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Multiple Levels of Steroid Hormone-Dependent Control of Osteocalcin During Osteoblast Differentiation: Glucocorticoid Regulation of Basal and Vitamin D Stimulated Gene Expression

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Abstract We have examined the contribution of transcriptional mechanisms to the pleiotropic effects of glucocorticoids on basal and vitamin D stimulated expression of the developmentally regulated bone-specific osteocalcin (OC) gene. OC expression was systematically investigated at the level of protein, mRNA, and newly synthesized transcripts during maturation of the bone cell phenotype in cultures of fetal rat calvarial-derived osteoblasts. Our results indicate that transcriptional control of basal and hormone-regulated OC expression predominates in immature osteoblasts prior to matrix mineralization. However, in mature osteoblasts OC expression is controlled primarily by posttranscriptional mechanisms reflected by elevated mRNA levels with a decline in transcription. Vitamin D, alone or in combination with Dex, is a significant factor contributing to mRNA stabilization in mature osteoblasts with a mineralized extracellular matrix. Transcriptional modifications in response to Dex are reflected by quantitative differences between proliferating and mature osteoblasts in the formation of glucocorticoid receptor binding complexes at the proximal OC glucocorticoid response element. Vitamin D and glucocorticoid receptor mRNA levels are significantly higher in mature osteoblasts than in early stage bone cells. However, receptor complexes do not appear to be rate limiting in proliferating osteoblasts when the OC gene is not transcribed. Our results indicate (1) developmental stage-specific effects of steroid hormone on transcriptional regulation of bone expressed genes, and (2) inverse relationships between levels of transcription and cellular representation of mRNA with OC message stabilized in mature osteoblasts. J. Cell. Biochem. 69:154–168, 1998.

Key words: transcription; mRNA stability; dexamethasone; gene regulation; glucocorticoid receptor; rat calvarial osteoblasts; osteopontin; vitamin D receptor

Osteocalcin (OC) is a developmentally regulated, tissue-restricted gene that encodes a major non-collagenous extracellular matrix protein of bone. It is expressed at maximal levels both in vivo [reviewed in Stein and Lian, 1993; Hauschka et al., 1989] and in vitro [Pockwinse et al., 1992; Owen et al., 1990; Stein and Lian, 1993] in mature osteoblasts with a mineralizing matrix. Consequently, osteocalcin serves as a marker of osteoblast differentiation. The OC gene is regulated by a plethora of hormones and growth factors, including glucocorticoids and $1,25(OH)_2D_3$ [reviewed in Stein and Lian, 1993, and references therein]. These steroid hormones regulate bone turnover in vivo by modulating growth, differentiation, and metabolic activities of both osteoclasts and osteoblasts [Lukert and Raisz, 1990; Lian and Stein, 1993; Suda et al., 1992]. In most species, vitamin D upregulates osteocalcin and other genes associated with development of the mature osteoblast phenotype [reviewed in Lian and Stein, 1992, 1993]. Glucocorticoids, such as dexamethasone (Dex), promote the differentiation of osteoprogenitor cells into mature osteoblasts [Shalhoub et al., 1992; Bellows et al., 1987; Bellows and Aubin, 1989]. Transient exposure of rat osteoblast cultures to glucocorticoids during the preconfluent developmental period results in an

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increased number and size of bone tissue-like nodules. The increased number of mature osteoblasts associated with mineralized nodules is correlated with and may be functionally linked to increased synthesis of osteocalcin [Shalhoub et al., 1992].

We experimentally addressed a paradox. Dex inhibits basal and vitamin D-stimulated OC mRNA expression through specific gene regulatory sequences of both the rat and human OC genes in ROS 17/2.8 cells [Schepmoes et al., 1991; Bortell et al., 1992; Morrison and Eisman, 1993; Morrison et al., 1989; Aslam et al., 1995; Heinrichs et al., 1993] and human osteoblasts [Subramaniam et al., 1992; Wong et al., 1990]. However, Dex promotes OC expression when acting as a potent differentiation inducing agent for marrow cells [Kasugai et al., 1991; Leboy et al., 1991; Cheng et al., 1996] or fetal rat calvarial derived osteoprogenitor cells [Bortell et al., 1993; Shalhoub et al., 1992]. Thus, we have examined the effects of acute and chronic Dex-treatment on basal and vitamin D induced OC expression (transcription, mRNA and protein synthesis) throughout the in vitro differentiation of fetal rat calvaria-derived cells. We compared primary cultures and passaged fetal rat calvarial derived osteoblasts that did not receive adequate signals to differentiate into bone nodules (representing control groups) to cultures chronically treated with dexamethasone, which develop mineralized nodules with a bone tissue-like organization.

The rat and human OC promoters contain multiple glucocorticoid responsive elements (GREs) [Stromstedt et al., 1991; Morrison and Eisman, 1993; Morrison et al., 1989; Aslam et al., 1995; Heinrichs et al., 1993]. In the rat gene, GREs in the distal (nt −697 to −683) and proximal promoter regions (nt −16 to −1 nt) bind purified glucocorticoid receptor and have been shown to modulate negatively the $1,25(OH)_2D_3$-induced transcription of osteocalcin in ROS 17/2.8 osteosarcoma cells [Aslam et al., 1995]. However, in normal diploid osteoblasts, the mechanisms by which OC mRNA and protein synthesis are increased during glucocorticoid treatment are unclear. The complexity of glucocorticoid responsiveness is also indicated by location of several GREs, each in domains overlapping other regulatory sequences within the OC gene promoter. The TATA box and a proximal GRE are partially overlapping in the human gene [Stromstedt et al., 1991] and contiguous in the rat gene [Heinrichs et al., 1993]. A half GRE site in the tissue-specific OC Box I domain in the rat promoter is potentially involved in GR binding [Aslam et al., 1995; Heinrichs et al., 1993]. In addition, glucocorticoid responsiveness of both the human and rat OC genes involves other, non-GRE, elements [Morrison and Eisman, 1993; Aslam et al., 1995]. How the independent and combined activities of these regulatory sequences interface with the multiple parameters that contribute to glucocorticoid regulation of OC gene transcription during osteoblast differentiation is unknown. In the present studies, DNA binding complexes formed at the OC GREs at three stages of osteoblast phenotype development are examined. The findings reveal differences in the formation of Dex-responsive complexes between early osteoblasts and mineralized cultures.

The OC promoter contains a well-characterized vitamin D responsive element (VDRE) at nucleotides −465 to −437, which binds the VDR/RXRα transactivation complex [MacDonald et al., 1993; Schrader et al., 1993; Staal et al., 1996]. Several reports indicate that osteocalcin synthesis and mRNA levels are upregulated by $1,25(OH)_2D_3$. The extent of induction is inversely related to the basal level of expression [Bortell et al., 1992; Owen et al., 1991, 1993]. Several molecular mechanisms contribute to this regulation and involve crosstalk (protein-protein interactions) between the VDR and TFIIB of the basal transcriptional machinery [Blanco et al., 1995; MacDonald et al., 1995; Guo et al., 1997], and vitamin D regulation of transcription factors [e.g., cfos: Candeliere et al., 1991, 1996; Msx-2 Hoffmann et al., 1994] that modulate osteocalcin basal expression. These observations raise questions related to modifications in vitamin D induction of transcriptional activity by glucocorticoids as a function of osteoblast differentiation.

