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Influence of Dexamethasone on the Vitamin D–Mediated Regulation of Osteocalcin Gene Expression

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Abstract The influence of dexamethasone on expression of the osteocalcin gene which encodes the most abundant non-collagenous and only reported bone-specific protein was examined in ROS 17/2.8 osteosarcoma cells which express a broad spectrum of genes related to bone formation. Consistent with previous reports, quantitation of cellular osteocalcin mRNA levels by Northern blot analysis, osteocalcin gene transcription by activity of the osteocalcin gene promoter fused to a chloramphenicol acetyl-transferase (CAT) mRNA coding sequence following transfection into ROS 17/2.8 cells, and osteocalcin biosynthesis by radioimmunoassay indicate that dexamethasone in a concentration range of 10^{-6} to 10^{-9} M only modestly modifies basal levels of osteocalcin gene expression. However, dexamethasone significantly inhibits these parameters of the vitamin D–induced upregulation of osteocalcin gene expression in both proliferating and in confluent ROS 17/2.8 cells. In this study, we observed that the extent to which abrogation of the vitamin D response occurs is dependent on basal levels of osteocalcin gene expression as reflected by a complete inhibition of the vitamin D–induced upregulation in a ROS 17/2.8K subline with low basal expression and only a partial reduction of the vitamin D stimulation in a ROS 17/2.8C subline with eightfold higher levels of basal expression. This effect of glucocorticoid appears to be at the transcriptional and post-transcriptional levels as demonstrated by a parallel decline in the cellular representation of osteocalcin mRNA, osteocalcin gene promoter activity, and osteocalcin biosynthesis. The complexity of the glucocorticoid effect on vitamin D–mediated transcriptional properties of the osteocalcin gene is indicated by persistence of sequence-specific protein-DNA interactions at two principal osteocalcin gene promoter regulatory elements, the osteocalcin (CCAAT) box which modulates basal level of transcription, and the vitamin D responsive element, where vitamin D–mediated enhancement of osteocalcin gene transcription is controlled.

Key words: glucocorticoid, transcription, mRNA stability, histone, differentiation, bone development, osteoblast, promoter factors, collagen, osteosarcoma cells

The biosynthesis of osteocalcin is stringently regulated during the progressive development and maintenance of the osteoblast phenotype. The selective expression of osteocalcin occurs during bone formation and remodeling [Lian and Stein, in press; Stein et al., 1990], in primary cultures of normal diploid osteoblasts following completion of proliferative activity when extracellular matrix mineralization is ongoing [Aronow et al., 1990; Owen et al., 1990a], and in several transformed osteoblastic and osteosarcoma cell lines which express genes associated with mature, differentiated bone cells [Lian et al., 1989; Rodan and Rodan, 1984].

Multiple lines of evidence support an influence of glucocorticoids on the extent to which the osteocalcin gene is expressed within the context of bone cell growth and differentiation, both in vitro and in vivo. In vitro, in bone organ cultures [Canalis, 1983], in outgrowth cultures from trabecular explants [Wong et al., 1990], in cultures of bone derived cells [Chen et al., 1986], and in osteosarcoma cell lines [Eilam et al., 1980], dexamethasone inhibits cell proliferation and promotes expression of several mature osteoblast phenotypic properties. While these include elevated alkaline phosphatase activity [Majeska et al., 1985] and enhanced responsiveness to parathyroid hormone and 1,25 $(OH)_2$ vitamin D_3 (vitamin D) [Rodan et al., 1985], an inhibition of osteocalcin biosynthesis has been observed [Morrison et al., 1989; Wong et al., 1990] in these same cultures with the exception of fetal rat derived calvarial cells [Chen et al., 1986]. Similarly, in vivo the skeletal effects of glucocorticoids, which include bone loss and increased

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incidence of spontaneous fractures, are associated with decreased osteoblastic activity and a decline in serum levels of osteocalcin [Ekenstam et al., 1988; Lukert et al., 1986]. However, molecular mechanisms by which expression of the osteocalcin gene is modulated by glucocorticoids remain to be established, necessitating a systematic analysis of the influence of glucocorticoids on regulation of the osteocalcin gene at the transcriptional and at a series of post-transcriptional levels.

We have initially addressed the effects of glucocorticoids on expression of the osteocalcin gene in ROS 17/2.8 osteosarcoma cells which express a broad spectrum of genes related to the mature osteoblast phenotype [Majeska et al., 1980; Rodan and Noda, 1991]. Our results indicate that dexamethasone is compatible with basal level expression of the osteocalcin gene in ROS 17/2.8 osteosarcoma cells. However, dexamethasone inhibits the vitamin D–induced upregulation of osteocalcin expression in both proliferating and in confluent ROS 17/2.8 cells. The partial or complete abrogation of vitamin D–enhanced osteocalcin gene expression by dexamethasone is dose dependent, related to the basal level of osteocalcin expression and mediated both transcriptionally and post-transcriptionally. This glucocorticoid effect is reflected by a parallel decline in cellular levels of osteocalcin mRNA, osteocalcin transcriptional activity, and osteocalcin biosynthesis. Yet, vitamin D receptor-dependent and other sequence-specific protein-DNA interactions are maintained at two principal osteocalcin gene promoter regulatory elements, the osteocalcin (CCAAT) box which modulates basal level of transcription, and the vitamin D responsive element (VDRE), where vitamin D regulation of osteocalcin gene transcription is controlled. Contributions of additional regulatory elements and/or further protein-DNA interactions at the osteocalcin box and VDRE to the vitamin D–mediated transcriptional properties of the osteocalcin gene are therefore indicated.

MATERIALS AND METHODS

Cell Culture

The studies reported in this paper were carried out using two sublines of ROS 17/2.8 cells, designated ROS 17/2.8C and ROS 17/2.8K. Both express phenotypic markers of the osteoblast that reflect parameters of bone formation (type I collagen, alkaline phosphatase, osteopontin, and osteocalcin) with vitamin D responsiveness and increased expression of these genes in confluent, compared with actively proliferating cells (Fig. 1). However, as indicated in Figure 1, expression of osteocalcin and type I collagen are significantly greater in the ROS 17/2.8C cells, exhibiting more than eightfold higher levels of osteocalcin mRNA and osteocalcin biosynthesis, and sixfold higher levels of type I collagen mRNA. Cells were grown to confluence, unless otherwise stated in the results, in F12 medium (Gibco, Grand Island, NY) supplemented with calcium [Majeska et al., 1980] and 5% (vol/vol) fetal calf serum. For maintenance and growth of the cultures, media was replaced every 48 hours. At the initiation of experiments to determine effects of steroid hormones on osteoblast-related and cell growth-associated gene expression, the media was exchanged for fresh media containing 10 nM $1,25(OH)_2D_3$ in 0.1% ethanol and/or 1 nM to 1 mM dexamethasone in 0.1% ethanol. The order of addition of hormones and duration of treatments are indicated in the results.

