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Glucocorticoids Promote Development of the Osteoblast Phenotype by Selectively Modulating Expression of Cell Growth and Differentiation Associated Genes

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Abstract To understand the mechanisms by which glucocorticoids promote differentiation of fetal rat calvaria derived osteoblasts to produce bone-like mineralized nodules in vitro, a panel of osteoblast growth and differentiation related genes that characterize development of the osteoblast phenotype has been quantitated in glucocorticoid-treated cultures. We compared the mRNA levels of osteoblast expressed genes in control cultures of subcultivated cells where nodule formation is diminished, to cells continuously (35 days) exposed to 10^{-7} M dexamethasone, a synthetic glucocorticoid, which promotes nodule formation to levels usually the extent observed in primary cultures. Tritiated thymidine labelling revealed a selective inhibition of internodule cell proliferation and promotion of proliferation and differentiation of cells forming bone nodules. Fibronectin, osteopontin, and c-*fos* expression were increased in the nodule forming period. Alkaline phosphatase and type I collagen expression were initially inhibited in proliferating cells, then increased after nodule formation to support further growth and mineralization of the nodule. Expression of osteocalcin was 1,000-fold elevated in glucocorticoid-differentiated cultures in relation to nodule formation. Collagenase gene expression was also greater than controls (fivefold) with the highest levels observed in mature cultures (day 35). At this time, a rise in collagen and TGFβ was also observed suggesting turnover of the matrix. Short term (48 h) effects of glucocorticoid on histone H4 (reflecting cell proliferation), alkaline phosphatase, osteopontin, and osteocalcin mRNA levels reveal both up or down regulation as a function of the developmental stage of the osteoblast phenotype. A comparison of transcriptional levels of these genes by nuclear run-on assays to mRNA levels indicates that glucocorticoids exert both transcriptional and post-transcriptional effects. Further, the presence of glucocorticoids enhances the vitamin D_3 effect on gene expression. Those genes which are upregulated by $1,25(OH)_2D_3$ are transcribed at an increased rate by dexamethasone, while those genes which are inhibited by vitamin D_3 remain inhibited in the presence of dexamethasone and D_3. We propose that the glucocorticoids promote changes in gene expression involved in cell-cell and cell-extracellular matrix signaling mechanisms that support the growth and differentiation of cells capable of osteoblast phenotype development and bone tissue-like organization, while inhibiting the growth of cells that cannot progress to the mature osteoblast phenotype in fetal rat calvarial cultures.

Key words: osteocalcin, osteopontin, collagen, c-*fos*, oncogene, histone, fibronectin, alkaline phosphatase, collagenase, steroid hormone, growth control, osteoblast differentiation

Glucocorticoids (GC) are physiological regulators of the growth and development of many organs including bone [reviewed in Cutroneo et al., 1986]. GC influence cellular proliferation and cell-cell or cell-matrix interactions. For example, GC inhibit, in general, cell proliferation in a variety of tissues and cells including adult rat hepatocytes in primary culture [Vintermyr and Doskeland, 1989] and fibroblasts [reviewed in Durant et al., 1986; Fagot et al., 1991] and osteoblasts [Canalis, 1983]. Examples of GC inducing phenotypic alterations include inhibition of chondrocyte differentiation [Silberman et al., 1983], and induction of fibronectin in transformed cells allowing them to appear more normal [Oliver et al., 1983] or the inhibition of

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neoplastic transformation [Birek et al., 1988] and induction of osteoblasts from progenitor cells [Leboy et al., 1991] and promotion of bone formation in vitro [Bellows et al., 1987].

Long-term GC administration in pharmacological doses for the treatment of chronic inflammatory diseases and metastases results in severe osteopenia [Gennari, 1985]. This decrease in bone mass derives from both direct and indirect actions of GC on bone cells. Thus, numerous studies have measured GC effects on bone cell parameters to understand GC control of bone turnover. GC act on osteoblasts directly [Raisz and Kream, 1983], generally to decrease collagen synthesis [Hahn et al., 1984; Leboy et al., 1991; Ng et al., 1989; Chyun et al., 1984; Canalis, 1983; Wong, 1979], as observed in hepatocytes [Weiner et al., 1987] and fibroblasts [Cutroneo et al., 1986; Perez et al., 1992]. However, there are reports of GC enhancement of collagen synthesis in bone cells [Gallagher et al., 1983; Canalis, 1983]. Thus bone cell responsiveness to GC is a function of both the culture system and upon the experimental conditions.

Studies performed on different bone cell systems reveal that although GC generally repress cellular proliferation [Eilam et al., 1980; Canalis, 1983; Chen et al., 1983a; Chyun et al., 1984; Wong et al., 1990], there are reports of enhanced [Chen et al., 1983a] and biphasic effects [McCulloch and Tenenbaum, 1986; Ng et al., 1989] on bone cell growth. Similarly, alkaline phosphatase also exhibits biphasic expression [Canalis, 1983] or inhibition [Chen and Feldman, 1979], but in most bone systems is increased by GC treatment [Hahn et al., 1984; Leboy et al., 1991; McCulloch and Tenenbaum, 1986; Wong et al., 1990; Majeska et al., 1985]. Vitamin D receptors also may be enhanced [Chen et al., 1983b] or repressed [Godschalk et al., 1992] in different bone systems. In addition, comparisons of GC action in bone with reports on other tissues are difficult because GC have separate modes of action in different tissues.

GC added to rat osteoblasts in primary or secondary cultures of fetal rat calvarial derived cells over a period of 30 days result in an increase in the size and number of nodules with a bone tissue-like organization [Bellows et al., 1987, Bellows and Aubin, 1989]. The demonstration that GC promotes osteoblast phenotype expression from marrow derived progenitors [Leboy et al., 1991] and have separate effects on cells derived from periosteum vs. central bone [Chyun et al., 1984] has led to the hypothesis that GC may act on osteoprogenitor cells to inhibit proliferation and cause them to differentiate, as well as on differentiated cell types to maintain or express higher levels of differentiated functions. Consequently, it is necessary to characterize the mechanism(s) of action of GC on gene expression during osteoblast growth and bone nodule formation.

We have characterized a temporal and functionally related expression of genes that reflect defined stages of osteoblast growth and differentiation in primary cultures of rat calvarial derived osteoblasts [Owen et al., 1990]. However, a systematic analysis of the expression of cell growth and osteoblast related genes at the mRNA level during progressive development of the osteoblast phenotype influenced by GC has not been carried out. The goal of the present study is to correlate the effects of dexamethasone (dex), a synthetic glucocorticoid, on rat osteoblast cell growth parameters and gene expression with its effect on promoting bone nodule formation. Studies were carried out in primary and subcultivated normal diploid osteoblasts where a dramatic enhancement of bone cell related gene expression and bone nodule formation occurs in the presence of GC, confirming the ability of dex to promote osteoblast differentiation. These osteoblast cultures were acutely treated with dexamethasone and, in both acute and chronic studies, mRNA levels were examined for several of the osteoblast expressed genes: cell cycle dependent histone, c-*fos*, type I collagen, TGFβ, fibronectin, alkaline phosphatase, osteopontin, osteocalcin, collagenase, and actin. The pattern of gene expression is shifted to one that reflects early nodule formation and more mature cells. Autoradiography of [^{3}H]-thymidine labelled nuclei was performed at different times in culture to analyze cell growth at the single cell level and reveals a selective inhibition and proliferation of cells. In some experiments regulation of $1,25(OH)_2D_3$ modulated genes in dex-treated culture was examined. We observed that mRNA levels result from GC control of gene expression at both the transcriptional or post-transcriptional levels.