In the present studies, regulation of osteocalcin gene expression was systematically examined at the levels of protein synthesis, mRNA, rate of transcription, in vitro protein-DNA interactions, and nuclear levels of the glucocorticoid and vitamin D receptors. The results indicate that basal and steroid hormone modulation of osteocalcin expression by both $1,25(OH)_2D_3$ and Dex are controlled by different mechanisms throughout the osteoblast developmental sequence. Transcriptional regulation predomi-

nates in immature osteoblasts where basal levels are minimal. In contrast, mRNA stability is the major component of OC gene regulation in mature osteoblasts with a mineralized matrix when basal expression is high.

MATERIALS AND METHODS

Cell Culture

Calvariae from fetal rats of 21 days gestation were isolated and subjected to sequential digestions of 20, 20, and 90 min at 37°C in 1 mg/ml collagenase P (Boehringer-Mannheim, Indianapolis, IN)/0.25% trypsin (Gibco, Grand Island, NY) [Aronow et al., 1990]. The cells of the first two digests were discarded, and those released from the third digestion were plated in minimal essential medium (MEM; Gibco) supplemented with 10% fetal calf serum (FCS) in 100-mm dishes (Corning, Corning, NY) at a density of 5×10^5 cells/dish. At confluence (day 7), cells received media supplemented with 10% FCS and 25 μg/ml ascorbic acid. On the following feedings, cells received media supplemented with 50 μg/ml ascorbic acid, 10 mM β-glycerol phosphate (βGP04). Cells were treated for 24 h with 10^{-8} M, $1,25(OH)_2D_3$ (gift of Dr. M. Uskokovic, Hoffmann-La Roche Inc., Nutley, NJ) or 10^{-7} M Dex (Sigma Chemical Co., St. Louis, MO) prior to harvest on the days indicated. Passaged cells were subcultivated from primary cultures on day 10 and grown as either control cells in the absence of Dex and βGP04 or in the continual presence of 10^{-7} M Dex in differentiation media (with 50 μg/ml ascorbate and 10 mM βGP04).

Osteocalcin Protein Quantitation

Aliquots of the medium were collected every 48 h throughout the developmental time course and assayed for the level of osteocalcin secreted by radioimmunoassay as previously described [Gundberg et al., 1984]. The values represent the mean of three independent samples collected from the same 100-mm plates used for RNA or nuclei preparations.

RNA Isolation

Cell layers were scraped and harvested at the days indicated and the pellets stored at −70°C until completion of the experiment. Total cellular RNA was isolated from each cell pellet by the method of Chirgwin [Chirgwin et al., 1979]. RNA preparations were quantitated by absorbance at 260 nm and intactness assessed by ethidium bromide staining after separation in 6.6% formaldehyde-1% agarose gels. Fractionated RNA was transferred by Northern blot to Zeta probe membrane (Bio-Rad, Richmond, CA). The blots were then probed with random primer-labelled (α-^{32}P)dCTP cDNA probes with a specific activity of at least 1×10^9 dpm/μg DNA [Feinberg and Vogelstein, 1983]. The osteocalcin probe is an insert from plasmid pOC3.4 [Lian et al., 1989]. The histone H4 probe is an insert from the plasmid pFO002 [Grimes et al., 1987]. The collagen [Genovese et al., 1984], osteopontin [Yoon et al., 1987], vitamin D receptor [Burmester et al., 1988], and glucocorticoid receptor [Alnemri et al., 1991] cDNAs were directly labeled. Autoradiographs were quantitated by scanning laser densitometry (LKB 2400 Gel Scan XL; LKB, Bromma, Sweden) within the linear range of signals and normalized to ribosomal 28S RNA levels from ethidium bromide-stained gels.

Nuclear Run-On Assay

Nuclei were isolated at days 3, 6, 14, and 24 by a described procedure [Dignam et al., 1983]. Equal amounts of (^{32}P)UTP labelled transcripts synthesized in vitro from 1×10^7 nuclei/run-on reaction were hybridized to excess DNA probes specific for 28S rRNA (28S) [Wilson et al., 1978]; pUC19 [Robertson et al., 1995]; histone H4 (H4) [Grimes et al., 1987]; osteocalcin [Lian et al., 1989]; vitamin D receptor [Burmester et al., 1988] and glucocorticoid receptor [Alnemri et al., 1991]. Hybridization signals were detected by exposing blots to XAR-5 X-ray film (Eastman Kodak Co., Rochester, NY) using a Cronex Lightning Plus (E.M. Parker Co., Inc., Wilmington, MA) screen at −70°C. Autoradiographs were quantitated by scanning laser densitometry (LKB 2400 Gel Scan XL) within the linear range of signals. The graph represents densitometer units minus pUC19 plasmid background signal normalized to the 28S rRNA levels.

Gel Mobility Shift Assay

Nuclear proteins were extracted by the method of Dignam et al. [Dignam et al., 1983]. Double-stranded oligonucleotides used as probes and competitors for the rat osteocalcin VDRE spanning nucleotides −468 to −440 and for the proximal (nt −26 to +11) and distal (nt −707 to −672) rat GREs are shown in Table I. The steroid mutant oligonucleotides have specific

base changes in the steroid half elements (indicated in boldface). Each strand was labeled with (γ-^{32}P)ATP with phage T4 polynucleotide kinase, and the strands were annealed. Gel mobility shift assays for analysis of nuclear extract protein-DNA interactions were performed as described by Aslam et al. [Aslam et al., 1995] for the GRE containing probes. Antibodies to the GR were generously provided by Dr. Gerald Litwack, Jefferson University Medical School, Philadelphia, PA [Robertson et al., 1995].

Statistics

The Student's *t*-test was applied to derive significant differences between the control and each hormone treated group. For mRNA and transcription, data was pooled from duplicate Northerns or runons from two independent experiments.

RESULTS

Osteocalcin Transcription, mRNA Accumulation, and Protein Synthesis During Development of the Osteoblast Phenotype in Primary Cultures and Dexamethasone-Treated Passaged Cells

Regulation of osteocalcin expression was examined in three experimental paradigms: rat calvarial-derived primary osteoblasts (ROBs), which in vitro proceed through a developmental sequence of differentiation; subcultivated ROBs maintained continuously in dexamethasone, which undergo osteoblast differentiation and mineralized ECM formation; and passaged cells, which lose the ability to produce a mineralized ECM and differentiate for parallel construct. OC mRNA levels and secreted osteocalcin protein, quantitated by radioimmunoassay in conditioned media, were assayed 24 h after feeding at three stages of osteoblast maturation. In primary osteoblast cultures (Fig. 1A), osteocalcin mRNA and protein were first detected when cells reached monolayer confluency on day 6 and increased thereafter during formation of bone nodules (day 14). A 100-fold increase in OC mRNA and secreted protein was observed from day 6 (1.5 $\pm$ 0.3 ng/ml) to day 20 (1,750 $\pm$ 125 ng/ml) during active mineral deposition. In passaged osteoblasts (Fig. 1B–D), the non-Dex treated control cultures, which do not differentiate, exhibited low levels and small increases in protein synthesis (Fig. 1B) and mRNA (Fig. 1C) from day 6 to day 24. Continuous treatment of these passaged cells with 10^{-7} M Dex restored OC synthesis to the high levels observed in primary cultures coincident with mineralized nodule formation (Fig. 1B,C). Chronic treatment of passaged cells with Dex resulted in 10- (day 6) to 23-fold (day 24) increases in accumulated mRNA compared to slowly differentiating passaged cells in the absence of Dex. In cells chronically treated with Dex, mRNA accumulation declines after day 24 (data not shown) as a result of accelerated differentiation and more advanced mineralization in Dex-treated cultures compared to primary cultures. This finding is consistent with the decline of osteocalcin expression (mRNA and protein) in mature mineralized cultures after day 28 observed in primary osteoblasts, demonstrated in previous studies [Shalhoub et al., 1994; Stein and Lian, 1993].