RNA Isolation and Analysis

Cells were harvested by scraping with rubber policemen from 150 mm plates in ice cold phosphate buffered saline (PBS) and pelleted by centrifugation at 600*g* for 3 min. Cytoplasmic RNA was prepared as described previously [Owen et al., 1990c]. RNA samples were quantitated by absorbance at 260 nm, and fractionated electrophoretically in 1.5% agarose–6.6% formaldehyde gels. Intactness of the RNA was assessed by ethidium bromide staining. Transfer and slot blotting to Zeta Probe membrane (BioRad, Richmond, CA), labeling of DNA probes, and hybridization were carried out according to Owen et al. [1990a]. Cloned DNA probes used for hybridization were rat osteocalcin [Lian et al., 1989], rat H4 histone [Grimes et al., 1987], and rat type I collagen [Genovese et al., 1984], labeled with ^{32}P-dCTP using a random primer kit obtained from Stratagene, Inc.

Osteocalcin Radioimmunoassay

Media from cell cultures were assayed directly for levels of osteocalcin by a radioimmunoassay previously described [Gundberg et al., 1984].

Preparation of Nuclear Extracts

Cells were harvested by scraping with a rubber policeman and pelleted as above. Nuclei were isolated and nuclear extracts prepared accord-

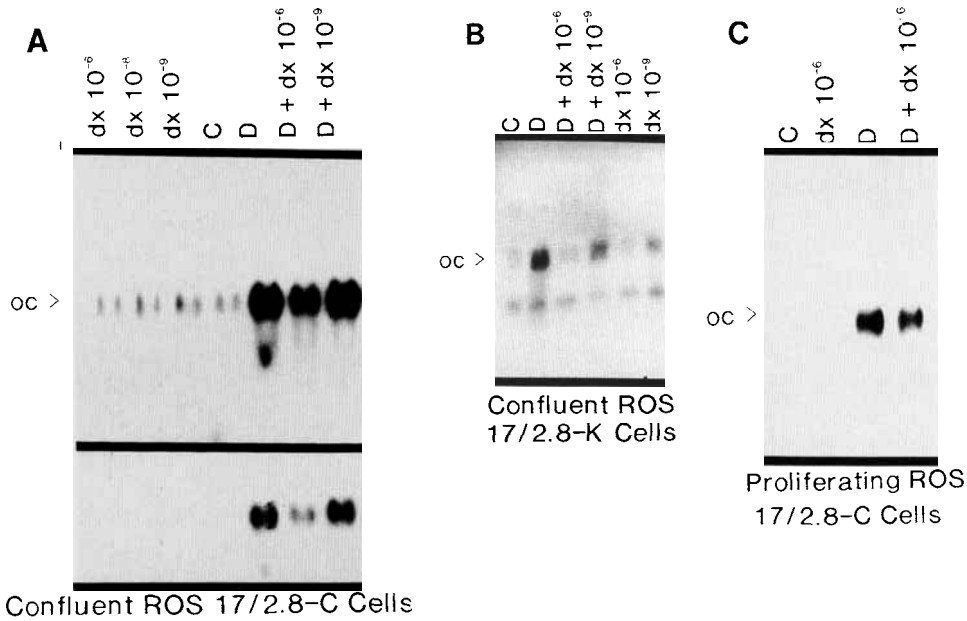

Fig. 1. Osteocalcin mRNA expression in confluent ROS 17/2.8 cells in response to treatment with dexamethasone (dx) and vitamin D (D), as determined by Northern blot analysis. Confluent cells were treated on day 6 with the concentrations of dexamethasone indicated above the lanes and with 10 nM $1,25(OH)_2D_3$ either alone (D) or together with dexamethasone (D + dx) where indicated. Cells were harvested 24 hours later. Control untreated cells (C) were harvested on day 7. **A:** ROS 17/2.8C subline. $1,25(OH)_2D_3$ stimulated mRNA sixfold over basal level. Dexamethasone inhibits the vitamin D–induced stimulation of mRNA production by about 50%, which is clearly evident in the lower panel. The lower panel shows a shorter autoradiographic exposure of the blot in the upper panel where the signal is in the linear range of sensitivity for the film emulsion. **B:** ROS 17/2.8K subline. With an eightfold lower basal expression relative to the 17/2.8C subline, there is a similar sixfold stimulation with $1,25(OH)_2D_3$, but dexamethasone completely inhibits the $1,25(OH)_2D_3$ response. **C:** Proliferating ROS 17/2.8C cells. Cells were treated with dexamethasone, $1,25(OH)_2D_3$ (at 10 nM), or both, as indicated above the lanes, on day 2½ during rapid growth, harvested 24 hours later, and mRNA was prepared. Dexamethasone inhibited the vitamin D stimulation of osteocalcin RNA.

ing to Dignam et al. [1983] and stored in aliquots at −70°.

Probes for Gel Mobility Shift Assay

The osteocalcin box is the sequence from nt −99 to −76, which includes the CCAAT motif [Lian et al., 1989; Markose et al., 1990; Owen et al., 1990b]. Probes for this region consisted of these 24 nucleotides alone (cloned into the SmaI site of pUC19), nt −101 to −43 or nt −142 to −43. The probe for the VDRE [Markose et al., 1990] was the −527 to −344 fragment of the rat osteocalcin gene promoter. The probe for the TATA box-containing proximal promoter was the −43 to +150 fragment. To label probes with ^{32}P, 10 μg of restriction enzyme digested pOC3.4 [Lian et al., 1989] plasmid DNA was treated with 2.5 units of calf intestinal alkaline phosphatase in 50 mM Tris (pH 8) for 1 hour at 50°, then inactivated for 45 min at 65° in 0.5% SDS, 0.1% EDTA. DNA was dissolved in 20 mM Tris (pH 9.5), 0.1 mM EDTA, 1 mM spermidine in a volume of 50 μl, heated at 70° for 5 min, and placed at 4°. 5 μl of a buffer consisting of 0.5 M Tris (pH 9.5), 0.1 M $MgCl_2$, 50 mM dithiothreitol, and 50% glycerol was added; then this DNA solution was added to 0.1 μC of lyophilized $\gamma^{32}P$-ATP. Twenty units of T4 polynucleotide kinase were added and the reaction was incubated at 37° for 30 minutes. After a second restriction enzyme digestion, the single end-labeled fragment was purified by polyacrylamide gel electrophoresis.

Gel Mobility Shift Assay

The protein/DNA binding reactions contained 4,500–5,000 cpm of end-labeled probe, 3 μg of poly(dI-dC)-poly(dI-dC), 50 mM KCL, 10 mM Hepes-NaOH (pH 8.0), 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 4 mM levamisole (a phosphatase inhibitor), and between 2.5 and 12 μg of nuclear protein extract. 20 μl reactions were incubated for 15 min at 20° and then fractionated electrophoretically in 4% polyacryl-

amide gel using a high ionic strength buffer [Ausubel et al., 1989]. The gels were dried and protein-DNA complexes were visualized by autoradiography.