MATERIALS AND METHODS

Cell Isolation

Calvariae were excised from 21 day fetal rats and the dura and periosteum removed. Cells were obtained from the calvariae by three se-

quential digestions of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase P (Boehringer Mannheim Biochemicals, Indianapolis, IN)/0.25% trypsin (Gibco, Grand Island, NY). Cells from digests one and two were discarded and cells from the third digest were grown in minimal essential medium (MEM; Gibco) supplemented with 10% fetal calf serum (FCS) in 100 mm dishes or six-well dishes (Corning, Corning, NY) at a density of 6.5×10^5 or 3.6×10^5 cells/dish, respectively. At confluence, cells were released by trypsin digestion, counted, and subcultivated in MEM at a density of 6.5×10^5 in 100 mm dishes. Depending on the experiment, 10^{-7} M dex (Sigma Chemical Co., St. Louis, MO) was added to the medium beginning at different times during a 30 day time course, or added to the medium at the time of replating. After the initial addition of dex, media changes were performed every 2 days and dex was included in the media. For both primary and passaged cells, the time course of mineralization was accelerated by the inclusion of 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate in media from day 5. In some experiments cells were treated with 10^{-8} M $1,25(OH)_2D_3$, a gift of Milan Uskokovic (Hoffman LaRoche, Nutley, NJ); control cultures contained hormone vehicle .01% ETOH.

[^{3}H] TdR Labelling

[^{3}H]-thymidine at a concentration of 1 μCi/ml was added to the medium of cells growing on 22 mm Thermonox coverslips (U.S.A. Scientific, Ocala, FL) for 24 h. Cells were then rinsed twice in ice-cold PBS and fixed in absolute methanol (−20°C). Coverslips were then air dried overnight. Autoradiography was performed using Ilford K-5 emulsion as described by Baserga and Malamud [1969]. Exposures were for 5–7 days at 4°C.

Histochemical Staining of the Cultures

For each experiment, cultures were monitored for alkaline phosphatase (APase) activity and mineralization. APase was detected after incubating the cells for 30 min at room temperature with shaking in 10 mM Tris HCl, pH 8.4, containing 20 mg/ml disodium napthol AS-MX phosphate and 40 mg/ml fast red TR salt (Sigma). Mineral was assayed by von Kossa staining of cultures (30 min in 3% $AgNO_3$) [Clark, 1981].

RNA Isolation and Analysis

Total cellular RNA was isolated from frozen cell pellets stored at −70°C by the Chirgwin procedure [Chirgwin et al., 1979]. RNA was resuspended in diethyl pyrocarbonate-treated water, quantitated by absorbance at 260 nm, and stored at −70°C. RNA samples were monitored with respect to representation of ribosomal RNA (18S and 28S) as internal standards, and the intactness of the RNA was ascertained by electrophoretic fractionation on 6.6% formaldehyde, 1% agarose gels and ethidium bromide staining. For some assays, 20 μg of total cellular RNA was fractionated as described above and Northern blot transfer performed using Zetaprobe membranes (Bio-Rad, Rockville Centre, NY). For other assays, 3 μg and 6 μg of total cellular RNA of each sample were immobilized on Zetaprobe membranes using a Minifold II slot blot system (Schleicher and Schuell, Keene, NH). RNA was cross-linked to filters by UV irradiation for 1 min and stored in plastic bags at 4°C. Probes were labelled with (α-^{32}P)dCTP to a specific activity of at least 1×10^9 dpm/μg DNA. In some cases plasmid gene probes were used. The human histone H2B probe was a plasmid, pcL120, which was cloned in this laboratory [Collart et al., 1991]. The type I collagen gene probe is a cDNA plasmid α1R1 [Genovese et al., 1984]. The actin probe is a human fibroblast cytoplasmic β actin genomic plasmid, pHFβA-1 [Gunning et al., 1983]. The human 18S ribosomal gene probe is a plasmid, LS2 [Wilson et al., 1978]. Other gene probes were gel purified inserts isolated from plasmid DNA. Homologous inserts included fibronectin [Schwarzbauer et al., 1983], osteocalcin [Lian et al., 1989], alkaline phosphatase [Noda et al., 1987], osteopontin [Oldberg et al., 1986], and the collagenase probe [Quinn et al., 1990]. Non-homologous inserts included mouse c-*fos* [Miller et al., 1984], and mouse TGFβ [Derynck et al., 1986]. The prehybridization and hybridization conditions were as described previously [Shalhoub et al., 1991]. Blots were exposed to preflashed XAR-5 x-ray film (Eastman Kodak Co., NY) using a Cronex Lightning Plus screen at −70°C. Autoradiograms were quantitated by scanning laser densitometry (LKB 2400 GelScan XL) within the linear range of signals. Data were expressed in densitometric units. All mRNA levels were normalized to the amount of 18S ribo-

somal RNA on the filters as determined by hybridization to the LS2 18S ribosomal gene probe.

Nuclear Transcription Assays

Nuclei were isolated from 25 day rat osteoblasts grown in secondary culture and stored at −70°C in 50 mM Tris, pH 8.3, 40% glycerol, 5 mM $MgCl_2$, and 0.1 mM EDTA. Nuclei isolation involved lysis in a buffer containing 10 mM Tris, pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, and 0.5% NP-40, and passage over 4 layers of cheesecloth to separate nuclei from extracellular matrix and mineral. Each reaction contained 10^7 nuclei, which were transcribed in the presence of ^{32}P-labelled UTP according to Greenberg and Ziff [1984]. Radiolabelled RNA was then hybridized to plasmid DNA containing 2 μg of the gene of interest which was immobilized onto Zetaprobe membranes according to Schleicher and Schuell. Each membrane was hybridized to 2×10^6 counts from 10^7 nuclei. Each experiment was performed in duplicate.

RESULTS

Dexamethasone Promotes Differentiation of Subcultivated Osteoblasts: Morphology and Biochemistry

Previously, we have shown that osteoblasts isolated from fetal rat calvariae and grown in culture over a 30 day period develop a collagenous extracellular matrix which supports the ordered deposition of mineral resulting in the three-dimensional characteristics of bone nodules [Bhargava et al., 1988; Pockwinse et al., 1992; Owen et al., 1990]. During the initial period of primary culture, cells proliferate and are characterized by high levels of expression of histone (which is coupled to DNA synthesis), type I collagen, TGFβ, and fibronectin [Owen et al., 1990]. As proliferation declines the cells undergo a period of matrix maturation which is characterized by the accumulation of collagen protein and increased levels of alkaline phosphatase mRNA and protein. A period of matrix mineralization ensues during which time the levels of osteocalcin and osteopontin rise and peak.