In contrast to the steady increase in osteocalcin mRNA during differentiation of primary isolated rat osteoblasts (Fig. 1A), nuclear run-on analyses demonstrated an inverse relationship with OC transcription in mature osteoblasts (day 24). Transcription increases during the early phase of osteoblast differentiation (day 6–14) and then declines (day 24) at the peak level of OC mRNA accumulation. The subcultivated control cell cultures (Fig. 1D) also exhibited decreased transcription with increasing mRNA levels, but the changes were modest in these passaged cells. In contrast, we observed that osteocalcin transcription continued to increase throughout osteoblast differentiation in passaged cells under the influence of chronic Dex (Fig. 1D), supporting the increased mRNA levels (Fig. 1C).

Steroid Hormone Regulation of Osteocalcin Transcription Is Related to the Stage of Osteoblast Differentiation

We examined the extent to which short-term effects of glucocorticoid modify basal and vitamin D-enhanced osteocalcin (OC) gene expression at different stages of osteoblast differentiation (Fig. 2). Ongoing transcription was measured directly during active proliferation (day 2, data not shown) and on day 7 (the onset of OC expression), day 14, and day 20 by harvesting the cells for nuclear run-on assays. Transcriptional effects of Dex were examined within 24 h of glucocorticoid treatment and we compared these findings to the effects of $1,25(OH)_2D_3$ in two independent experiments. From day 2 to 3, OC transcription was barely detected in control cells and after the 24 h of Dex treatment; and, no OC mRNA or protein

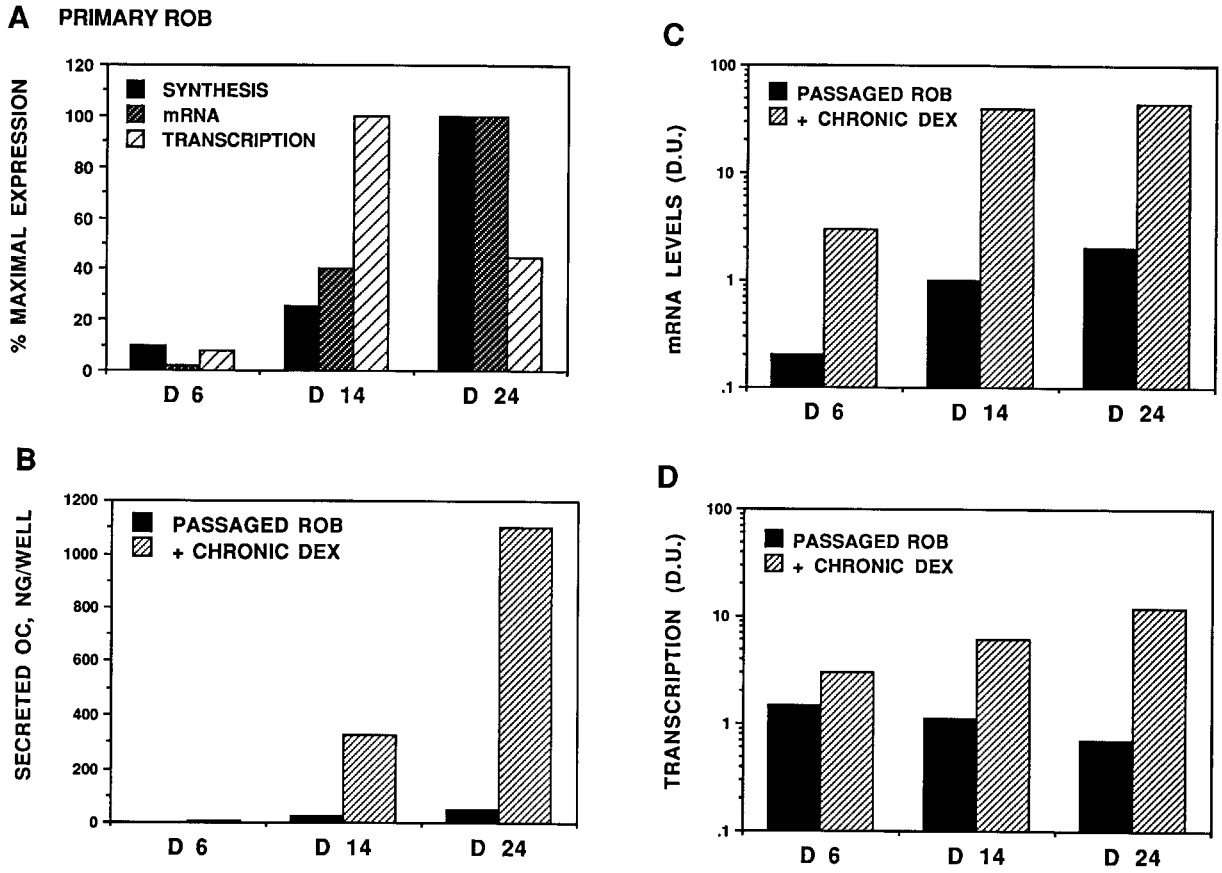


Fig. 1. Regulation of osteocalcin gene expression during osteoblast differentiation. **A:** Primary fetal rat calvarial-derived osteoblasts were harvested at the indicated days 24 h after feeding for determinations of osteocalcin synthesis (secreted protein in media), mRNA, and level of transcription (nuclear run-ons). Values were normalized to total DNA for osteocalcin protein in n = 3 wells and to representation of 18S ribosomal RNA for mRNA and transcription. The data are presented as % maximal expression based on determinations from 2 independent experiments. B–D: Regulation of OC gene expression in passaged cells (solid bars) and in passaged cells maintained in the presence of 10^{-7} M dexamethasone (+ chronic Dex) (hatched bars). **B:** Osteocalcin synthesized in 48 h quantitated by radioimmunoassay of media from n = 3 wells, **(C)** mRNA, and **(D)** transcription. Data are expressed on a log scale to emphasize the developmental changes in OC gene expression in the absence of Dex because levels are low in passaged cells. In C and D, the data from one experiment are shown and the trend reproducible in a second experiment.

was observed (data not shown). On day 7, transcription of OC in response to Dex was increased 7–10-fold compared to control levels, but mRNA and protein levels were increased only 1.8-fold (Fig. 2). On day 14 (postconfluent stage), Dex did not significantly modulate OC expression, and in mature day 20 osteoblasts, Dex significantly decreased OC transcription and mRNA levels.

$1,25(OH)_2D_3$ resulted in a 14- and 8-fold transcriptional increase on days 7 and 14, respectively, but only a 28% increase in transcription on day 20. Thus, compared to Dex, vitamin D is a more potent enhancer of OC transcription in the early stage of osteoblast maturation. The extent to which $1,25(OH)_2D_3$ increased OC mRNA levels also declined during osteoblast differentiation (Fig. 2) as basal OC levels increased (Fig. 1). In mature cells, when osteocalcin expression is at maximal levels, vitamin D regulation of OC is minimal, suggesting both posttranscriptional and posttranslational feedback regulation on osteocalcin expression in osteoblasts.