Transfection and CAT Assays

pOCZCAT is a PGEM 7f+ based plasmid containing the initial 1120 bp (−11 to −1131) of the rat osteocalcin gene promoter fused to the protein-coding sequence of the CAT gene. ROS 17/2.8 cells were plated at 3.0×10^5/100 mm plate and after 24 h the cells were transfected with 20 μg of pOCZCAT using an overnight calcium phosphate coprecipitation method [Chen and Okayama, 1987]. Twenty-four hours post-transfection, the media was replaced and 48 hours later the cells were harvested and assayed for CAT activity [Gorman et al., 1982]. Acetylated ^{14}C chloramphenicol derivatives were separated by thin layer chromatography, exposed to x-ray film for 20 hrs and quantitated by densitometry.

RESULTS

Dexamethasone Inhibits the Vitamin D–Mediated Upregulation of Osteocalcin Biosynthesis and Cellular mRNA Levels

The influence of glucocorticoids on osteocalcin gene expression was examined by determining the effect of acutely (48 h) treating ROS 17/2.8 osteosarcoma cells under two distinct metabolic conditions, actively proliferating and confluent, with several concentrations of the synthetic glucocorticoid, dexamethasone. The effect of dexamethasone on both basal expression and on the upregulation of osteocalcin expression by vitamin D, a physiological mediator, was systematically determined by quantitation of 1) cellular mRNA levels using Northern blot and/or slot blot analysis; 2) osteocalcin gene transcription by activity of the osteocalcin gene promoter fused to a CAT mRNA coding sequence following transfection into ROS cells; and 3) osteocalcin biosynthesis by radioimmunoassay.

The Northern blot, shown in Figure 1A and quantitated in Table I, indicates that dexamethasone in the concentration range of 10^{-9} M–10^{-6} M does not significantly modify basal levels of osteocalcin mRNA in confluent ROS 17/2.8C cells where the gene is maximally expressed. In the ROS 17/2.8K cells where basal levels are low even at confluency, while 10^{-6} M dexamethasone also had no effect as in ROS 17/2.8C cells, treatment with 10^{-9} M dexamethasone resulted in higher (2–3 times) cellular levels of osteocalcin mRNA (Fig. 1B). However, 10^{-6} M dexamethasone reduced the sixfold vitamin D induction of osteocalcin mRNA by 50% in ROS 17/2.8C cells, and in ROS 17/2.8K cells completely abrogates the sixfold vitamin D upregulation of cellular osteocalcin mRNA content when the confluent ROS 17/2.8K cells are simultaneously treated with 10^{-6} M dexamethasone and vitamin D (Figs. 1A,B). At 10^{-9} M dexamethasone in the presence of vitamin D, a 50% decrease was observed. In proliferating ROS 17/2.8C cells, where basal osteocalcin mRNA levels are lower than those found following the completion of proliferation (Fig. 1 and Table I) and similar to osteocalcin mRNA levels in confluent ROS 17/2.8K cells, again, treatment of the cells with 10^{-6} M dexamethasone together with vitamin D reduces the fivefold vitamin D–mediated stimulation of osteocalcin mRNA.

The parallel influence of dexamethasone on both osteocalcin mRNA level and on osteocalcin

TABLE I. Quantitation of Osteocalcin mRNA and Synthesized Protein and Histone and Type I Collagen mRNA in ROS 17/2.8C Cells in Response to Vitamin D and Glucocorticoid Treatment*

	Osteocalcin		Histone	Collagen
Sample	mRNA	Synthesis	mRNA	mRNA
Confluent Cells				
C	1	1	1	1
D_3	6.6	5.90	1.3	0.56
$dx10^{-6}$	0.73	1.20	0.5	0.14
$dx10^{-8}$	0.84	0.93	0.6	0.77
$dx10^{-9}$	1.22	1.65	0.9	0.90
$D_3 + dx10^{-6}$	3.40	2.55	0.5	0.42
$D_3 + dx10^{-9}$	6.12	5.73	1.2	0.88
$dx10^{-6} \rightarrow D_3$	3.41	2.20	0.7	0.40
$dx10^{-9} \rightarrow D_3$	7.60	5.80	1.6	0.58
Proliferating Cells				
C	1	1	1	1
D_3	5	5.71	1	0.75
$dx10^{-6}$	0.67	1.60	0.3	0.62
$dx10^{-6} \rightarrow D_3$	3.70	4.20	0.6	0.88

*Northern blots were scanned using the β-Scope (Betagen Co.). Osteocalcin synthesis was measured as secreted protein in 48 h by RIA. Numbers are the -fold increase or decrease relative to the control value which is designated as 1. The confluent and proliferating samples are designated as follows: control (C), $1,25(OH)_2D_3$-treated (D_3), dexamethasone-treated (dx), treatment with dexamethasone together with $1,25(OH)_2D_3$(dx + D_3), dexamethasone treatment followed by $1,25(OH)_2D_3$ treatment ($D_3 \rightarrow$ dx).

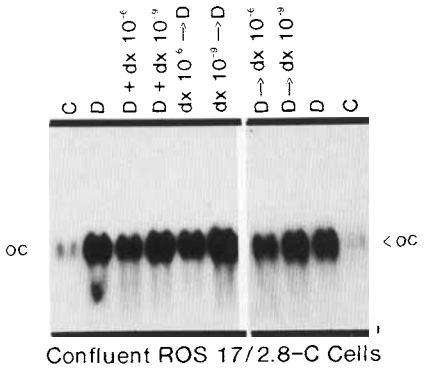

Fig. 2. Northern blot analysis showing effect on vitamin D–stimulated osteocalcin mRNA expression of pretreatment or subsequent treatment with dexamethasone. dx → D: Cells were treated at confluence for 24 hours with dexamethasone, then for the following 24 hours with vitamin D in fresh media. D → dx: Cells were treated for 24 hours with vitamin D, then for the next 24 hours with dexamethasone. Concentrations of dexamethasone are indicated above lanes. The vitamin D concentration is 10 nM. First two lanes, C and D, day 6 control and vitamin D–treated; last two lanes D and C, day 7 vitamin D–treated and control.