Figure 1A shows the typical mineralized nodule observed throughout the primary cultures on day 28. Primary cells passaged after confluency [Aronow et al., 1990] and allowed to grow under mineralization conditions demonstrated a dramatic reduction in nodule formation and mineralization (Fig. 1B). However, osteoblasts

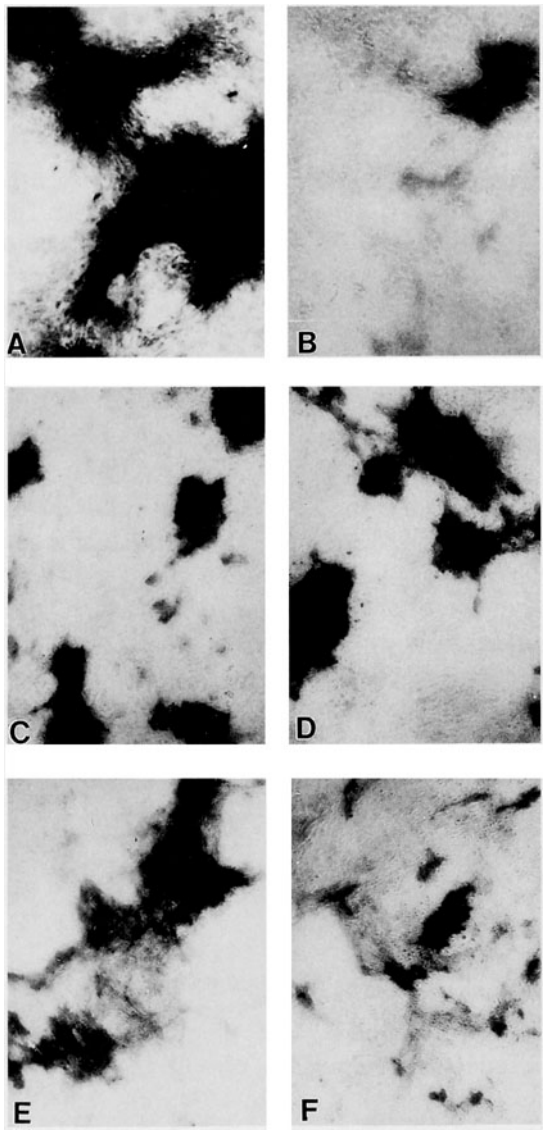

Fig. 1. Comparative morphology of primary, second passage, control, and dexamethasone-treated rat osteoblast cultures. Panels show von Kossa and alkaline phosphatase stained nodules after 28 days of culture. **A:** Primary cultures. **B:** Cells subcultivated from primaries on day 11. **C–F:** Passaged cells treated with 10^{-7} M dexamethasone initiated on day 2 **(C)**, day 7 **(D)**, day 14 **(E)**, or day 21 **(F)** after plating. Note the increased number and size of mineralized nodules in cultures treated with dexamethasone early, day 2, 7, and day 14 compared to the control and later treated cultures.

subcultivated in the presence of the synthetic glucocorticoid, dexamethasone (dex), retained the ability to form mineralized nodules (Fig. 1C–F). Subcultivated cells were treated with 10^{-7} M dex initiated at days 2, 7, 14, or 21 after plating and assayed histochemically for nodule formation on day 28. Except for treatment initiated at day 21 (Fig. 1F), dex-treated cultures

developed more mineralized nodules which were more focal in nature than controls, indicating that dex is effective in promoting mineralized nodule formation for up to 2 weeks after plating (compare panels C or D to panel A in Figure 1).

Biochemical markers of nodule formation and mineralization were assayed as shown in Figures 2 and 3. Osteocalcin synthesis, representative of the mature osteoblast phenotype and previously documented to correlate highly ($r = 0.9$) with mineral deposition [Aronow et al., 1990] is a quantitative reflection of mineralized nodule formation. Figure 2A shows the increases in osteocalcin in relation to time of addition of dexamethasone which correlated with the number and size of nodules shown in Figure 1. Dex was most effective when added to the passaged cells immediately after subcultivation (day 2) or within the first week (day 7). In control cultures, osteocalcin was detectable at the sensitivity threshold of the radioimmunoassay on day 12, whereas on day 12 significantly higher levels were synthesized in the day 2 and day 7 dex-treated cultures (approximately tenfold higher). Dex added to cultures on day 14 or 21 resulted in slight increases in osteocalcin (from 10–15%) several days later (day 19 and 23). When dex was removed from the cultures following nodule formation (Fig. 2B)—for example, either on day 18 or day 21—expression of osteocalcin (measured every 48 h) persisted and only declined as a function of the natural developmental time course (compare to control in Fig. 2B and to primary cultures [Owen et al., 1990]).

In subsequent studies dex was added to the passaged cells at the first feeding (day 2). Figure 3 demonstrates the effects of dex on total DNA, alkaline phosphatase (APase) collagen, and osteocalcin accumulation throughout the culture period. Again early detection of osteocalcin was observed in the dex-treated cultures (day 7) with the onset of cellular multilayering in nodule areas. At this early stage of the cultures, total cell number (data not shown) or DNA (Fig. 3A), collagen accumulation (Fig. 3B), and APase enzyme levels (Fig. 3C) were 20%, 50%, and 60% decreased, respectively, in dex-treated cultures. In the following weeks of culture while DNA and APase remained unchanged in control cultures, these parameters in dex-treated cultures became significantly elevated over control; DNA increased twofold by day 28; APase was twofold greater by day 21. The pattern of APase enzyme

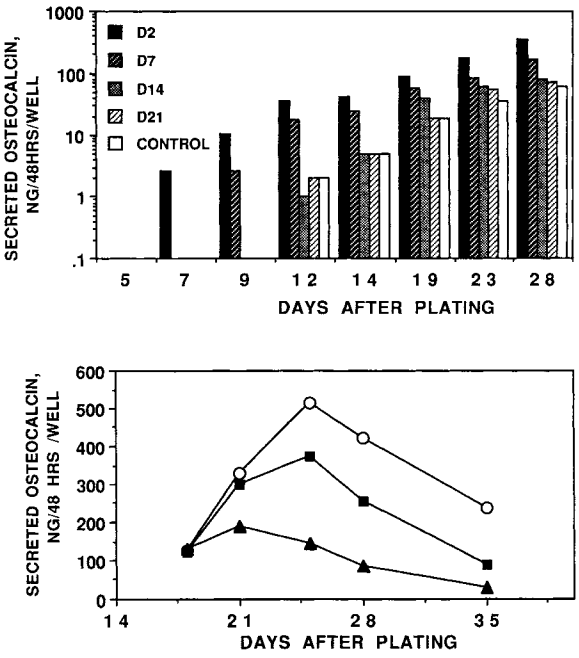

Fig. 2. Biosynthesis of osteocalcin in dexamethasone-treated cultures. **Top panel:** The effects of addition of dexamethasone at the indicated times (days 2, 7, 14, and 21) compared to nontreated control levels. An early induction of osteocalcin in day 2 and day 7 dexamethasone-treated cultures is seen correlating with the early appearance of nodules. Note the log scale and the absence of osteocalcin expression in actively proliferating cells (day 5). **Lower panel:** The effect on osteocalcin synthesis in cultures where dexamethasone has been removed at day 18 (solid triangles) or day 21 (solid squares) compared to those cultures that have had continuous dexamethasone (open circles). Note that osteocalcin synthesis is sustained at the level observed when dexamethasone was removed until levels declined after day 25 following the natural time course.

activity was identical to that observed in mineralizing primary cultures [Aronow et al., 1990; Owen et al., 1990], declining in heavily mineralized dex-treated cultures, reflected by the high levels of osteocalcin synthesis (Fig. 3D). Collagen accumulation increased in both control and dex-treated cultures to approximately the same extent. However, in control cultures a slower rate of mineralization reflected by low levels of osteocalcin and consistent with von Kossa silver stain for calcium (Fig. 1) was observed. This accounted for the absence of significant changes in APase levels in the control cultures.