The combined effects of Dex and vitamin D on OC expression are also related to the stage of osteoblast maturation. At all stages of osteoblast phenotype development (days 7, 14, and 20), the transcriptional induction by vitamin D (D) is clearly suppressed by dexamethasone. At the early stages of osteoblast development (days 7 and 14), mRNA levels were also significantly

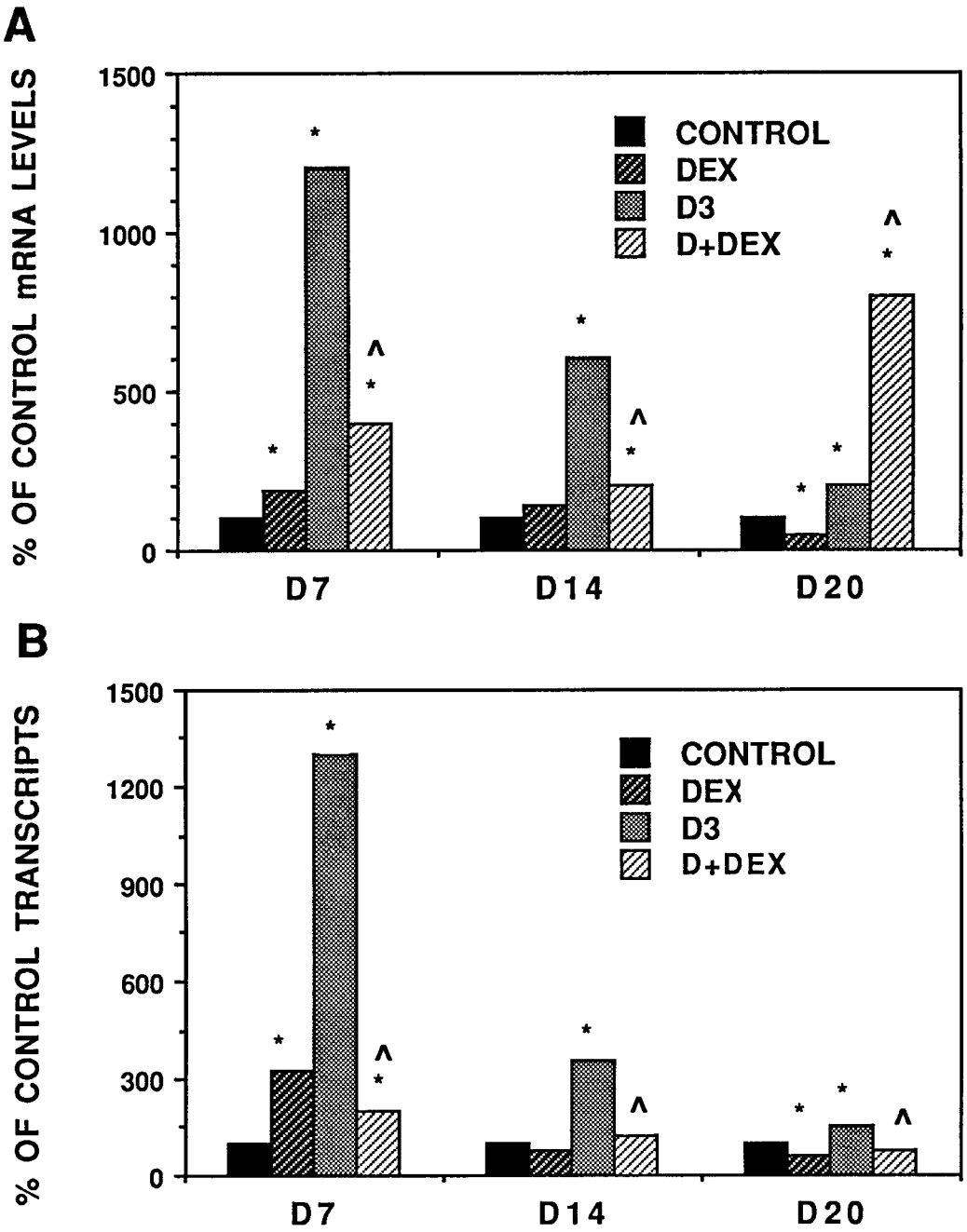


Fig. 2. Regulation of osteocalcin gene expression by hormones during osteoblast phenotype development. The effect of 24-h hormone treatments [10^{-7} M dexamethasone (Dex), 10^{-8} M $1,25(OH)_2D_3$ (D_3) or both (D + Dex)] on **(A)** osteocalcin mRNA levels (from Northern analysis) and **(B)** transcription (from run-on assays) is shown. The data are represented as percent of control values at each time point: day 7, monolayer confluency; day 14, nodules present; day 20, mineralization period. Asterisk denotes statistically significant differences in 2 independent experiments of the treated groups compared to controls ($P < .05$); the D + Dex groups significantly differed from the D_3 group ($P < .001$), designated by the arrowheads.

reduced from the vitamin D-stimulated level. However, in mature cells (day 20), the presence of Dex enhanced the vitamin D stimulation of OC mRNA levels. The striking decrease in transcription relative to the increased mRNA levels in the Dex + D treated cells on day 20 suggests the OC message level is being stabilized. Synthesized osteocalcin protein secreted into the media reflected the changes in mRNA levels (data not shown).

We examined whether this significant effect on stabilization of OC mRNA in cultures treated

with vitamin D alone or with vitamin D and Dex extends to other osteoblast expressed genes known to be regulated by these hormones (Figs. 3 and 4). Transcription and mRNA levels were compared after 4 and 24 h of treatment with the hormones. Figure 3 shows examples of Northern blots probed for expression of the histone and osteopontin (OP) gene for comparison to expression of osteocalcin. Histone gene expression (reflecting DNA synthesis) from day 7 to day 20 exhibited a parallel decline in levels of mRNA and transcription (Fig. 4). Dex significantly increased H4 expression (transcription and mRNA) after 24 h on day 7, consistent with nodule formation, but not on day 20 (Figs. 3 and 4). OP mRNA levels are regulated to a greater extent by either Dex or vitamin D in osteoblasts on day 7 compared to mature cells on day 20 (Fig. 3), but transcription does not parallel the changes in mRNA levels (Fig. 4). Similar to OC (Figs. 2 and 3), a synergistic increase in OP mRNA was observed in the Dex + D treated cultures, while transcription was inhibited (Fig. 4). Together these findings of OC and OP gene regulation support the concept that the combination of both steroid hormones affects stabilization of mRNAs in mature osteoblasts.