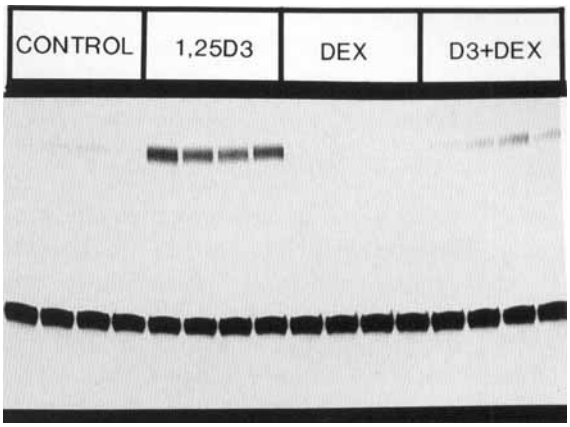

Fig. 3. ROS 17/2.8C cells were transfected with the osteocalcin gene promoter–CAT construct pOCZCAT and assayed for CAT activity 48 hour post-transfection with A) 0.1% ethanol, B) 10 nM $1,25(OH)_2D_3$, C) 1.0 μM dexamethasone, or D) 10 nM $1,25(OH)_2D_3$ + 1.0 μM dexamethasone. Relative density of acetylated chloramphenicol bands/80 μg protein extract were A) 0.78 ± 0.17, B) 3.76 ± 0.85, C) 0.65 ± 0.13, and D) 2.32 ± 0.11. The results from CAT assays of four independent transfections for each parameter are shown.

protein biosynthesis, reflected by radioimmunoassay (Table I), suggests that a rate limiting step in the modulation of osteocalcin gene expression of glucocorticoids is the stability of osteocalcin mRNA. Basal levels of osteocalcin mRNA appear to be minimally affected by dexamethasone but, particularly evident at higher concentrations of the glucocorticoid (10^{-6} M) and most pronounced in confluent ROS 17/2.8C cells where vitamin D elicits a maximal stimulation of osteocalcin gene expression, the extent to which dexamethasone treatment results in a reduction in the vitamin D upregulation of osteocalcin gene expression is dampened.

To further pursue mechanisms by which dexamethasone influences the vitamin D–mediated expression of osteocalcin, confluent ROS 17/2.8C cells were pretreated with vitamin D for 24 hours prior to addition of dexamethasone to the cultures. As shown in Figure 2, while dexamethasone treatment of ROS cells together with $1,25(OH)_2D_3$ reduced the vitamin D–associated increase in osteocalcin mRNA by 49%, when dexamethasone is introduced into cultures of confluent ROS 17/2.8C cells 24 hours after treatment with vitamin D, at which time osteocalcin mRNA levels are elevated sixfold, a 42% decline in osteocalcin mRNA is observed 24 hours later. Also shown in Figure 2 is that pretreatment of the cells with dexamethasone results in a dampened vitamin D response. Together, these results support at least two effects of dexamethasone on the vitamin D–mediated regulation of osteocalcin gene expression: first, by selectively or partially restricting the accumulation of osteocalcin mRNA, and second, by decreasing vitamin–D stimulated osteocalcin mRNA levels.

The extent to which the dexamethasone effect on the upregulation of osteocalcin gene expression by vitamin D is modulated transcriptionally was directly assayed by activity of the osteocalcin gene promoter fused to a CAT mRNA coding sequence transfected into ROS 17/2.8C cells. As indicated in Figure 3, the 4.8-fold increase in transcriptional activity following vitamin D treatment is reduced 40% by 10^{-6} M dexamethasone. These results indicate that, in addition to the influence of dexamethasone on the stability and cellular levels of osteocalcin mRNA that are retained in response to vitamin D treatment, glucocorticoid similarly downregulates the elevated levels of osteocalcin gene transcription that are vitamin D–mediated.

Influence of Glucocorticoids on Gene Expression Associated With Osteoblast Growth and Differentiation

To assess the influence of glucocorticoids on other parameters of gene expression associated with the osteoblast phenotype, cellular levels of mRNA transcribed from a cell growth regulated gene (H4 histone) and a bone extracellular ma-

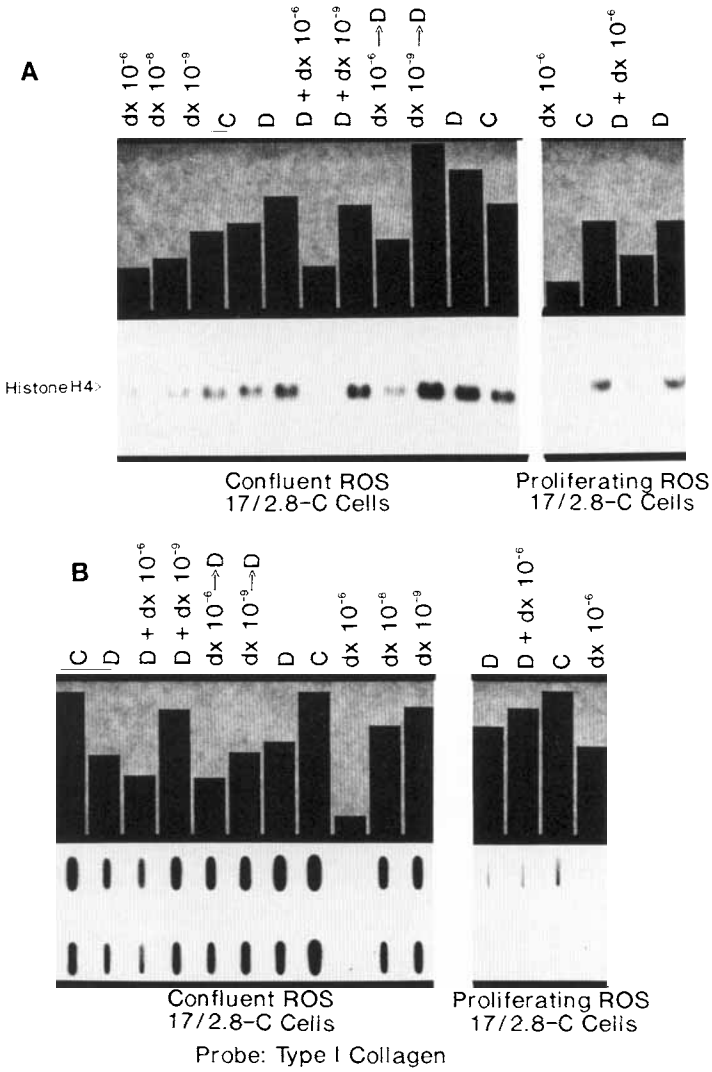

Fig. 4. Expression of cell growth and differentiation-related genes in confluent and proliferating 17/2.8C cells in response to vitamin D and dexamethasone. **A:** H4 histone mRNA expression. The Northern blots in Figs. 1–3 were rehybridized with an H4 histone gene probe. The blots were quantitated by scanning with a β-Scope, and the values are represented in the bar graph in the upper panel. **B:** Type I collagen mRNA expression. RNAs from the experiments in Figs. 1–3 were slot-blotted, hybridized with a Type I collagen gene probe, and scanned with β-Scope. Upper panel is a bar graph representation of the quantitation values.

trix gene (type I collagen) were determined in actively proliferating and in confluent ROS 17/2.8C cells. H4 histone mRNA levels were assayed since histone gene expression is tightly coupled with DNA replication [Baumbach et al., 1984; Holthuis et al., 1990; Plumb et al., 1983; Shalhoub et al., 1989; Stein et al., 1975], thereby providing a marker for proliferation and expression of a gene expressed in a cell cycle dependent manner. As shown in Figure 4A, acute treatment (48 hours) of both actively proliferating and confluent cells results in decreased H4 histone gene expression. In confluent cells, a dose dependent decline in H4 histone mRNA levels is observed (10%, 60%, and 50% at 10^{-9} M, 10^{-8} M, and 10^{-6} M concentrations of dexamethasone, respectively) while in actively proliferating ROS 17/2.8C cells, dexamethasone-associated inhibition in histone mRNA levels is more pronounced (70%). Dexamethasone 10^{-6} M treatment in the presence of $1,25(OH)_2D_3$ resulted in a similar reduction of H4 histone mRNA levels in both proliferating and confluent cells as occurred in cells treated with dexamethasone alone, while 10^{-8} M $1,25(OH)_2D_3$ alone had only a minimal effect. Interestingly, when a 24 hour treatment

with vitamin D (10^{-8} M) was initiated in confluent cells following the completion of a 24 hour treatment with dexamethasone, the glucocorticoid-related inhibition of H4 histone gene expression was partially (10^{-6} M dexamethasone) or completely (10^{-9} M dexamethasone) alleviated (Table I and Fig. 4A).