The formation of increased nodules was examined at the single cell level by analysis of cell proliferation using [^{3}H]-thymidine labeling and autoradiography. Figure 4 shows the proliferating population of cells in control and 10^{-7} M chronic dex-treated cultures of subcultivated rat osteoblasts. Nodules were more defined by day

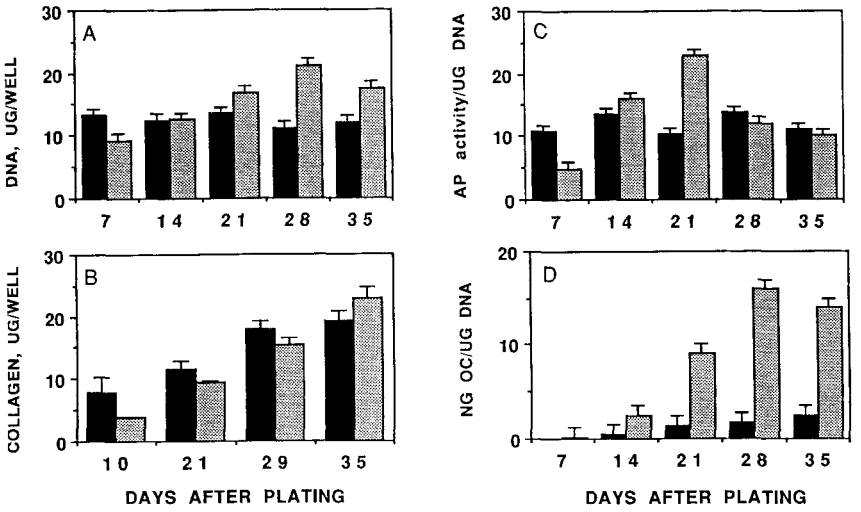

Fig. 3. Biochemical parameters of dexamethasone-treated cultures. Each point represents n = 3 independent samples and duplicate determinations. The range of values was less than 10% for each point. Primary cultured cells, subcultivated after day 12, were treated with dexamethasone beginning on day 2 and DNA content (A), total collagen (B), alkaline phosphatase enzyme activity/DNA (C), and media osteocalcin normalized to DNA (D) were quantitated at the indicated days. Solid bars represent control and hatched bars the dexamethasone-treated cultures. Note that in control cultures, DNA and AP/DNA were not significantly changed from post-confluency day 7 through day 35 since there was a very low representation of nodules in this experiment.

11 in dex-treated cultures. By day 15 mineralization was evident in dex-treated cultures, in contrast to control cultures where proliferation continued in nodule areas. There was less internodular cell proliferation in dex-treated cultures, particularly in the first 2 weeks, but at all times when compared to controls. Proliferation had clearly ceased in the center of the nodule of the dex-treated cultures which were heavily mineralized by 3 weeks in contrast to control cultures (see day 23). At day 28 in control cultures, the nodules are APase positive with mineral deposition just being initiated. Mineralized nodule area and number of nodules are distinctly greater in dex-treated cultures. The nodules are characterized by peripheral cell proliferation (day 23, dex-treated; day 28, control) indicating continued growth of the nodule as tissue-like organization further develops. Since more nodules are present in the dex-treated cultures, this observation is consistent with the increased DNA content of late cultures (Fig. 3).

Analysis of Gene Expression

We normalized levels of gene expression to ribosomal RNA (Fig. 5) since we observed that actin, a commonly used parameter, is significantly altered during the differentiation time course with development of the osteoblast phenotype. This is unquestionably a reflection of modifications in cytoskeletal biosynthesis to accommodate differentiation-mediated changes in cell size and shape. Initially we examined in primary cultures the effects of acute dex treatment at three stages of development of the osteoblast phenotype to determine responsiveness of the different genes in these rat derived osteoblasts (Fig. 6). The effects of acute dex treatment on histone, APase, osteocalcin, osteopontin, type I collagen, and fibronectin mRNA levels in primary cultures are shown in Figure 6 for the post-proliferative period at the initial stage of nodule confluency (day 13), the period of active mineral deposition in nodules (day 20), and heavily mineralized mature cultures (day 34). The cells were treated with 10^{-7} M dex 48 h prior to harvesting at the indicated times. Since days 13 through 34 represent the stages following the peak period (days 1–7) of proliferative activity, cellular levels of H2B histone, fibronectin, and collagen are significantly downregulated and maintained at a relatively constant level in control cultures. Acute dex treatment of cells stimulated cellular proliferation on day 13 and day 20 but not on day 34, as reflected by histone mRNA levels. No significant changes in the levels of mRNA for fibronectin and type I collagen were detected. In contrast, osteopontin mRNA is upregulated at all times in the dex-treated cultures, but with greater than twofold increases on day 13 and day 34 when basal levels are low. Osteocalcin expression which is linked

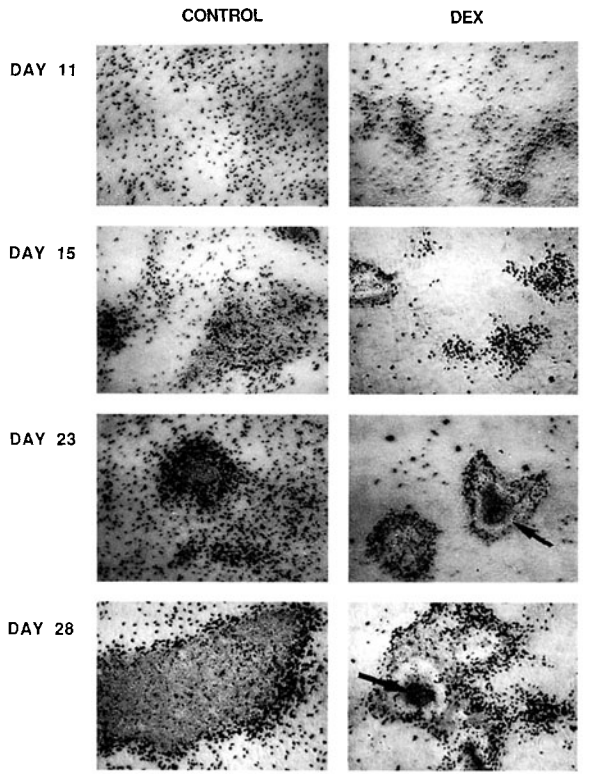

Fig. 4. Cellular proliferation in control and glucocorticoid treated cultures. Subcultivated cells were cultured in the presence of 10^{-7} M dexamethasone from day 2 and pulsed with 1 μCi/ml [^{3}H]-thymidine for 24 h prior to fixation at the indicated days. Cultures were stained for alkaline phosphatase activity prior to autoradiography which reveals actively proliferating cells with thymidine labelled nuclei. As early as day 11, focal areas of proliferation are more apparent in the dex-treated cultures. Internodule proliferation is clearly not present by day 15, whereas nodule maturation continues in control cultures. Proliferation continues to be evident at the periphery of the nodules (day 23 and day 28) in both control and the dex-treated cultures. Arrows indicate mineralizing apical region of nodules in dex-treated cultures. Note the delay in mineralization within the alkaline phosphatase positive nodules of control cultures (day 28).

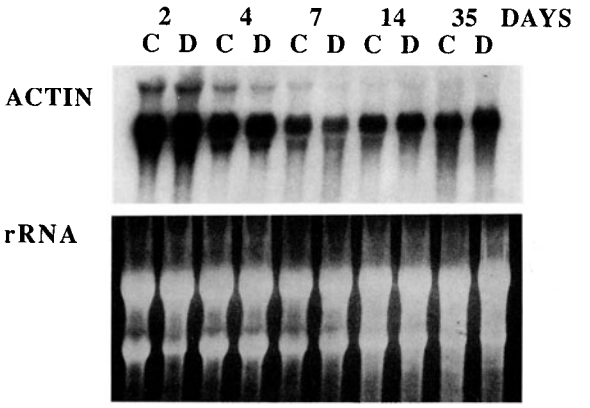

Fig. 5. Comparison of 18S ribosomal RNA (by ethidium bromide staining) and actin mRNA levels (by Northern blot analysis) in control and dexamethasone-treated cultures. Note the changes in actin mRNA levels throughout the developmental sequence, from proliferation (days 2–4) to post-proliferative cells (day 14) and differentiated cells in a mineralized matrix (day 35).

to mineralization also exhibited increased cellular mRNA levels following dex treatment, but only at times when it was already expressed in control cultures. APase mRNA levels were decreased by dex at 13 days and increased at days 20 and 34 when compared to control. Thus, APase mRNA levels were either up- or downregulated by glucocorticoids as a function of the stage of differentiation of the cultured cells.