Influence of VDR and GR Levels on Modification of Osteocalcin Gene Transcription by Dexamethasone and 1,25(OH)$_2$D$_3$

We examined regulated expression and DNA binding activity of the glucocorticoid and vitamin D receptors during osteoblast differentiation and the influence of hormonal treatments on expression of these receptors as a potential contributing factor to observed modifications in OC transcription. Both VDR and GR basal mRNA levels increased from day 7 (proliferation stage) to day 20 (differentiated osteoblasts) (Fig. 5). Dex significantly increased VDR transcription on days 7 and 20, but this resulted in a small increase in VDR mRNA accumulation only on day 7, and a decrease on day 20. Vitamin D alone and together with Dex dramatically increased VDR mRNA levels while transcription decreased; this suggests stabilization of VDR mRNA in mature osteoblasts. GR receptor mRNA levels and transcription were significantly upregulated by Dex only in day 7 cultures. In contrast, vitamin D suppressed GR transcription (day 7) while mRNA levels remained equivalent to the control. Transcriptional control of the GR in mature cells (day 20)

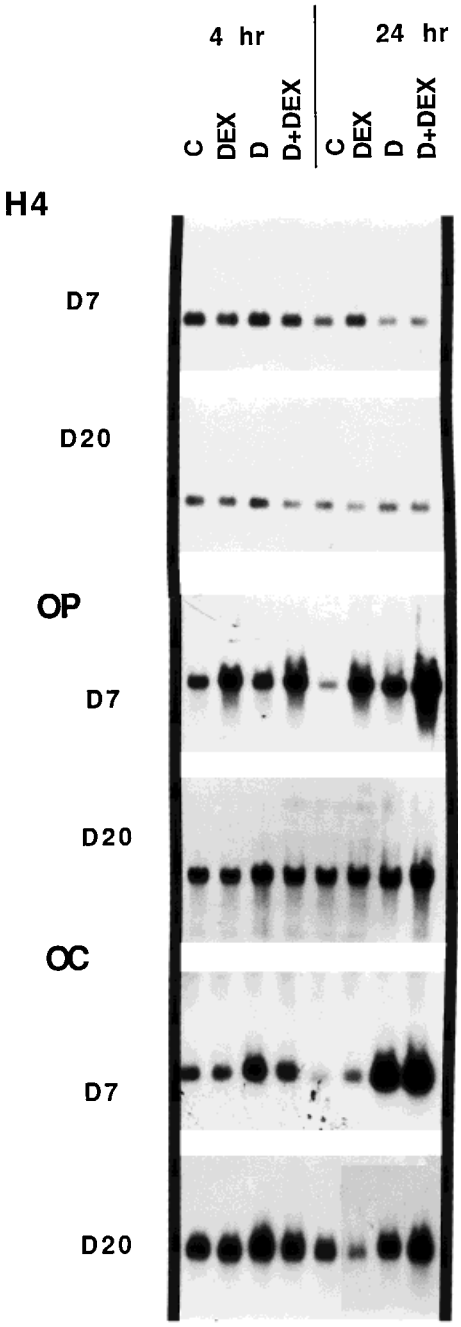


Fig. 3. Cellular mRNA levels of osteoblast expressed genes after 4 and 24 h of hormone treatment. Northern blot analysis for histone (H4), osteopontin (OP), and osteocalcin (OC) using 10 μg of total cellular RNA per lane from fetal rat calvarial-derived osteoblasts at the indicated time and days in culture. Ethidium bromide staining of each gel demonstrated intactness and similar quantities of RNA (not shown). Exposure times for H4 on days 7 (late proliferation) and 20 (differentiation) are equivalent; OP and OC on day 20 required a shorter exposure time compared to day 7 to reveal differences among the group's quantitation because of the increased representation of these transcripts in the differentiated osteoblasts.

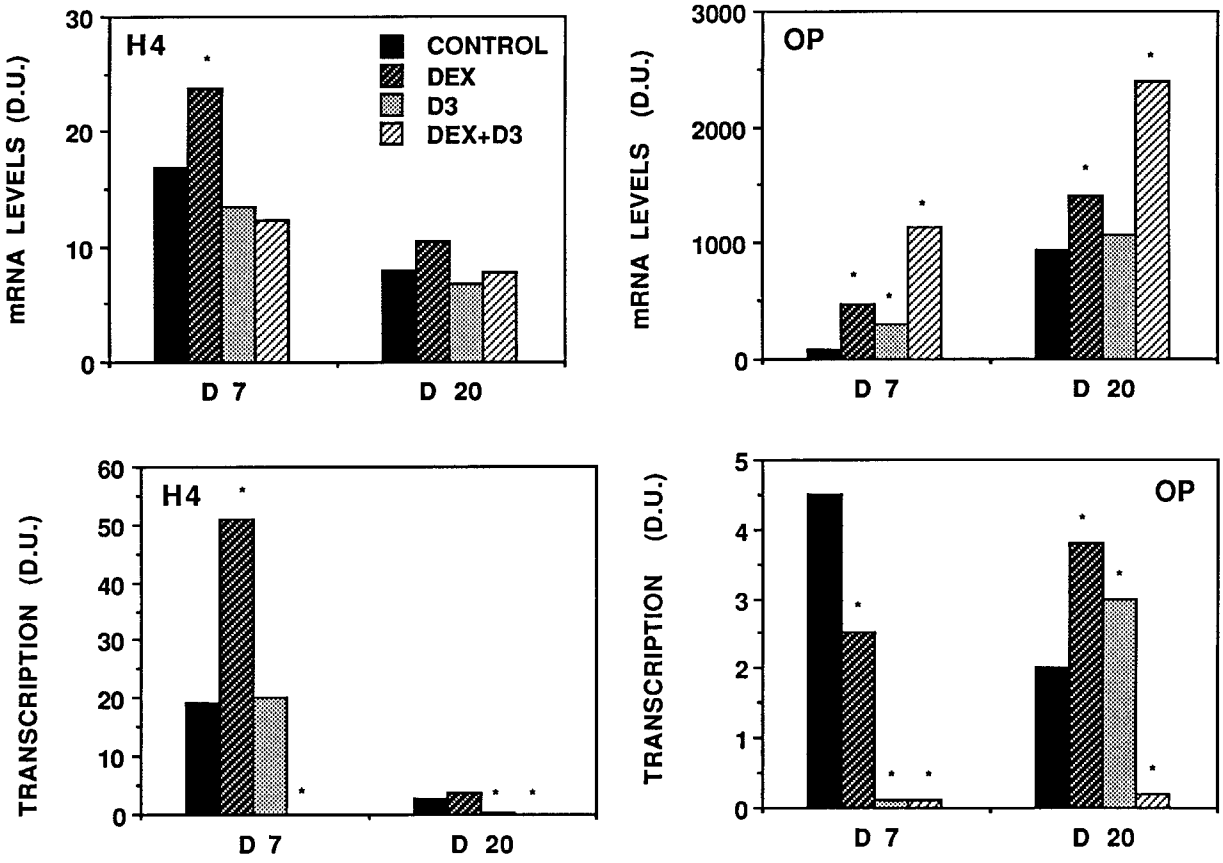


Fig. 4. Quantitation of hormonal regulation (mRNA and transcription) of histone (H4) and osteopontin (OP) expression as a function of osteoblast differentiation. Data represents cells harvested 24 h after feeding and hormone treatment. On day 7, growth period, and day 20, the mineralization period, histone H4 **(left)** and OP **(right)** mRNA levels were quantitated from Northern blot analysis **(top)** and transcription from nuclear run-on assays **(bottom)** at equal exposure and normalizing to 28S ribosomal RNA. Total cellular RNA and nuclei were prepared as described in Materials and Methods. For Northern analysis, 10 μg/lane were applied; and for run-on analysis, 1×10^{-7} nuclei per slot were applied for hybridization with the specific probes.

was either very low or not observed in independent experiments. However, the combined effects of Dex + D_3 consistently reduced transcription to nondetectable levels compared to the control, but the mRNA levels were stabilized.