Cellular levels of type I collagen mRNA (Fig. 4B) were dramatically reduced in confluent (86%) and in proliferating (38%) ROS 17/2.8C cells by *acute treatment* (48 hours) with 10^{-6} M dexamethasone and, to a significant but lesser extent, at lower glucocorticoid concentrations [Hodge and Kream, 1988]. Also observed was a dose dependent biphasic and synergistic influence of dexamethasone on the well-documented vitamin D–mediated downregulation of collagen gene expression [Genovese et al., 1984; Owen et al., 1991a]. Confluent ROS 17/2.8 cells treated 48 hours with both 10^{-8} M $1,25(OH)_2D_3$ and 10^{-6} M dexamethasone maintained reduced collagen mRNA levels than result from treatment with vitamin D alone. However, when the cells were treated for 48 hours with 10^{-8} M vitamin D together with 10^{-9} M dexamethasone, the inhibitory effect was less than that observed in cells treated only with $1,25(OH)_2D_3$. ROS 17/2.8 cells treated with 10^{-6} M dexamethasone for 24 hours prior to treatment with $1,25(OH)_2D_3$ continued to exhibit type I collagen mRNA levels in the range of cultures treated with vitamin D alone or with vitamin D and 10^{-6} M dexamethasone. This indicates that vitamin D has the ability to reduce the dexamethasone-related downregulation of collagen gene expression (60% with 10^{-6} M dexamethasone), a phenomenon which we similarly observed but in a less pronounced manner in proliferating ROS 17/2.8C cells.

Influence of Dexamethasone on Protein-DNA Interactions in Primary Regulatory Elements of the Osteocalcin Gene

Having established that dexamethasone reduces or totally eliminates the vitamin D–mediated upregulation of osteocalcin gene expression, as reflected by osteocalcin biosynthesis, cellular mRNA levels, and transcription, we further examined the glucocorticoid effects on molecular mechanisms operative in osteocalcin gene transcription in proliferating and confluent ROS 17/2.8 cells by investigating protein-DNA interactions at two key regulatory elements of the osteocalcin gene promoter. We examined nuclear factors binding at the osteocalcin box, a principal 5′ regulatory element responsible for basal transcription of the osteocalcin gene [Lian et al., 1989; Markose et al., 1990; Owen et al., 1990b]. This is a highly conserved element in the proximal promoter region of both the rat (−99 to −76) and human osteocalcin genes with a CCAAT motif as a central core [Lian et al., 1989; Markose et al., 1990; Owen et al., 1990b]. Binding of nuclear factors at the VDRE which resides at −462 to −437 [Demay et al., 1990; Lian et al., 1989; Markose et al., 1990; Owen et al., 1990b] was also determined. This element functions as an enhancer type sequence mediating the extent to which the osteocalcin gene is transcribed. Binding of the vitamin D receptor complex independently, as well as in a synergistic and/or antagonistic relationship with a series of other proteins, such as the oncogene encoded fos and jun proteins [Owen et al., 1990b] and yet-to-be-characterized accessory factors [Liao et al., 1990], appears to influence both vitamin D receptor interactions and to modulate transcriptional activity.

Our experimental strategy for addressing the influence of glucocorticoids on promoter factor binding at the osteocalcin box was to construct a series of probes, most including the OC box and several with a specific subset of 5′ and/or 3′ flanking sequences (schematically illustrated in Fig. 5) for assessment of protein-DNA interactions by electrophoretic mobility shift (gel retardation) analysis. The results presented in Figure 6A clearly indicate that using a −99 to −76 probe, which includes only the osteocalcin box, dexamethasone at a concentration of 10^{-6} M, either alone or together with 10^{-8} M $1,25(OH_2D_3$, does not modify the binding of nuclear factors to the "minimal osteocalcin box" sequence. This consistency in protein-DNA interactions at the osteocalcin box was found in confluent ROS 17/2.8K and ROS 17/2.8C cells (Fig. 6A) and the same pattern of promoter factor binding is maintained in actively proliferating ROS 17/2.8C cells (Fig. 6B). These results provided an initial indication that dexamethasone does not modify the association of transcription factors with a primary promoter regulatory element responsible for basal transcription of the osteocalcin gene. When protein-DNA interactions of nuclear factors from confluent ROS 17/2.8K and ROS 17/2.8C cells were examined using a probe spanning the −101 to −43 sequences of the osteocalcin gene promoter, again dexamethasone, independently or together with

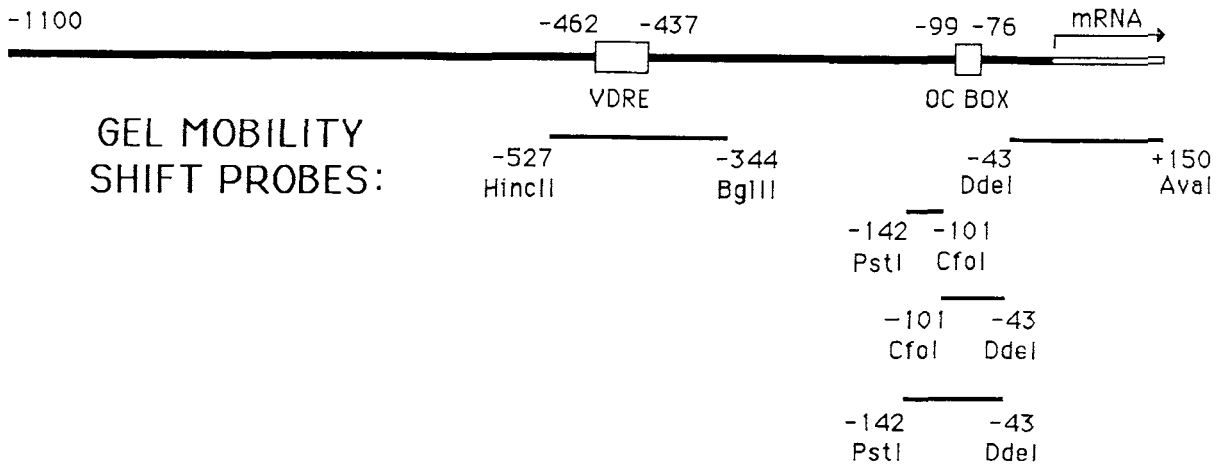

Fig. 5. Schematic representation of probes used to examine in vitro protein-DNA interactions in the osteocalcin gene promoter. The position and sequence of the CCAAT-motif-containing OC box and of the VDRE are shown at the top. Thin lines represent the fragments used as probes.