The effects of chronic dex treatment on growth and differentiation parameters of subcultivated rat calvarial derived osteoblasts in culture for 35 days are shown in Figure 7. Cells, passaged into media containing 10^{-7} M dex, were always harvested 24 h after feeding for a strict comparison

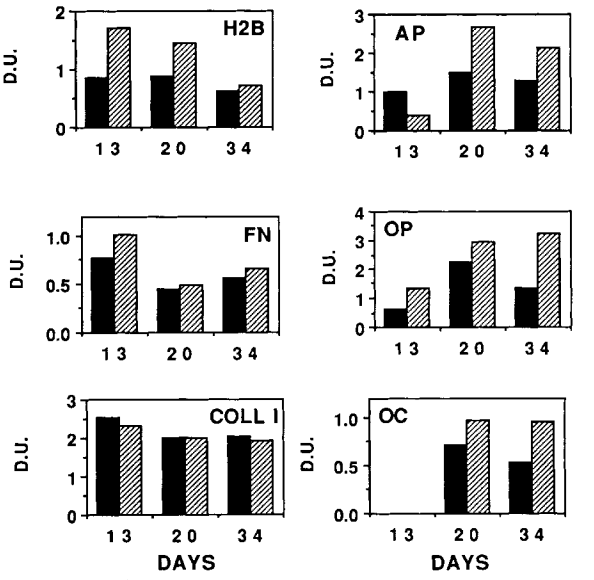

Fig. 6. Acute effects of dexamethasone (10^{-7} M) on expression of osteoblast genes. Primary cultures of fetal rat calvarial derived osteoblasts were examined for glucocorticoid responsiveness of osteoblast markers expressed at different stages of osteoblast phenotype differentiation. Cells were treated with 10^{-7} M dex 48 h prior to harvest. H2B, histone; AP, alkaline phosphatase; FN, fibronectin; COLL, collagen; OP, osteopontin; OC, osteocalcin. Densitometric units (D.U.) were normalized to 18S ribosomal mRNA. Solid bars, control; hatched bar, dexamethasone. The data is representative of 2 independent experiments. Each bar represents the average of duplicate determinations.

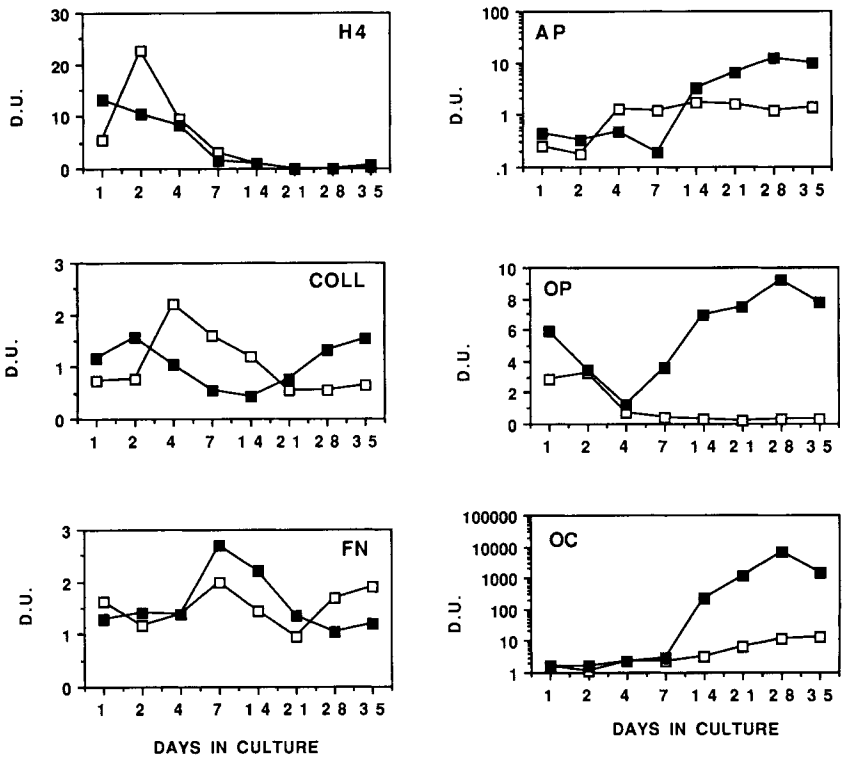

Fig. 7. Expression of osteoblast cell growth and differentiation related genes in glucocorticoid continuously treated cultures compared to control passaged cells. Total cellular RNA was prepared from cells harvested 24 h after feeding at the indicated days. All control and dex-treated samples were hybridized together for each gene to allow for direct comparison of cellular mRNA levels. Note the biphasic effects after plating particularly with respect to cell proliferation (histone H4), collagen (COLL), fibronectin (FN), and alkaline phosphatase (AP). Osteocalcin (OC) and osteopontin (OP) are elevated in relation to nodule formation in the dex-treated cultures compared to their low level of expression in the control cultures in which nodule formation is reduced and mineralization is delayed. Open square, control; closed square, dex-treated.

of mRNA levels which may selectively downregulate as a function of time after feeding. Histone mRNA levels were lower during the period of active proliferation compared to controls except at day 1 when the dex-treated samples showed elevated levels. Compared to control, c-*fos*, an early response gene [Birek et al., 1991], whose product is known to interact with the GC receptor complex [Yang-Yen et al., 1990; Schüle et al., 1990] or be regulated by GC [Birek et al., 1991] was expressed at elevated levels during the proliferation period (see Fig. 8 for Northern blot analysis of c-*fos* and histone).

Type I collagen mRNA transcripts were initially elevated in dex-treated cultures (about two times control) at days 1 and 2 (Fig. 7). By day 4 the level of type I collagen transcripts began to decrease below control and continued to decline; the mRNA levels remained lower than control values until day 14, after which time they rose gradually to reach their highest level at day 35. The level of transcripts for

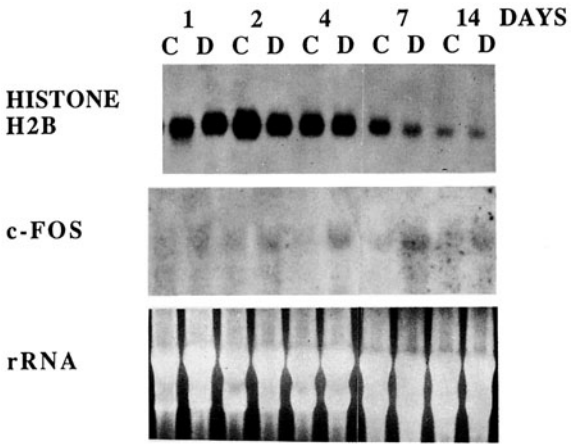

Fig. 8. Northern blot analysis of proliferation related genes in control and dexamethasone-treated subcultivated cell cultures. c-*fos* is detected during the period of active proliferation reflected by histone H2B expression and there is a higher level of c-*fos* expression in the dexamethasone-treated cultures. Total cellular RNA was isolated from cells harvested 24 h after feeding at the indicated days. Ethidium bromide staining indicates constant amount of total RNA which was hybridized.

fibronectin were similar in control and dex-treated cultures at days 1, 2, and 4, but rose above control values at days 7, 14, and 21. By day 28 the mRNA levels for fibronectin had declined to less than control values, a pattern opposite to that seen for collagen. Such significant changes in these genes were not observed in the short term (48 h) dex-treated cells (Fig. 6). While TGFβ expression (data not shown) was slightly higher in the dex-treated cultures, the increase was significant (1.5-fold) on days 28 and 35 when collagen mRNA levels were also elevated in dex-treated cultures. Expression of TGFβ has been shown to be temporally and perhaps functionally coupled with type I collagen gene expression during osteogenesis both in vivo [Bortell et al., 1990] and in vitro [Owen et al., 1990].

