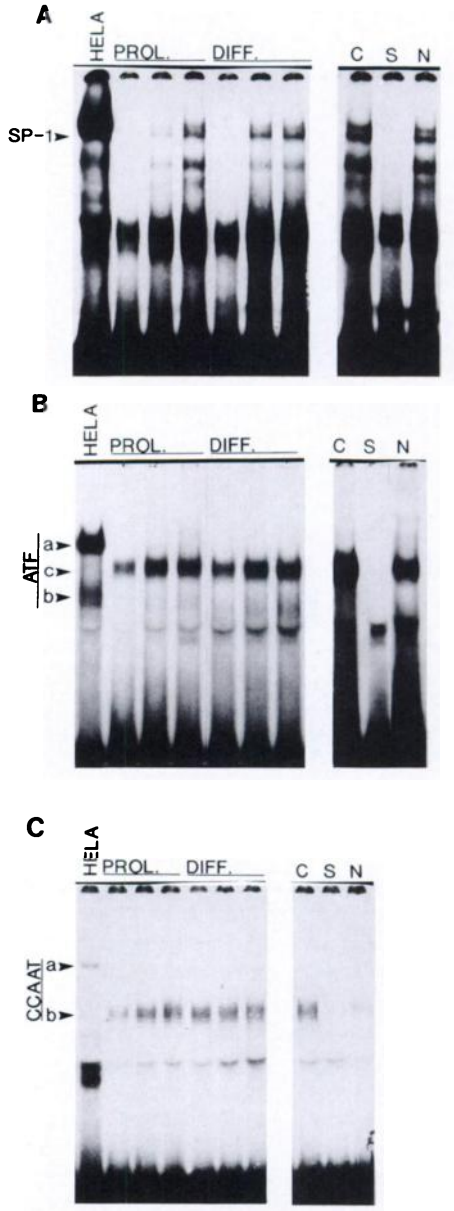


Fig. 8. Gel mobility shift analysis monitoring abundance of general transcription factors in nuclear extracts derived from proliferating (day 2) and differentiated (day 30) ROB cells. In *A*, the radiolabeled oligonucleotide containing the Sp1 binding site (36) was incubated with increasing amounts of nuclear extracts derived from HeLa S3 cells (*HELA*, 4 μg of protein), proliferating rat osteoblasts (*PROL.*, 3, 6, and 9 μg), and differentiated rat osteoblasts (*DIFF.*, 2, 4, and 8 μg). The *last 3 lanes on the right* represent a competition analysis, using 12 μg protein from proliferating rat osteoblasts. Reactions were performed in the absence of competitor DNA (*C*) or in the presence of specific competitor Sp1 (*S*) or the nonspecific competitor AP-1 (*N*). Additional sequence-specific complexes observed for this probe may reflect posttranslationally modified forms of Sp1 (*e.g.*, oligomeric, glycosylated, and/or phosphorylated variants) (46). *B*, ATF binding activity was present in both proliferating (*PROL.*, 0.9, 1.9, and 2.8 μg of nuclear extract) and differentiated (*DIFF.*, 1.3, 1.9, and 2.5 μg) rat osteoblasts. *Lanes C, S,* and *N*, a competition assay in the absence (*C*) or in the presence (*S*, specific ATF competitor DNA; *N*, nonspecific, Sp1 oligonucleotide) of oligonucleotide competitor DNA. *Lane HELA*, 5 μg of HeLa nuclear protein. The probe spans the distal part of H4-site I (spanning nt −154 to −128 in the H4 gene promoter). In *C*, the CCAAT-binding element present in the H3 histone gene promoter interacts with nuclear proteins derived from HeLa S3 cells (*Lane HELA*, 2 μg), proliferating rat osteoblasts (*PROL.*, 1, 1.9, and 2.9 μg), and differentiated rat osteoblasts (*DIFF.*, 1.4, 2.1, and 2.8 μg). The *last 3 lanes on the right* show a competition analysis in the presence of no competitor DNA (*C*), with CCAAT (*S*) and ATF (*N*) as competitors. The probe used in this assay was identical to the fragment used in Fig. 4*C*.