We have previously shown differences in the properties of the VDRE binding complex between proliferating and differentiated osteoblasts by gel mobility shift assays. These findings are consistent with absence and presence of vitamin D regulation of OC [Shakoori et al., 1994; Bortell et al., 1993]. Based on these results, during osteoblast differentiation we examined GR interactions at the multiple GREs, which occur in the proximal promoter, and at an upstream distal GRE [Aslam et al., 1995]. At the distal GRE, one predominant protein-DNA complex forms, which is constitutively expressed and not regulated by Dex during osteoblast development (Fig. 6A). However, differences between proliferating (day 2) and differentiated osteoblast extracts (days 11 and 20) were observed in formation of GR receptor complexes at the proximal GRE. The major specific complex observed at the distal GRE also forms at the proximal GRE, but at reduced levels. However, other bands of slower mobility are observed. One complex forms at significant levels in postproliferative osteoblasts, day 11 and day 20 (Fig. 6, arrowhead). This complex was represented at lower levels in the more differentiated day 20 cells and was increased by Dex on day 20. We verified that these complexes involve specific GR interactions by antibody and oligonucleotide competition analysis (Fig. 6B). At the proximal GRE, the presence of GR in the complexes was demonstrated by a block shift of the slower mobility complex and a

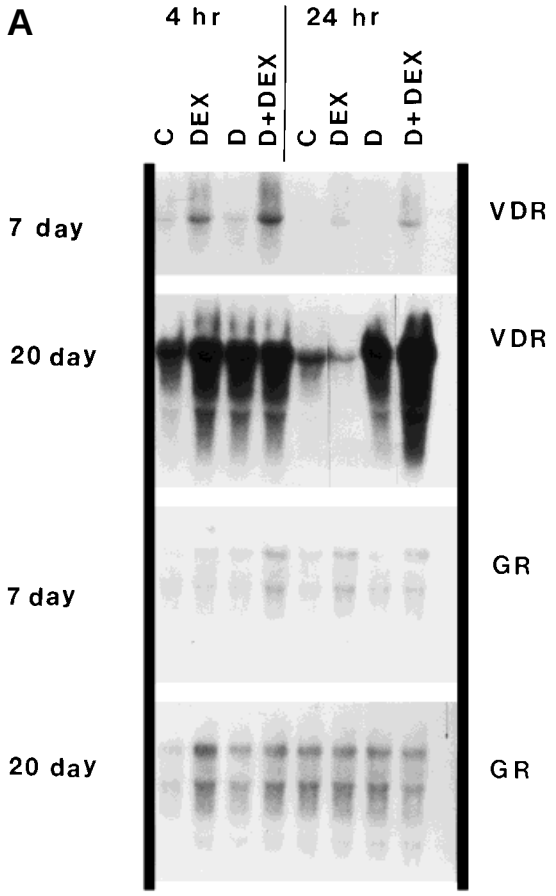
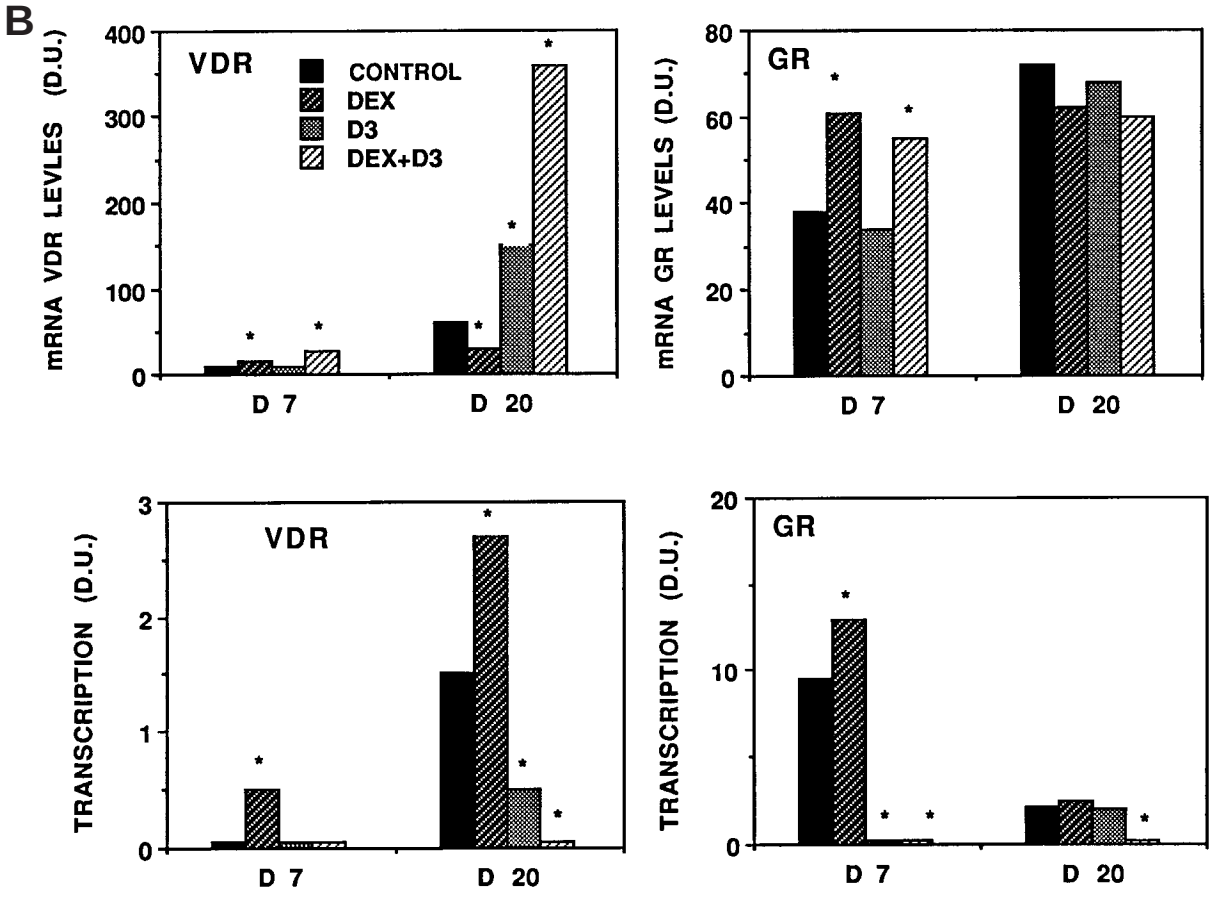


Fig. 5. Regulation of vitamin D receptor (VDR) and glucocorticoid receptor (GR) expression during osteoblast differentiation. **A:** Cells were harvested at the indicated days following 4 and 24 h of feeding with addition of each hormone 10^{-8} M $1,25(OH)_2D_3$ (D) and 10^{-7} M dexamethasone (Dex) or both (D + Dex) on days 7 and 20. Total cellular RNA (10 µg) was used for Northern blot analysis. **B:** Quantitation of Northern blot analysis of the 24-h mRNA levels **(top)** and nuclear run-on assays for transcription **(bottom)** of the vitamin D and glucocorticoid receptors. Asterisks designate significant differences from controls from two independent experiments after 24 h of hormone treatment.