Three osteoblast parameters increased significantly with nodule formation in dex-treated cultures (Fig. 7). APase transcripts were low initially, both in actively proliferating (days 1 and 2) control and dex-treated cultures. In dex-treated cultures, APase remained lower than control in the first week; then, after day 14, there was a progressive increase in the levels of mRNA transcripts. Since control levels remained unchanged, a significant fold increase over control levels occurred. APase mRNA levels correlated well with enzyme activity and maturation of the nodules (Fig. 3). Osteopontin mRNA levels were higher in dex-treated cultures at all times, but the magnitude of difference between control and treated cultures was greatest after day 7, coincident with nodule formation. Osteocalcin mRNA levels were dramatically elevated over controls after day 7 through day 35 with the highest level achieved at day 28 (greater than 1,000-fold).

Collagenase, a product of osteoblastic cells [Sakamoto and Sakamoto, 1984; Partridge et al., 1987] and a gene regulated by glucocorticoid [Brinckerhoff et al., 1986], was examined in control and dex-treated cultures. Figure 9A shows that levels of collagenase mRNA were detectable at day 7 in control cultures and increased on days 14 and 35 when mineralized nodules were evident. In the presence of chronic 10^{-7} dex treatment, collagenase expression was sixfold greater on day 14 and eightfold greater on day 35 paralleling the proportional increase in nodules in the dex-treated cultures. To ascertain if collagenase gene expression in osteoblasts is dex responsive and not merely a reflection of increased nodule formation, mRNA levels were quantitated after a 48 h exposure to 10^{-8} M and 10^{-7} M on day 20 of primary cultures, observing a stimulation of collagenase mRNA transcripts (Fig. 9B).

Influence of Vitamin D on Glucocorticoid Modulated Gene Expression

Because $1,25(OH)_2D_3$ regulates several osteoblast gene products [Lian and Stein, 1992b], and since $1,25(OH)_2D_3$ receptor levels are modulated by dex [Chen et al., 1986], we examined whether the regulation of these genes was similar in the presence of dexamethasone. In this experiment both mRNA levels and transcription rates affected by the hormone(s) were monitored to determine the level at which control is mediated (Fig. 10). These experiments were carried out in differentiated cells in a mineralized matrix to evaluate steroid hormone effects under conditions that are analogous to in vivo bone. Further cultures were treated for 48 h to evaluate the influence sustained by the cells in response to hormone treatment rather than transient effects. Transcription did not consistently parallel the changes in mRNA for each hormone group relative to control. For histone, both hormones independently or together increased transcription while mRNA levels were decreased. In these mature osteoblast cultures (day 25) exposed to hormone 48 h, basal transcription was very low for APase and osteopontin. Dexamethasone (10^{-7} M) resulted in an increase in mRNA levels for collagen and osteocalcin, while transcription was not significantly changed, suggesting stabilization of the message. Osteopontin, on the other hand, showed slightly increased transcription. These changes were small (10%) but reproducible in several experiments, and taken together all these effects suggest that dex regulates genes at both transcriptional and post-transcriptional levels (e.g., by altering mRNA stability).

When dex was added together with $1,25(OH)_2D_3$ (10^{-8} M), multiple effects were observed. For those genes where $1,25(OH)_2D_3$ inhibited expression and dex increased levels of mRNA transcripts (e.g., histone, collagen, and APase), the combined effect was inhibition of the dex effect by $1,25(OH)_2D_3$. For the osteocalcin and osteopontin genes, where each hormone increased mRNA transcripts, together the effect was synergistic with an even higher level of mRNA and transcription. Thus, the effects of $1,25(OH)_2D_3$ predominate.

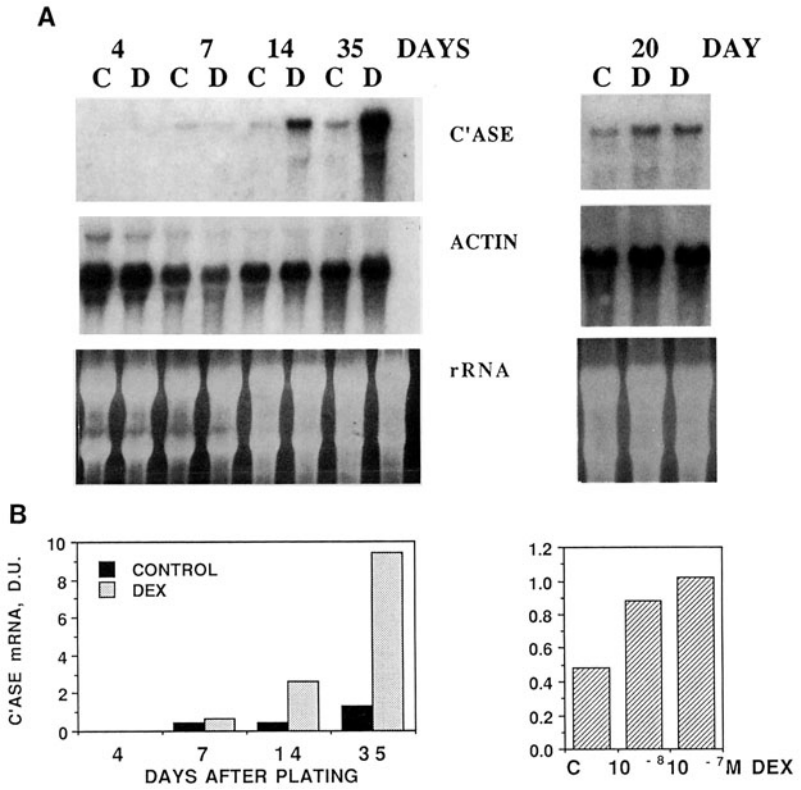

Fig. 9. Induction of collagenase gene expression in dexamethasone-treated rat osteoblast cultures. **A:** Northern blot analysis of collagenase and actin mRNA and 18S and 28S ribosomal visualized by ethidium bromide staining. **B:** Quantitation of collagenase dexamethasone differentiated cultures (hatched bar) compared to control (solid). Left panels show results of continuously treated passaged cells and the right panels show the short-term (48 h) acute effects in primary cultures on day 20.

DISCUSSION

Primary cultures of fetal rat calvarial derived osteoblasts multilayer and develop areas of cells in an extracellular matrix (nodules) having a bone-like tissue organization. In between these nodules are APase positive cells. Subcultivation results in decreased nodule formation and delayed mineralization of existing nodules. In this study we have shown that dex promotes maintenance of the bone nodule forming cell in subcultivated cells derived from fetal rat calvarial osteoblasts by both selective inhibition and stimulation of growth of cells in the population. Although there was an initial burst of proliferation (within the first 24 h), continuous exposure of the cells to 10^{-7} M dex resulted in an overall decrease in histone H2B compared to control during the period of nodule growth. This biphasic effect is consistent with an initial increase in cell proliferation with subsequent inhibitory effects seen in GC treated fetal rat bone organ cultures [Chyun et al., 1984]. A similar effect was described for UMR 201 rat nontransformed clonal cells [Ng et al., 1989] at the same dex concentration of 100 nM which was used in the present study. Also, similar to our results, in dex-treated embryonal chick periosteal explants, proliferation was increased within three days followed by cessation of proliferation, resulting in a clustering of cells visualized by autoradiography in that study [McCulloch and Tenenbaum, 1986]. The earlier cessation of proliferation in the nodule center and deposition of mineral in dex-treated cultures suggest that the dividing population was pushed to differentiate earlier in the presence of dex. Furthermore, continued growth of the nodule seemed apparent from the single row of proliferating cells at the periphery of the nodule. Since many more nodules (greater than thirtyfold) were present in dex-treated cultures, this may account for the significant increase in total DNA accumulated by day 28.