$1,25(OH)_2D_3$, did not result in altered factor binding (Fig. 6C). Similarly, when nuclear factor binding was examined in the region of the osteocalcin gene promoter immediately upstream from the osteocalcin box (−142 to −101) in confluent ROS 17/2.8 cells, there was no evidence of a dexamethasone effect (Fig. 6D). Likewise, when a −142 to −43 probe was used, which includes sequences immediately upstream from the osteocalcin box, again protein-DNA interactions in these proximal promoter sequences were unaltered by either dexamethasone alone or dexamethasone and vitamin D treatment of confluent ROS 17/2.8C and ROS 17/2.8K cells (Fig. 6E).

Figure 7 shows results from an analysis of protein/DNA binding in the −527 to −344 region of the osteocalcin gene promoter containing the VDRE, using nuclear factors from ROS 17/2.8C and K cells. As expected, in both proliferating and confluent cells, binding of the vitamin D receptor is dependent on treatment of the cells with $1,25(OH)_2D_3$. Dexamethasone alone does not stimulate vitamin D receptor binding and vitamin D promotes factor binding to the VDRE even in the presence of 10^{-6} M dexamethasone. When added to cell cultures, together with $1,25(OH)_2D_3$, dexamethasone (10^{-6} M) is compatible with full induction of vitamin D receptor binding to the VDRE. Further support for the compatibility of dexamethasone with formation of vitamin D–VDRE complexes is provided by the initiation of hormone-VDRE interactions when ROS cells, pretreated with dexamethasone, are subsequently treated with vitamin D (Fig. 7A).

To further explore the extent to which protein-DNA interactions in the osteocalcin gene are influenced by glucocorticoids, we determined whether changes in nuclear factor binding are observed in a segment of the gene containing the TATA box (−43 to +150) following treatment of confluent and proliferating ROS 17/2.8 cells with dexamethasone. Figure 8 shows that neither dexamethasone alone nor dexamethasone together with vitamin D significantly modify protein-DNA interactions.

DISCUSSION

Modifications in gene expression associated with osteoblast growth and differentiation are mediated by steroid hormones [reviewed in Lian et al., 1991b, and Stein et al., 1990]. These physiological modulators of gene expression exert an influence on regulation at both the transcriptional and a series of post-transcriptional levels, acting independently and in combination to influence the extent to which bone cell growth and tissue related genes are expressed [Owen et al., 1991a]. For the osteocalcin gene, the level of transcription is controlled by the coordinated utilization of a series of modularly organized regulatory elements in the promoter [Demay et al., 1990; Kerner et al., 1989; Lian et al., 1989;