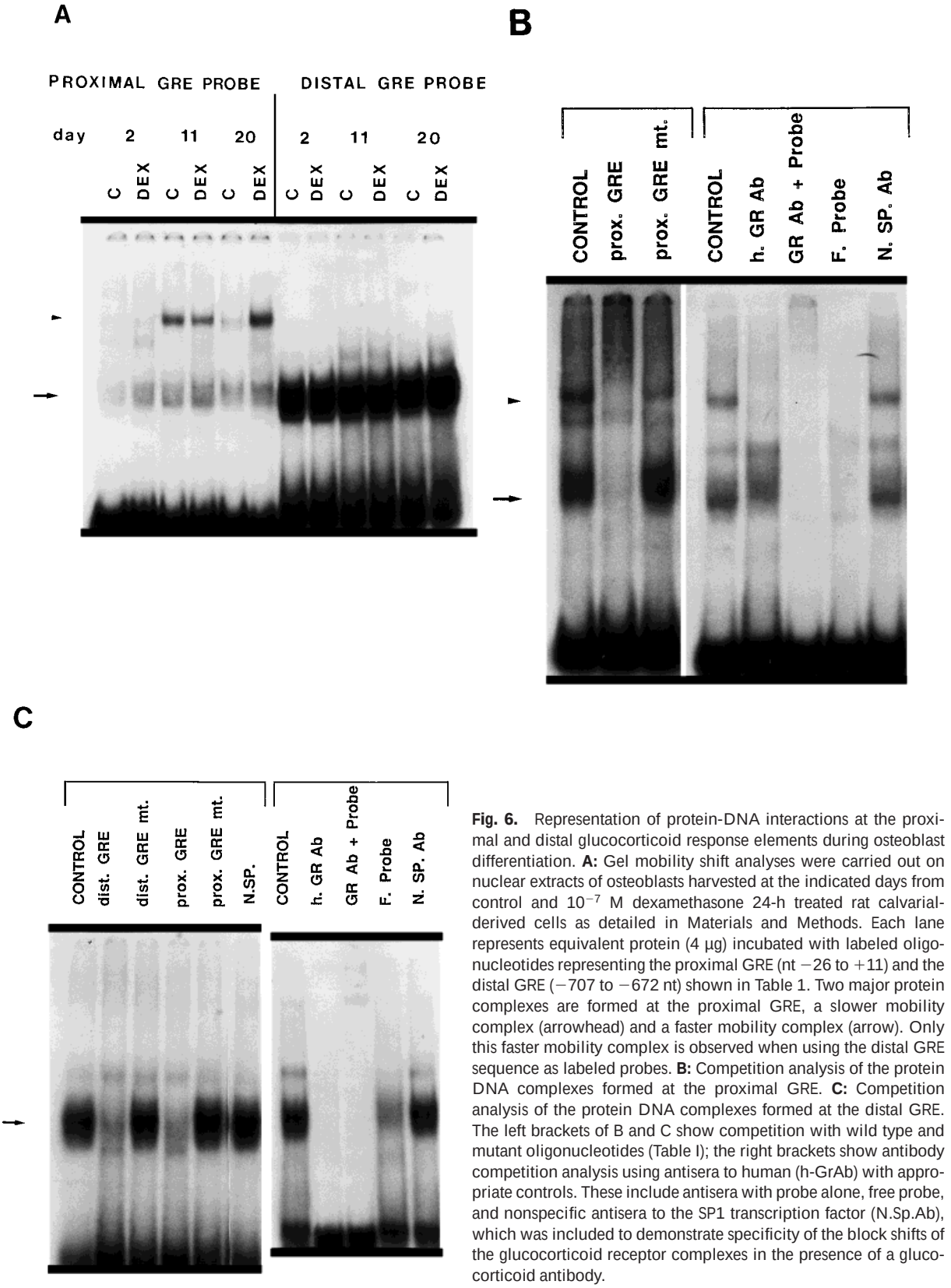


Fig. 6. Representation of protein-DNA interactions at the proximal and distal glucocorticoid response elements during osteoblast differentiation. **A:** Gel mobility shift analyses were carried out on nuclear extracts of osteoblasts harvested at the indicated days from control and 10^{-7} M dexamethasone 24-h treated rat calvarial-derived cells as detailed in Materials and Methods. Each lane represents equivalent protein (4 μg) incubated with labeled oligonucleotides representing the proximal GRE (nt −26 to +11) and the distal GRE (−707 to −672 nt) shown in Table 1. Two major protein complexes are formed at the proximal GRE, a slower mobility complex (arrowhead) and a faster mobility complex (arrow). Only this faster mobility complex is observed when using the distal GRE sequence as labeled probes. **B:** Competition analysis of the protein DNA complexes formed at the proximal GRE. **C:** Competition analysis of the protein DNA complexes formed at the distal GRE. The left brackets of B and C show competition with wild type and mutant oligonucleotides (Table I); the right brackets show antibody competition analysis using antisera to human (h-GrAb) with appropriate controls. These include antisera with probe alone, free probe, and nonspecific antisera to the SP1 transcription factor (N.Sp.Ab), which was included to demonstrate specificity of the block shifts of the glucocorticoid receptor complexes in the presence of a glucocorticoid antibody.

supershift of the fast mobility complex by addition of GR antibody. Notably, the slower mobility complex is unique to the proximal GRE sequence since the distal GRE when used as competitor does not modify DNA binding (data not shown). Both complexes at the proximal GRE remained bound to DNA in the presence of the mutant GRE oligonucleotide, suggesting specific protein interactions at the steroid half motifs (Fig. 6B). However, a slight decrease in the slower mobility complex is observed in the presence of the mutant oligonucleotide, suggesting that the flanking sequences may also contribute to binding. The faster mobility complex formed with the distal GRE sequence (Fig. 6C) clearly involves specific GR binding to the steroid half elements because a complete blocking of the complex occurred in the presence of GR antibody.

DISCUSSION

Post-Transcriptional Control of Gene Expression Predominates in Mature Osteoblasts

We have used the osteocalcin gene as a model to examine the molecular mechanisms contributing to steroid hormone responsive gene regulation operative during development of the osteoblast phenotype. Our results show that the extent to which steroid hormone regulation of the OC gene is transcriptionally and/or post-transcriptionally controlled is dependent upon the stage of osteoblast differentiation. The predominance of transcriptional regulation of osteocalcin in developing osteoblasts at the onset of osteocalcin gene expression is evident by the high level of transcription in control cells and the large increases induced by vitamin D and chronic treatment with dexamethasone coinciding with the end of the proliferative period. In more differentiated cells, regulation of osteocalcin production occurs at a transcriptional level but with dominant posttranscriptional control. This is observed in both primary osteoblast cultures and passaged cells in the absence of Dex. Our results show that in mature osteoblasts the dominant posttranscriptional mechanism ultimately results in osteocalcin gene expression, as reflected by protein biosynthesis. The transition from transcriptional to posttranscriptional control as the osteoblast differentiates is consistent with the high rate of OC transcription observed in young rat bone (up to 4 weeks postnatally). OC transcription then declines sharply in older rats [Shalhoub et al., 1994]. In adult rats (8–16 weeks), OC mRNA and protein synthesis are maintained at a steady state level [Shalhoub et al., 1991]. Thus OC mRNA levels appear to be stabilized in mature osteoblasts in agreement with observations in ROS 17/2.8 cells [Mosavin and Mellon, 1996]. The combinatorial effects of vitamin D and dexamethasone in mature osteoblasts (day 20) emphasize the significant accumulation of OC mRNA in the absence of increased transcription compared to early stage osteoblasts in a nonmineralized matrix. The striking increase in the VDR mRNA in mature osteoblasts in the presence of both hormones with decreased transcription support the concept of mRNA stabilization as an important mechanism of gene regulation in differentiated osteoblasts. However, osteopontin mRNA levels were also significantly stabilized by the combined activity of these hormones in day 7 osteoblasts.