A careful examination of the changes in gene expression may provide insight into factors promoting nodule formation. Acute dex treatment

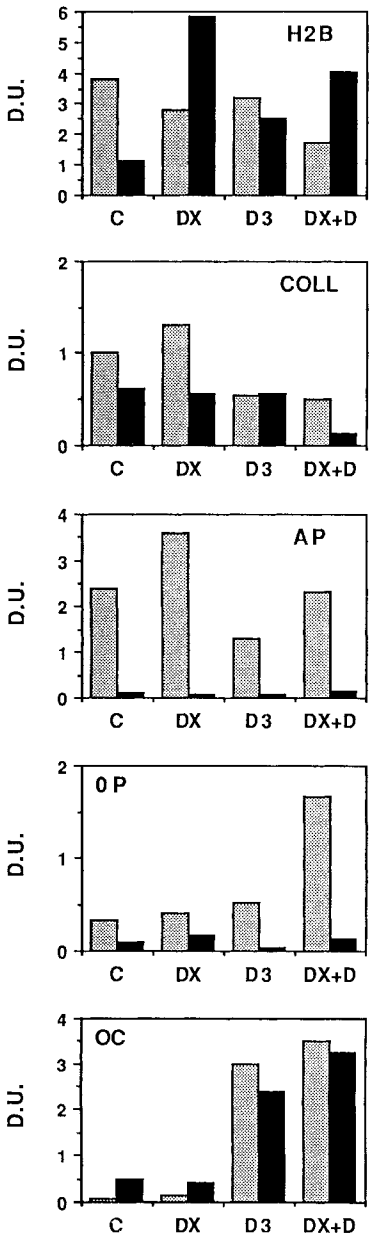

Fig. 10. Hormonal effects on osteoblast mRNA and transcription for vitamin D_3 and glucocorticoid modulated gene expression. Passaged osteoblasts, differentiated in the presence of dexamethasone, were examined for vitamin D and glucocorticoid responsiveness. Glucocorticoids were removed from the media for 48 h (one feeding) and media replaced with either 10^{-8} M $1,25(OH)_2D_3$ (D_3), or 10^{-7} M dexamethasone (DX), or both (DX + D) for 48 h. Solid bars represent transcription and hatched bars mRNA levels. Note the low level of transcription for several genes in these mature osteoblast cultures but the significant and hormone responsive changes in mRNA levels. Histone (H2B); osteocalcin (OC); osteopontin (OP); collagen (COLL); alkaline phosphatase (AP).

of osteoblasts (Fig. 6, 48 h; Figure 8, 24 and 48 h; Fig. 9, 48 h) demonstrates dex rapidly regulates several genes. Slightly higher levels of collagen, APase, and osteopontin mRNAs in dex-treated cultures within the first 48 h suggest the messages have been stabilized in the subcultivated cells, contributing to maintenance of the bone cell phenotype. In the acute primary culture studies, dex enhanced the levels of proliferation, c-*fos*, osteocalcin, and osteopontin mRNAs. APase transcript levels are reduced at 13 days but elevated at later times, revealing that the effects of dex on a gene are dependent upon the differentiated state of the cell, similar to what has been reported for vitamin D [Owen et al., 1991]. The increase in proliferation with either 24 h (Fig. 8) or 48 h (Fig. 6) GC treatment is consistent with the findings of Chen and Feldman [1979], who reported an increase in cell growth and a decrease in APase levels also consistent with our day 13 results in primary rat osteoblast cultures treated with dex at confluency. That GC may have effects on both bone precursor and differentiated cells has been suggested [Chyun et al., 1984] and here we show, with respect to APase and proliferation, different regulation in the young osteoblast (day 13) compared to the mature osteoblast in a mineralized matrix (day 34). Osteopontin and osteocalcin were upregulated at all developmental stages but to different degrees depending on the basal level of expression. Osteopontin and osteocalcin were minimally stimulated on day 20 when basal levels were highest. Note that the hormone was an effective enhancer only when a gene was expressed, indicated, for example, by osteocalcin regulation. The increases in osteopontin and osteocalcin in fetal rat derived osteoblasts in response to dex are in contrast to studies in transformed ROS 17/2.8 cells [Schepmoes et al., 1991] and human osteoblasts [Wong et al., 1990] where dex treatment was found to decrease osteocalcin or vitamin D stimulated osteocalcin synthesis and mRNA and osteopontin mRNA levels [Oldberg et al., 1989]. These observations are consistent with the hypothesis that glucocorticoid effects on gene expression are a function not only of species but of the phenotypic stage of the cell and ongoing levels of gene expression. The studies of Turksen and Aubin [1991] which demonstrate that APase negative osteoprogenitors respond thirtyfold greater to dexamethasone than more mature APase positive osteoblasts in forming bone nodules also support the

concept that steroid effects are related to the developmental stage of the osteoblast.

Chronic 10^{-7} M dex treatment of subcultivated cells during a 38 day developmental period promotes cells to form nodules and express higher levels of differentiation markers. The pattern of gene expression for growth and differentiated related osteoblast parameters is dramatically altered by continuous dex treatment, compared to untreated controls. These changes result in early formation of nodules and expression of genes reflecting osteoblast differentiation concomitant with nodule formation. c-*fos* has been implicated as a factor that plays a role in signal transduction during proliferation as well as differentiation of osteogenic bone cells [Birek et al., 1991]. Elevations in c-*fos* mRNA during proliferation and nodule formation with dex treatment in this study support such a hypothesis. In addition to selective effects on cellular proliferation, the question arises as to what signaling mechanisms involving cell-matrix or cell-cell interactions are involved in nodule formation.

Osteopontin mRNA levels are induced early during the initial stages of nodule formation (days 4–7) as is fibronectin. Both are R-G-D containing proteins and may promote cell-cell interaction for nodule formation. In other systems, dex increases fibronectin protein in rat hepatocytes [Marceau et al., 1980] and human fibrosarcoma [Oliver et al., 1983]. Osteopontin is a phosphoprotein and it has been hypothesized that phosphoproteins are nucleators of mineralization [reviewed in Boskey, 1989]. Thus, these results raise the question whether the early expression of osteopontin could also be related to the early deposition of mineral in the dex-induced nodule.

The reciprocal relationship with an r value of -0.9 exhibited by type I collagen and fibronectin between days 1 and 7 in dex-treated cultures also suggests factors involved in nodule formation. The decrease in type I collagen by day 4 when cells reach monolayer confluency may allow for focal proliferation. Once the cells are grouped into nodules, collagen matrix accumulation occurs, to support mineralization and further expression of differentiated genes (e.g., osteocalcin). By day 21 when nodules are abundant and actively mineralizing, collagen synthesis is higher than controls. The biphasic effects on type I collagen mRNA levels observed in the subcultivated cells continually exposed to dex were also seen in rat organ culture—an early GC stimulation followed by inhibition [Canalis, Hahn et al., 1984]. The inhibitory period is also consistent with decreases in type I collagen protein synthesis with the greatest effect seen in confluent primary ROB cells after at least two days treatment with 130 nM dex reported by Chen et al. [1986]. APase mRNA levels also exhibit biphasic changes in dex-treated cultures compared to control cultures where APase levels are constant. In the early culture period (up to day 7) AP levels are suppressed during the proliferative phase of nodule formation. By day 14 levels rise an order of magnitude above controls with the initiation of nodule mineralization. These increases are concomitant with elevations in osteocalcin and osteopontin mRNA levels.