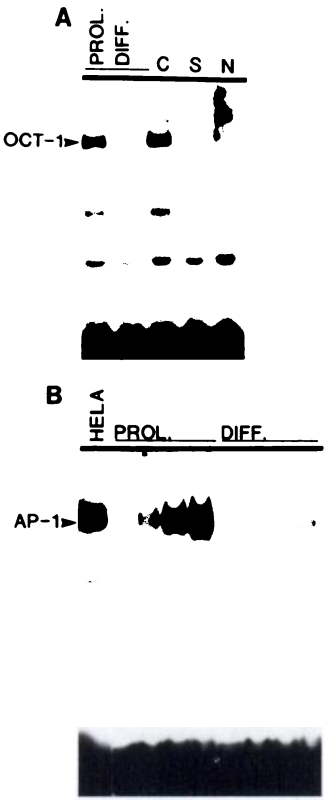


Fig. 9. *A*, regulation of OCT-1 binding activity to the H2B histone gene promoter during differentiation of normal rat osteoblasts. Each lane contains 1 μg of nuclear protein. *Lanes C, S,* and *N*, competition with 1 μg of HeLa nuclear extract. *Lane 3*, no competitor (*C*); *Lane S*, OCT-1 oligonucleotide as specific competitor; *Lane N*, with Sp1 as nonspecific competitor oligonucleotide. The same probe and competitor DNA fragments were used as described in Fig. 5*A*. *B*, regulation of AP-1 binding activity during osteoblast differentiation. *Lane HELA*, 4 μg of HeLa nuclear extract. *PROL.*, 1.6, 3.1, 4.7, and 6.3 μg of proliferating ROB cells; *DIFF.*, 1.1, 2.2, 3.3, and 4.4 μg of differentiated ROB nuclear extracts. The probe spans an AP-1 consensus sequence (see Fig. 5*B*).

interaction changes during the up-regulation of OC expression when ROS 17/2.8 cells cease to divide.

The down-regulation of TATA region complex A observed in ROS 17/2.8 cells at the cessation of proliferation (Fig. 6*C*) is similar to the down-regulation of OCT-1 (Fig. 5*A*) and HiNF-D (Fig. 3*A*). When ROS 17/2.8 cells reach confluency, these cells develop a partially multilayered stratum of cells embedded in a dense collagen type I extracellular matrix. However, in some experiments we have observed that ROS 17/2.8 cells partially dissociate from the culture vessel during the later stages (day 7 to day 10) of the culturing period (data not shown). Nuclear protein preparations from these cultures did not show a decreased abundance of the factor mediating TATA region complex A, but no decrease was observed for OCT-1 or HiNF-D binding activity either. Although the reasons for cell dissociation remain to be established, cell cultures subject to this phenomenon displayed signs of continued cell division as visually evidenced by the abundance of mitotic cells (data not shown). Presumably, cell proliferation in these cultures may reflect the colonization of areas on the surface of the culture vessel that were rendered vacant due to cell dissociation.

Selective Down-Regulation of Several H4 Gene Promoter Factors during Normal Rat Osteoblast Development. It is of considerable interest to assess the extent to which the modification in transcription factor binding activities during ROS 17/2.8 cell growth inhibition reflect events occurring during normal diploid osteoblast differentiation. Similar to ROS 17/2.8 cells (4), normal diploid ROB cells modify cell-stage-specific patterns of gene expression in part by modulating gene transcription rates (13). We have previously shown that transcriptional control of the histone H4 gene during osteoblast differentiation is reflected by selective modifications in interactions of *trans*-activating factors with both proximal and distal *cis*-acting elements. For example, the interactions of HiNF-D with the proximal cell cycle control domain H4 Site II and H4UA-1 with the distal stimulatory domain H4 Site III are down-regulated upon cessation of histone gene transcription during the onset of differentiation in osteoblasts (13, 33), as well as in other cell types (45). However, the developmental regulation of several recently described H4 promoter factors, including HiNF-M and HiNF-P/H4TF-2 (17, 36), has not been addressed.

To gain further insight into the regulation of both H4 gene transcription and the corresponding control of H4 promoter factors, it is necessary to analyze H4 promoter DNA binding activities during both the proliferative and differentiated stages of osteoblast phenotype development using the same nuclear protein preparations. We performed gel mobility shift assays with promoter segments that span the cell cycle control region of a prototypical H4 gene (Fig. 7). The data show that nuclear factors HiNF-D (Fig. 7*A*) and HiNF-P/H4TF-2 (Fig. 7*B*) are detected only in proliferating ROB cells, whereas HiNF-M (Fig. 7*B*) is present in both rapidly dividing and differentiated osteoblasts. These results suggest that HiNF-D and HiNF-P/H4TF-2, but not HiNF-M, are cell growth-controlled binding activities and are selectively down-regulated in parallel at the onset of differentiation. Most importantly, the regulation of these histone DNA-binding activities is remarkably similar to that observed during ROS 17/2.8 cell growth inhibition.