Multiple GREs and Selective Protein-DNA Interactions in the OCN Promoter Supports Positive and Negative Transcriptional Control During Osteoblast Differentiation

The influence of Dex on OC transcription is dependent on the stage of osteoblast maturation. In proliferating, early stage osteoblasts, Dex can increase osteocalcin transcriptional rates, although the stimulatory effect is modest and does not compare to the 10-fold enhancement by vitamin D. Notably, the most dramatic positive effect of Dex on OC at the transcriptional level occurs only in cells continuously treated with this steroid for long periods (Fig. 1). In mature cells, acute treatments with Dex result in decreased transcription and mRNA levels.

The distal GRE of the OC gene, which has been characterized as a negative GRE, may contribute largely to the dominant negative transcriptional regulation of OC, as suggested by constitutive association of a GR complex with this element during all stages of osteoblast differentiation (Fig. 6). The proximal GRE of the rat OC promoter, which lies in close proximity to the TATA domain, shows developmentally dependent protein-DNA interactions that are responsive to glucocorticoids. Binding of this complex appears to correlate to the strong repression of OC observed at the transcriptional level in mature osteoblasts in response to acute Dex. Notably, the formation of this complex is absent and is not induced by Dex in

early osteoblasts when inhibition of OC transcription by Dex is not observed, but rather a slight increase occurs. These modifications in formation of GR-containing complexes suggest that the proximal GRE is sensitive to cellular requirements for either activation or suppression of osteocalcin transcription. Our findings support the concept that transcriptional control of osteocalcin in cultures chronically treated with dexamethasone is secondary to the differentiation-promoting affects of the hormone because transcriptional regulation differs between the response to acute vs. chronic treatments with Dex.

Expression of Steroid Hormone Receptors Is Regulated During Osteoblast Differentiation

Basal VDR and GR mRNA levels increase with differentiation. The more dramatic upregulation of VDR mRNA in osteoblasts producing a mineralized matrix in vitro is consistent with the relative abundance of VDR detected in osteoblasts in bone in situ [Davideau et al., 1996]. Dexamethasone has been reported to increase the sensitivity of osteoblasts to $1,25(OH)_2D_3$ as demonstrated by an increase or stabilization in vitamin D receptors in the rat cells and tissues [Godschalk et al., 1992; Hirst and Feldman, 1982; Chen et al., 1986a] and by a decreased vitamin D requirement to reach maximal stimulation of osteocalcin production [Chen et al., 1986b]. Our studies show that Dex leads to a rapid increase in vitamin D receptor mRNA levels only in young osteoblasts (day 7), which parallels increased transcription. This finding is consistent with biochemical assays of VDR levels reported previously [Chen et al., 1986b]. Vitamin D receptor levels are 20- to 30-fold higher in differentiated cells. Consequently, the increases in vitamin D receptor in early stage osteoblasts in response to Dex may reflect the differentiation-promoting properties of Dex and contribute to the increased expression of osteocalcin.

In contrast to the significant extent of developmental and hormonal regulation of the vitamin D receptor, the increase in glucocorticoid receptor mRNA levels are modestly increased. The relatively constitutive levels throughout osteoblast differentiation may relate to the constitutive complexes formed at the distal and proximal GREs. GR mRNA levels can be increased by Dex only in day 7 cultures to levels observed in mature osteoblasts (day 20). Glucocorticoid receptor and OC basal levels are primarily regulated at the transcriptional level in early stage osteoblasts by ligand, but GR transcription is minimal in mature osteoblasts.

The post-transcriptional regulation of GR and VDR in differentiated osteoblasts and the switch from transcriptional to posttranscriptional control of OC by these steroid hormones is not unprecedented. Glucocorticoids have also been suggested to play a role in the developmental regulation of the cysteine-rich intestinal protein (CRIP). Glucocorticoids increased the levels of CRIP protein, mRNA accumulation, and ^{65}Zn binding to CRIP during neonatal development throughout the suckling period in the small intestine [Levenson et al., 1993]. Glucocorticoids can stabilize human growth hormone [Paek and Axel, 1987] or destabilize interleukin/β mRNA [Lee et al., 1988]. Estrogens have also been shown to stabilize the mRNAs of the very low density apolipoprotein II and vitellogenin genes [Nielsen and Shapiro, 1990; Cochrane and Deely, 1988]. Stabilization of VDR mRNA by ligand is documented [Arbour et al., 1993]. Thus, there is precedence for Dex and vitamin D-mediated increases in mRNA levels that are not accounted for by RNA synthesis or increased receptor levels.

The stabilization of GR mRNA levels has also been reported to be tissue restricted [Kalinyak et al., 1987]. A tissue-specific accumulation of GR mRNA after adrenalectomy was found in brain (40% increase) and kidney (80% increase). In contrast, Dex decreased GR mRNA levels 40–60% in all soft tissues examined. In a separate study, Dex also decreased steady stage GR mRNA levels by 50% in human IM-9 lymphocytes and pancreatic acinar AR42J cells; the mechanism of this decrease was determined to be at the level of transcription [Rosewicz et al., 1988]. In osteoblasts, we observe that Dex increases GR mRNA levels and as early as 4 h following treatment. Dex increased GR transcription only in early stage developing osteoblasts where GR levels are at a lower level than in mature bone cells. Clearly, GR mRNA levels are maintained in differentiated osteoblasts with little ongoing transcription. Another example where steroid hormone regulation is predominantly modulated by mRNA stabilization in osteoblasts (UMR201 cells) is the effect of retinoic acid on alkaline phosphatase expression. Here, retinoic acid transiently causes a modest 2-fold increase in transcription with

continual mRNA accumulation for hours after cessation of transcription [Zhou et al., 1994]. Stabilization of OC or other mRNAs may be regulated via decreased message degradation [Brock and Shapiro, 1983], changes in mRNA and chromatin structure [Reik et al., 1991], or modifications of the nuclear matrix [van Steensel et al., 1995; Kirsch et al., 1986], which may contribute to elevated levels of gene expression [Stein et al., 1991; Goldberg et al., 1996]. Activated glucocorticoid receptor can bind to the nuclear matrix and has been shown to be associated with nuclear matrix-based DNA threads along with matrix associated by visualization with activated colloidal gold probe [Kirsch et al., 1986].

In summary, during development of the osteoblast phenotype, osteocalcin gene expression can be regulated by transcriptional and post-transcriptional mechanisms. Both $1,25(OH)_2$ vitamin D_3 and dexamethasone can control the rate of transcription during the transition from the proliferative period to the postproliferative phenotype development period. Later in the mineralization period, accumulated basal mRNA levels are stabilized and transcriptional effects of the hormones are minimal within 24 h. However, the chronic dexamethasone influence on osteoblast maturation maintains transcriptional control of osteocalcin production. These data demonstrate that particular environmental stimuli may allow ultimate commitment to development and maintenance of the mature osteoblast phenotype. This may occur by selection of cells that are osteoprogenitor in nature [Bellows et al., 1990]. Pre-osteoblasts may be activated by soluble effectors such as vitamin D and dexamethasone, which initiate the transcription of bone-related genes and allow the cell to continue the programmed osteogenic pathway. The combination of functional and structural changes provides a cell environment in which osteocalcin mRNA levels accumulate posttranscriptionally during progression to a mature osteoblast that synthesizes and organizes a three-dimensional bone-like tissue.

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