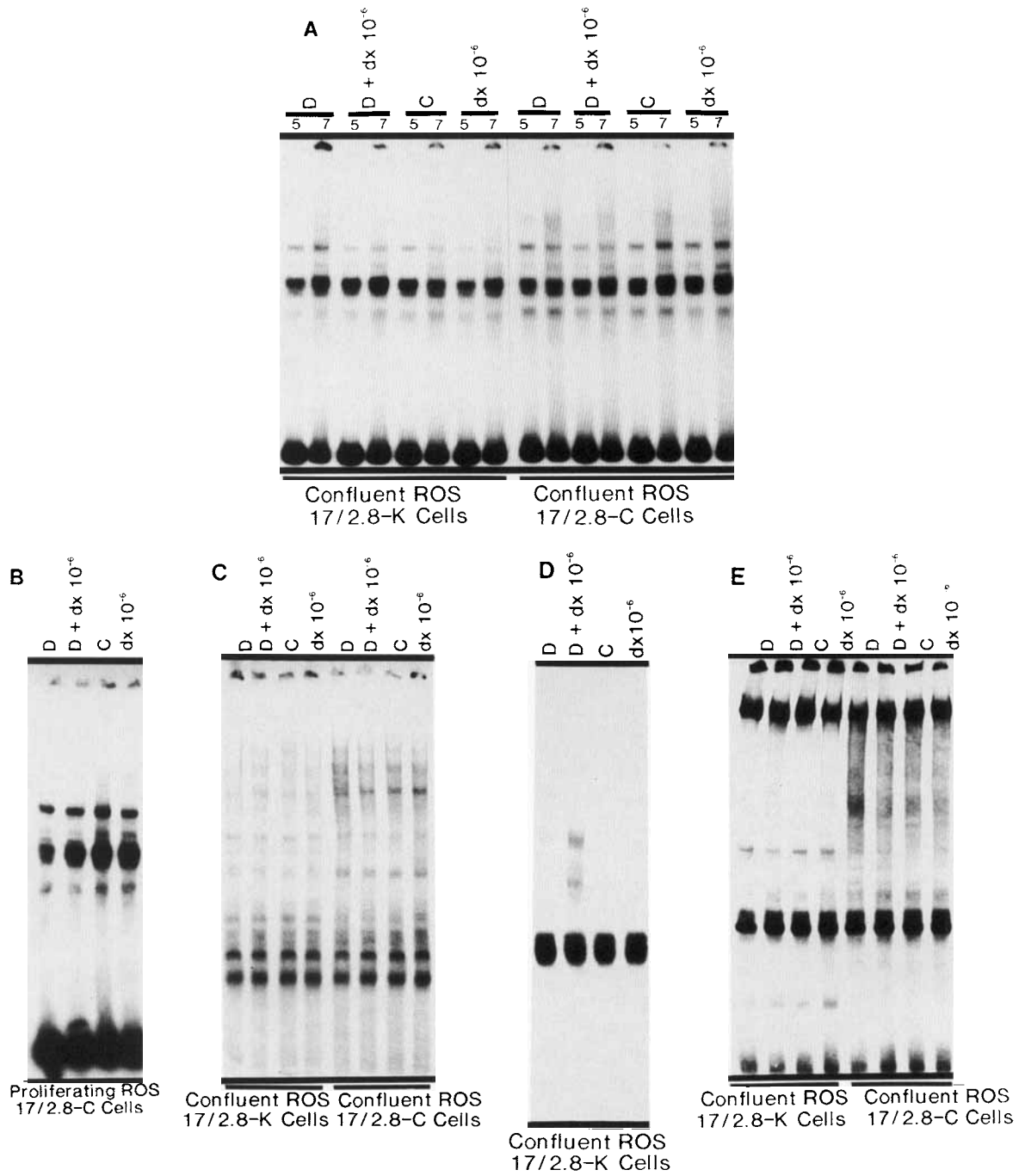

Fig. 6. Sequence-specific nuclear protein factor binding in the OC box, the promoter region regulating basal transcription level, does not change in response to vitamin D or dexamethasone treatment. **A:** ROS 17/2.8K and 17/2.8C cells were grown to confluence and treated with vitamin D, dexamethasone, or both for 24 hours, and nuclear proteins were prepared. Specific binding of proteins to the 24 base pair OC box probe −99 to −76 were assessed by gel retardation assay, resolving the protein/DNA complexes in a 4.5% native polyacrylamide gel. **B:** Binding of nuclear factors from proliferating ROS 17/2.8C cells to the 24 bp OC box probe. Cells were treated with vitamin D and dexamethasone during proliferation, and nuclear factors prepared and assayed by gel retardation analysis as in Fig. 6A. **C:** Binding of nuclear proteins from confluent 17/2.8C and 17/2.8K cells to the −101 to −43 probe, which contains the OC box and additional flanking sequences. **D:** Binding of nuclear proteins from confluent cells to the −142 to −101 probe, which contains only sequences immediately upstream of the OC box. **E:** Binding of nuclear proteins from confluent cells to the −142 to −43 probe, which contains the OC box and additional upstream and downstream flanking sequences.

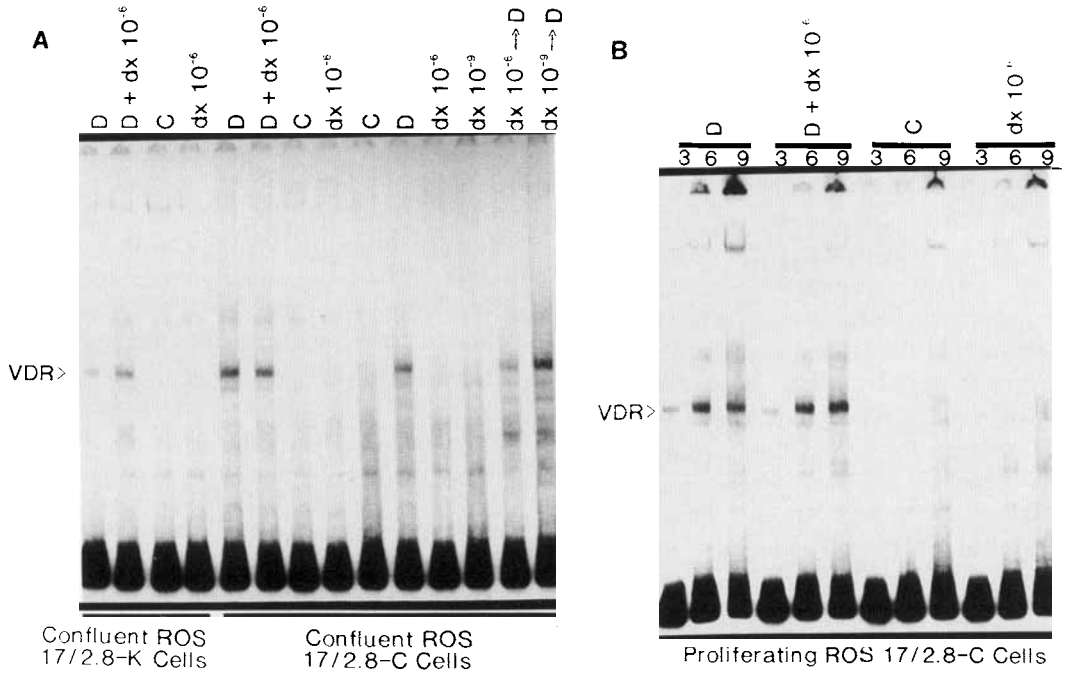

Fig. 7. Sequence-specific protein/DNA binding at the VDRE in response to vitamin D and dexamethasone treatment assessed by the gel retardation assay. The probe spanned the −527 to −344 region of the OC gene promoter which includes the VDRE. An arrow marks the complex of the probe with the vitamin D receptor. **A:** Binding of nuclear extracts from confluent ROS 17/2.8C and 17/2.8K cells. **B:** Binding of nuclear extracts from proliferating 17/2.8C cells.

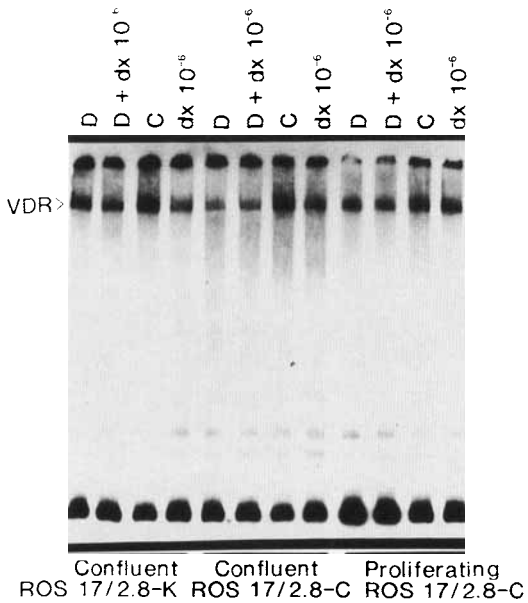

Fig. 8. Protein-DNA interactions in the TATA box region in confluent 17/2.8K and 17/2.8C, and proliferating 17/2.8C. Extracts were assayed for sequence-specific binding to the region −43 to +150 by gel retardation analysis.

Markose et al., 1990; Owen et al., 1990b]. Here, regulation reflects multiple copies of specific steroid responsive elements [Bortell, 1991; Lian et al., 1989; Markose et al., 1990], occupancy of several types of steroid responsive elements simultaneously by their cognate hormone-receptor complexes [Markose et al., 1990; Owen et al., 1990b], and the ability of different steroid receptor complexes to exhibit recognition and interaction with specific steroid responsive elements [Bortell et al., 1991; Schüle et al., 1990]. Post-transcriptionally, regulation of osteocalcin gene expression may be mediated by control of mRNA processing, mRNA translatability, and/or post-translational modifications and secretion of osteocalcin.

In the present studies, we focused on the influence of glucocorticoids on osteocalcin gene expression based on the well-documented effects of this steroid hormone on bone cell growth and differentiation, both in vivo as well as in vitro [Canalis, 1983; Chen et al., 1977; Eilam et al., 1980; Ekenstam et al., 1988; Lukert et al., 1986; Majeska et al., 1980, 1985; Morrison et al., 1989; Rodan et al., 1984; Wong et al., 1990]. Our results from studies carried out with two ROS

17/2.8 osteosarcoma cell sublines indicate that while dexamethasone does not significantly influence basal levels of osteocalcin gene expression as reflected by osteocalcin biosynthesis, cellular levels of osteocalcin mRNA, and osteocalcin gene transcription, dexamethasone reduces or abrogates the vitamin D upregulation of the osteocalcin gene. The glucocorticoid effect on the vitamin D–mediated upregulation of osteocalcin gene expression appears to be transcriptional as well as post-transcriptional since dexamethasone coordinately reduces the stimulation of osteocalcin biosynthesis, mRNA levels, and promoter activity. Such an effect of dexamethasone on the ability of vitamin D to enhance expression of the osteocalcin gene is consistent with observations of Morrison et al. [1989], who also examined osteocalcin biosynthesis and transcription in ROS 17/2.8 cells, and Wong et al. [1990] in studies of osteocalcin biosynthesis in bone cells derived from trabecular explants.