Constitutive Presence of Ubiquitous Transcriptional DNA-binding Activities and Down-Regulation of OCT-1 and AP-1 during Cessation of Osteoblast Proliferation. To provide internal standards for the presence and integrity of nuclear factors in our protein preparations, we monitored the DNA-binding activities of transcription factor families with broad promoter-binding specificity. The nuclear abundance of promoter factors binding to Sp1, ATF, and CCAAT elements is similar in proliferating and differentiated ROB cells (Fig. 8). The presence of these DNA-binding activities at both developmental stages is consistent with their postulated roles in the transcriptional activation of a plethora of genes (46). These results establish that the changes in the nuclear abundance of histone promoter factors are selective and not due to quantitation errors or major differences in the quality of our various nuclear protein preparations.

Our results also show (Fig. 9) that OCT-1 and AP-1 are present at high levels in rapidly dividing ROB cells and are not present in differentiated ROB cells. The down-regulation of OCT-1 and AP-1 during osteoblast differentiation corresponds with, respectively, the cessation of cell cycle-dependent histone H2B gene transcription (59, 60) and the up-regulation of OC transcription (4) established in previous studies. Comparison of results obtained with H4 gene promoter factors HiNF-D and HiNF-P/H4TF-2 (Figs. 3 and 7), H2B gene promoter factor OCT-1, and AP-1 binding activity (Figs. 5 and 9) suggests that these factors are regulated in concert and at least in part subject to stringent growth regulation.

DISCUSSION

Osteosarcoma cells (ROS 17/2.8) (1, 2), unlike normal diploid osteoblasts, do not form a mineralized extracellular matrix, the hallmark of the mature osteoblast phenotype. Thus at least one key transition point signaling the ultimate onset of osteoblast maturation is clearly not operative. Moreover, expression of osteoblast-related genes, which is functionally restricted to the postproliferative stages of normal osteoblast development (3, 4), occurs at low levels in proliferating ROS 17/2.8 cells. Hence, these cells display relaxation of

gene-suppressive mechanisms that are operative on bone-related genes during the proliferative period of normal diploid osteoblasts. However, density-inhibited growth of ROS 17/2.8 cells results in the shutdown of proliferation-specific genes and up-regulation of bone-related genes from minimal levels by transcriptional mechanisms (4). Here, we have studied this partial maintenance of the proliferation/differentiation relationship in ROS 17/2.8 cells.

Our results show that shutdown of histone H4 and H2B gene expression in confluent ROS 17/2.8 cells involves a corresponding down-regulation of at least four key histone promoter factors. Two of these, HiNF-D and HiNF-P/H4TF-2, interact with a highly conserved transcriptional element present in most histone H4 genes. This element (H4 Site II) mediates basal level and periodic, G_1/S-phase-specific modulations of transcription during the cell cycle (22). Similarly, OCT-1 interacts with an analogous H2B promoter element (51, 54, 61). The fourth factor, H4UA-1, is involved in the enhancement of histone gene transcription by a distal activating mechanism (33). The correlation between the shutdown of histone gene expression in ROS 17/2.8 cells and specific modifications in a select subset of histone promoter-binding activities interacting at both proximal and distal domains supports our model for transcriptional regulation of individual histone genes (14, 17). This model postulates contribution of modularly organized promoter regulatory elements exhibiting selective occupancy by a series of transcription factors in both a cell cycle- and a cell growth-responsive manner. We have discussed previously that at least three of these factors (HiNF-D, HiNF-M, and H4UA-1) may represent heteromeric proteins and postulated that the regulated activities of the putative components involve phosphorylation events (17, 36). Thus, down-regulation of these factors may reflect the dissociation or inactivation of (possibly H4 gene-specific) transcription factor complexes, rather than decreases in abundance of the individidual components. The current results are consistent with either possibility.