Figure 11 compares the time course of expression of cell growth and bone related genes in primary cells cultured without dex to subcultivated cells in both the presence and absence of dex. As reflected by histone mRNA levels, proliferation ceases earlier; subcultivated cells come to confluency faster than primary cultures seeded at the same density. In subcultivated cells, since nodule formation is delayed and mineralization initiates after 3 weeks, the temporal pattern observed in primaries does not occur. In dex-treated cultures elevated osteopontin expression precedes that found in primary cultures. However, the pattern of APase and osteocalcin is essentially similar to primary cultures since competency for osteoblast phenotype development (formation of mineralized nodules) is maintained. That cells are pushed to differentiate is supported by the decrease in collagen I mRNA levels at early times and the increases in the mRNA levels after day 7 concomitant with induction of osteocalcin, osteopontin, and APase genes that reflect the mature osteoblast. Notably, these genes are regulated by vitamin D. Thus, these responses could be mediated through increased vitamin D receptor levels which had been demonstrated in rat osteoblasts in response to dex treatment in cells [Chen et al., 1986].

Collagen matrix formation is related to bone cell differentiation [Owen et al., 1991; Quarles et al., 1992]. Rat osteoblasts produce collagenase [Sakamoto and Sakamoto, 1984], whose major substrate is collagen and is a necessary component of bone remodelling [Sakamoto and Sakamoto, 1988]. Therefore, it was of interest to examine the effects of GC on collagenase gene expression in rat osteoblasts. Phorbol myristate

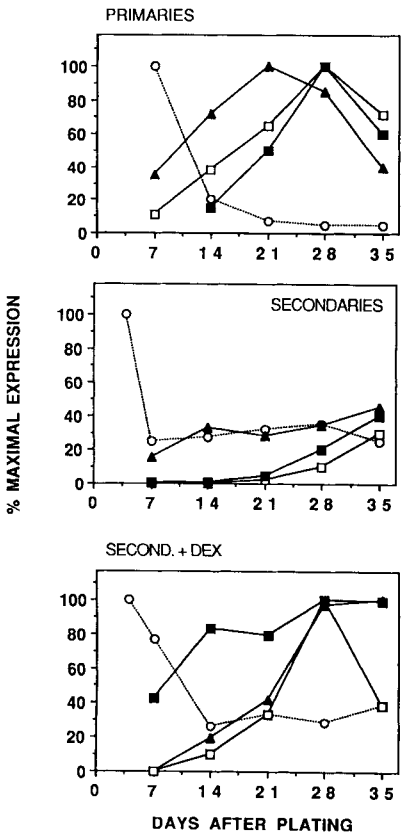

Fig. 11. Comparison of gene expression in primary osteoblasts, subcultivated cells, and dexamethasone differentiated subcultivated cultures. The normal pattern of gene expression, plotted as percent maximum for each gene, reflects progressive development of the osteoblast phenotype in primary cultures (PRIMARIES) of fetal rat calvarial derived osteoblasts (**top panel,** from Owen et al. [1991]). This temporal pattern of histone H2B (open circles), alkaline phosphatase (triangles), and osteopontin (open squares) or osteocalcin (closed squares) is representative of the growth, maturation, and mineralization stages of bone formation. This temporal sequence is lost in subcultivated cells (SECONDARIES; **middle panel**) over the same culture period since mineralization of the nodules is delayed. **Lower panel:** The presence of 10^{-7} M dexamethasone (SECOND + DEX) restores the temporal expression of genes to reflect formation of mineralized bone nodules and expression of differentiation related genes, osteocalcin, and osteopontin. The level of gene expression in the middle panel is represented as % maximum relative to the dexamethasone treated cultures (lower panel) and is taken from Fig. 6.

acetate (PMA) induction of the collagenase gene is repressed by GC in rabbit synovial cells [Brinckerhoff et al., 1986]. In fibroblasts GC inhibited both basal and PMA induced collagenase by interference with AP-1 activity [Jonat et al., 1990]. This is due to direct protein interaction between GC receptor and c-*jun* or c-*fos* [Yang-Yen et al., 1990] and this may be one of the mechanisms by which GC act as antiarthritic agents [Parillo and Fauci, 1979] and antitumor agents [Viaje et al., 1977]. The finding contrasts with GC mechanism of action in these dex-treated cultures of rat osteoblasts, both acute and chronic, where the presence of GC elevates collagenase mRNA levels. Notably, c-*fos* is also elevated in our dex-treated cultures. In continuously treated cultures, the increase in collagenase paralleled the other differentiation markers (AP, OC, OP) and appeared to be related to nodule formation with maximal expression in mature cultures (day 35). The elevated levels of collagenase may also be related to a phase of turnover of the matrix and expansion of bone nodules as reflected by an elevation of histone, collagen, and TGFβ transcripts and by proliferating cells around the periphery of the nodules late in the culture (Fig. 4). Here the release of growth factors from the extracellular matrix through the action of collagenase may stimulate osteoblast proliferation and type I collagen production. The acute studies of dex in primary cells on day 20 (Fig. 9) indicated that in mature osteoblasts, in contrast to synovial cells [Brinckerhoff et al., 1986], rat osteosarcoma cells (Connolly, Clohisy, Partridge and Quinn, unpublished observations), or rat calvaria in organ cultures [Delaisse et al., 1988], GC acts to increase collagenase mRNA levels.

Our observations of GC effects on gene expression in relation to osteoblast growth and differentiation are indeed complex and may be operative at multiple levels of gene control. Glucocorticoids enhance or repress gene expression directly or indirectly through the classic interaction of steroid-hormone-receptor complexes with specific DNA sequences in the promoter region of genes termed glucocorticoid response elements [Weiner et al., 1987]. Other modes of action, unrelated to DNA-hormone-receptor binding and involving GC receptor protein-protein factor interactions, have also been implicated as alternate and/or supplemental modes of GC action—for example, as in collagenase gene expression [Yang-Yen et al., 1990; Schüle et al., 1990]. The presence of glucocorticoid response elements (GRE) in the human [Stromstedt et al., 1991] and rat [Heinrichs et al., 1992] osteocalcin gene and the results in this paper for OC transcription demonstrate one mechanism is by direct hormone-receptor interaction on the promoter. Additionally, based on the synergistic increase in transcription in the presence of $1,25(OH)_2D_3$ and dex reported in

this paper, together with identification of vitamin D mediated enhanced protein-DNA interactions at the overlapping GRE/TATA element in the rat OC gene [Owen et al., 1992], other mechanisms of transcriptional control by GC are indicated. GC may act on another gene to produce a factor that affects transcription or stabilizes or destabilizes [Weiner et al., 1987; Philippe and Missotten, 1990] mRNA. This mechanism would be consistent with changes in mRNA levels for H2B histone or collagen and APase in this study. In conclusion, our results have shown 1) both acute and early responses of the developing osteoblast phenotype to glucocorticoid as well as 2) changes in gene expression reflecting mature osteoblasts and 3) an interplay of GC and $1,25(OH)_2D_3$ regulation of transcription of several osteoblast parameters. Thus, there may be multiple scenarios of physiological actions of dexamethasone at the transcriptional and/or post-transcriptional levels contributing to promotion of osteoblast growth and differentiation.

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