Interestingly, despite the selective effects of dexamethasone we have demonstrated on vitamin D–mediated transcriptional properties of the osteocalcin gene, we found no evidence for modifications in the sequence specific interactions of promoter binding factors at either the osteocalcin box or at the VDRE. Since these two promoter elements coordinately and possibly by a cooperative interaction bind the vitamin D receptor complex and contribute to the vitamin D–mediated enhancement in osteocalcin gene transcription [Bortell et al., 1991], an influence of dexamethasone on other promoter regulatory elements is suggested. Indeed, the findings of Morrison et al. (22) from deletion mutation analysis suggest that the glucocorticoid repression of $1,25(OH)_2D_3$ enhanced osteocalcin gene transcription is at least in part through a regulatory element(s) outside of the vitamin D responsive element residing between −196 and +134 (human osteocalcin gene). The recent identification of a glucocorticoid responsive element in association with the TATA motif of the human osteocalcin gene further supports this interpretation [Strömstedt et al., 1991]. A comprehensive understanding of the specific osteocalcin gene regulatory elements that are compromised in ability to facilitate the vitamin D responsiveness remains to be established and requires a systematic examination of protein-DNA interactions at sequences that contribute to transcriptional control by a broad spectrum of hormonal and other physiological mediators of osteocalcin gene expression.

Defining molecular mechanisms by which glucocorticoids modulate expression of the osteocalcin gene can provide valuable insight into development of the osteoblast phenotype. Perhaps the most compelling basis for this expectation is several lines of evidence which indicate that dexamethasone promotes osteoblast phenotype development in osteoprogenitor cells and that dexamethasone suppresses proliferation and is functionally related to activation and/or enhancement of specific genes expressed post-proliferatively during osteoblast differentiation. This ability of dexamethasone to promote expression of the bone cell phenotype is particularly evident in cultures of normal diploid calvarial derived osteoblasts where the steroid dramatically increases formation of nodules exhibiting a tissue-like organization of osteocytic cells in a mineralized extracellular matrix [Bellows et al., 1987; Tassinari et al., 1991].

The primary effect of glucocorticoids on the vitamin D–mediated enhancement, rather than on the basal levels of osteocalcin gene expression in osteosarcoma cells, is compatible with the well-documented physiological control of the osteocalcin gene by several steroid hormones which determine the level of transcription, rather than the ability of the gene to be transcribed. In contrast, glucocorticoids appear to more directly influence expression of other osteoblast phenotypic genes and signalling mechanisms in ROS 17/2.8 cells. For example, dexamethasone increases alkaline phosphatase expression [Majeska et al., 1985] and the activity of β-adrenergic receptors [Rodan and Rodan, 1986] and adenylate cyclase activity [Rodan and Rodan, 1986; Rodan et al., 1987]. Taken together, these results suggest that glucocorticoids exert pleiotropic effects on expression of genes associated with the osteoblast phenotype in ROS 17/2.8 cells, potentially involving unique methods for modifying expression at multiple levels and at several stages of osteoblast phenotype development. At the transcriptional level, the extent to which dexamethasone influences expression of specific genes may in part reflect the location of glucocorticoid responsive elements in the promoter. Transcriptional control may additionally reflect the interaction of a series of transcription factors including steroid receptor complexes at single or multiple steroid responsive elements. Here, synergistic and/or antagonistic effects of

steroid hormones may result from cooperative interactions between activities at independent promoter regulatory elements [Ankenbauer et al., 1988; Jantzen et al., 1987; Schüle, et al., 1988, 1990]. Such a regulatory mechanism is consistent with recent results indicating the presence of two sequence-specific and antibody sensitive vitamin D receptor binding sites in the osteocalcin gene promoter residing between −462 and −440 and within the osteocalcin box, the CCAAT-motif containing proximal elements principally involved with control of basal level expression [Bortell et al., 1991].

Several additional lines of evidence implicate a glucocorticoid influence on osteocalcin gene expression at multiple levels. A parallel effect on the extent to which dexamethasone reduces the vitamin D stimulation of osteocalcin gene transcription, mRNA levels, and protein synthesis is clearly consistent with transcriptional and post-transcriptional actions of dexamethasone. Post-transcriptional regulation is further suggested by the ability of dexamethasone to reduce osteocalcin mRNA levels and protein synthesis to a similar extent when glucocorticoid treatment is initiated following upregulation of the osteocalcin gene by vitamin D. The complexity of the influence of glucocorticoids on osteocalcin gene expression is further illustrated by the observation that the extent to which dexamethasone inhibits the vitamin D–mediated upregulation is reduced in a ROS 17/2.8 cell line exhibiting elevated levels of basal expression. These results suggest a maximum extent to which dexamethasone can abrogate the vitamin D enhancement of the osteocalcin gene.

It is important to consider molecular mechanisms associated with osteocalcin gene expression within the context of general regulatory mechanisms operative during the progressive development of the osteoblast phenotype. A reciprocal relationship between proliferation and expression of genes for mature osteoblast parameters is one component of this differentiation process [Aronow et al., 1990; Dworetzky et al., 1990; Owen et al., 1990a; Stein et al., 1990]. In transformed osteoblasts and osteosarcoma cells, key elements of the stringently regulated control of proliferation are abrogated as reflected by constitutive binding of transcription factors to the promoters of cell cycle regulated genes [Holthuis et al., 1990; Stein et al., 1990; van Wijnen et al., 1991]. In proliferating osteosarcoma cells, the constitutive expression of osteoblast related genes that are sequentially expressed post-proliferatively in normal diploid osteoblasts is indicative that the proliferation/differentiation relationship which is fundamental to bone tissue development has been compromised [Lian et al., 1991a; Stein et al., 1990].

It is therefore noteworthy that in contrast to the inhibition of vitamin D upregulation of osteocalcin gene expression by dexamethasone in ROS 17/2.8 osteosarcoma cells, in the normal diploid calvarial derived osteoblasts, dexamethasone increases levels of osteocalcin gene transcription [Owen et al., 1991b]. Furthermore, together with vitamin D, dexamethasone synergistically upregulates osteocalcin gene expression to levels severalfold above those resulting from vitamin D alone [Owen et al., 1991b]. Such differences in hormonal responsiveness of normal and transformed osteoblasts may in a restricted sense reflect modifications in regulation of the osteocalcin gene in response to steroid hormones. However, in a broader biological context, these observed differences may be a reflection of modifications in steroid hormone-mediated signalling mechanisms that are functionally related to the stringently regulated expression of genes during bone cell growth and differentiation.

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