With respect to specific mechanisms operative at the multipartite cell cycle control element H4 Site II, we have studied the complete set of H4 Site II binding activities within the same nuclear protein preparations as a function of the level of proliferative activity in both ROS 17/2.8 and normal diploid osteoblasts. Our results demonstrate that apart from HiNF-D a second H4 Site II binding activity (*i.e.*, HiNF-P/H4TF-2) is proliferation dependent in both cell types. However, unlike HiNF-D and HiNF-P/H4TF-2, HiNF-M binding activity is not coupled to cell proliferation. Thus, the H4 Site II transcriptional element may integrate two types of intracellular gene-regulatory signals: those monitoring cell cycle progression and those mediating general transcriptional competency.

Coordinate cell cycle regulation of histone genes in normal diploid osteoblasts involves collective modulation of protein/DNA interactions involving HiNF-D in histone H4, H3, and H1 gene promoters with respect to S phase (43, 62). However, these protein/DNA interactions are deregulated in concert and are constitutive during the ROS 17/2.8 cell cycle (43, 62). Hence, in addition to abrogated developmental regulation of critical transition points normally observed during osteoblast maturation, ROS 17/2.8 cells display relaxation of cell cycle check points capable of modulating the level of HiNF-D binding activity during the onset and following completion of S phase. Similar deregulation of histone promoter factors occurs during the cell cycle of HL60 promyelocytic leukemia cells, as well as other tumor-derived and transformed cell types (43, 62). HL60 cells are capable of down-regulating HiNF-D at the cessation of proliferation and onset of differentiation (45). Unlike HL60 cells, ROS 17/2.8 cells have lost the competency to fully differentiate but are also capable of down-regulating the elevated, cell cycle-independent levels of HiNF-D binding activity. These results suggest that regulation of HiNF-D is not only coupled to cell cycle oscillatory mediators but also to regulatory proteins capable of suppressing cell growth. Although we observed dramatic changes in the expression of cell growth-related histone genes and the cognate promoter binding activities during density inhibition of ROS 17/2.8 cells, no major modifications in protein/DNA interaction patterns were observed in the OC promoter at the VDRE and OC box under the conditions examined here during the up-regulation of OC expression from minimal basal levels. The constitutive binding activities interacting with these elements in the OC promoter may maintain basal levels of OC transcription in both proliferating and confluent osteosarcoma cells. However, we observed a novel nuclear factor interacting with a proximal promoter element of the OC gene (nt −43 to −16) that contains a putative steroid receptor recognition sequence overlapping with the TATA box consensus element. This factor is preferentially observed in proliferating ROS 17/2.8 cells and is down-regulated when the OC gene is up-regulated. Because this factor is most abundant when OC gene transcription is minimal (4) and interacts in close proximity to the TATA element specifying the initiation of mRNA synthesis, it is possible that this novel protein may represent a member of the steroid hormone receptor superfamily acting as a repressor of basal OC gene transcription.

The enhancement of the minimal level of OC expression during ROS 17/2.8 proliferation arrest may also involve different mechanisms, *i.e.*, posttranslational modifications of constitutive promoter factors or modulation of factors interacting at regions outside those examined. Also, up-regulation of OC expression from minimal basal levels in ROS 17/2.8 cells is coupled with down-regulation of AP-1 activity. This event may relieve a component of transcriptional control that suppresses the OC gene during active osteoblast proliferation (3, 5, 26). Regardless, the constitutive protein/DNA interactions occurring at the OC promoter, as well as the regulation of AP-1 activity, provide a physiological context for the alterations in histone gene protein/DNA interactions during ROS 17/2.8 density inhibition.

Direct comparison of results obtained during ROB development and ROS 17/2.8 cell growth inhibition shows that the regulation of the full complement of histone-specific and general promoter factors is similar to that occurring during normal osteoblast differentiation. Because ROS 17/2.8 cells do not differentiate into mature osteocytic cells in a mineralized extracellular matrix, these events appear to be a direct consequence of the cessation of proliferation rather than the onset of differentiation. Thus, the partial maintenance of the proliferation/differentiation relationship in ROS 17/2.8 cells, reflected by the reciprocal regulation of the histone and OC genes, is directly related to cell growth regulatory mechanisms that control the transcription of proliferation-specific histone genes by a selective decline in the levels of the associated promoter DNA-binding activities. In conclusion, the results presented in this study support a mechanistic molecular model for the regulation of histone gene transcription (14) in bone-derived cells. The observed differences in regulation of histone promoter binding activities during the cell cycle (43, 62) and similarities in control at the cessation of proliferation in both normal diploid and tumor-derived bone cells provide new avenues for further studies on the molecular basis of neoplastic transformation in bone tissue.

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