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Glucocorticoid Regulation of Alkaline Phosphatase, Osteocalcin, and Proto-Oncogenes in Normal Human Osteoblast-Like Cells

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Abstract In humans, glucocorticoids are known to have marked effects on bone metabolism and function, including the significant regulation of osteoblast cells. To aid in the understanding of the mechanism of glucocorticoid action on normal human osteoblasts (hOB), confluent cells were analyzed for the presence of glucocorticoid receptors (GR) as well as for the effects of the glucocorticoid dexamethasone (Dex) on the expression of both the rapid responding nuclear proto-oncogenes and the late responding structural genes for bone matrix proteins. The interactions between Dex and 1,25 dihydroxy vitamin D_3 (1,25 D_3) on the gene expression in these cells were also examined. Using a functional receptor assay, a mean of 11,600 functional nuclear bound glucocorticoid receptors (range 6,000–22,000) was measured in fifteen separate cell strains. Northern blot analysis with a cDNA probe to the human GR was used to demonstrate the presence of a 7Kb transcript which is a candidate mRNA for GR in these cells. In agreement with previous studies, treatment of the hOB cells with Dex increased the steady state mRNA levels for alkaline phosphatase (AP) but displayed little or no effect on the mRNA levels for osteocalcin (OC) and glyceraldehyde phosphate dehydrogenase (GAPDH). Interestingly, the 1,25 D_3 inductions of mRNA levels for OC were blocked by Dex but enhanced for AP. The above effects of Dex on AP and OC gene expression, including the interaction with 1,25 D_3, were also shown to occur at the level of protein. The effect of Dex on the mRNA levels of the nuclear proto-oncogenes c-myc, c-fos, and c-jun was also investigated, since the oncoproteins (Fos/Jun) appear to play a role in the delayed glucocorticoid regulation of structural genes. Interestingly, Dex increased the steady state levels of c-myc, c-fos, and c-jun mRNAs in nonproliferating (confluent) hOB cells by 3.5-, 10-, and 2.0-fold, respectively, over control (untreated cells) values within one h of steroid treatment. The Dex-induced mRNA levels were transient and returned to basal values within 24 h of the steroid treatment. A reduced but qualitatively similar pattern of response was found in proliferating hOB cells. The pattern of response of these genes to glucocorticoids in hOB cells mimics the response in avian liver cells but not in reproductive cells. These results support the theory that hOB cells are target cells for glucocorticoids, and that as a primary event glucocorticoids rapidly regulate the expression of the nuclear oncoproteins Fos/Jun in these cells.

Key words: steroid hormone, glucocorticoids, osteocalcin, alkaline phosphatase, glucocorticoid receptor, nuclear proto-oncogenes, c-myc, c-fos, c-jun, human osteoblast cells, mRNA levels

Glucocorticoid hormones have long been known to regulate calcium and phosphate metabolism and other functions in bone [Gennari, 1985]. High concentrations of glucocorticoids were shown to inhibit the growth and activities of isolated osteoblasts including decreases in cell multiplication as well as in total RNA, collagen levels, and other protein synthesis [Chen et al., 1977; Peck et al., 1967, 1969; Choe et al., 1978]. Recent studies have indicated that glucocorticoids regulate many other processes in bone tissue.

The action of glucocorticoids on bone tissue was originally thought to occur indirectly by inhibiting Ca^{++} absorption in the gastrointestinal tract [Younoszai and Gishan, 1978]. They were also reported to potentiate the ability of parathyroid hormone (PTH) to stimulate cyclic AMP (cAMP) production in bone cells [Chen and

Abbreviations used: mRNA, messenger RNA; DMEM, Dulbecco's modified Eagle's medium; Ci, curie; hOB, human osteoblast-like; OC, osteocalcin; AP, alkaline phosphatase; Dex, dexamethasone.

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Feldman, 1978]. However, glucocorticoid receptors have been identified in transformed osteosarcoma cell lines [Haussler et al., 1980; Mulkins et al., 1983] and osteoblast-like cells from rodent sources [Chen and Feldman, 1979; Chen et al., 1977, 1986]. Subsequent studies supported glucocorticoid stabilization of receptors for hormones like 1,25 D_3 in bone cells and in bone tissue culture [Chen et al., 1982; Manolagas et al., 1979] about the same time they were shown to regulate the levels of their own receptors in other target cells [Cidlowski and Cidlowski, 1981].

Glucocorticoids elicit specific molecular responses in vitro. Investigations using various osteoblast-like cell model systems have demonstrated that glucocorticoids induce alkaline phosphatase (AP) activity [Gallagher et al., 1982; Manologas et al., 1981; Majeska et al., 1985], the attenuation of osteocalcin (OC) release [Gallagher et al., 1982; Beresfold et al., 1984], and an enhanced cAMP formation after PTH treatment [Rodan et al., 1984; Silve et al., 1989]. In contrast, early studies showed that glucocorticoids inhibited collagen synthesis [Wong, 1979] and the cell proliferation in isolated osteoblast cell in vitro [Chen et al., 1977], as well as the 1,25 D_3 induction of the osteocalcin gene expression [Beresford et al., 1984]. Recently, glucocorticoids have also been shown to inhibit fibronectin synthesis in fetal rat parietal bones, an effect which may contribute to altered osteoblast organization and activity during bone formation [Gronowicz et al., 1991]. Long-term treatment of Dex to human bone-derived cells increased PTH-stimulated cyclic AMP production, AP activity, and 1,25 D_3-induced AP activity but decreased 1,25 D_3-stimulated OC production and cell proliferation [Wong et al., 1990].

Morrison et al. [1989], using transfected deletion constructs, demonstrated that glucocorticoids repressed the transcription of OC gene via a specific promoter response element in rat osteosarcomal cell line (ROS 17/2-8). Recently, extensive investigation by Stein, Lian, and coworkers [Schepmoes et al., 1991] has expanded the work of Morrison et al. [1989] to show that Dex regulation of the OC promoter occurs at the transcriptional and post-transcriptional levels and involves two promoter elements which bind transcription factors. Further, they demonstrated that Dex action on the OC promoter depends on the cell line which is used in the transfection studies.

Glucocorticoids have been shown to regulate the expression of nuclear proto-oncogenes in both reproductive and non-reproductive target cells. Dexamethasone (Dex), a potent synthetic glucocorticoid, has been shown to decrease c-myc mRNA levels in lymphosarcoma cells (P1798) in culture [Forsthoefel and Thompson, 1987] and decrease c-myc, c-myb, and c-ras mRNA levels in a mouse lymphoma cell line [Eastman-Reks and Vedeckis, 1986]. A rapid reduction in c-myc mRNA levels in the oviducts of estrogen treated immature chickens by treatment with dexamethasone has been reported by this laboratory [Rories et al., 1989]. Other steroid hormones, including progesterone and estrogens, have also been reported by this laboratory and others to rapidly regulate the expression of nuclear proto-oncogenes, e.g., c-myc and c-jun, in the avian oviduct and other tissues [Fink et al., 1988; Rories and Spelsberg, 1989; Lau et al., 1990; Webb et al., 1990; Murphy et al., 1987].

Although transformed osteosarcoma cell lines and rodent osteoblast cells are useful as osteoblast-like model systems, results obtained in nonhuman OB cell lines, especially transformed OB cells, must be confirmed in normal hOB cells since differences between animal species and transformed vs. nontransformed OB cells have been reported. Therefore, we have cultured normal human osteoblast-like cells (hOB) which maintain the osteoblast phenotype, including the stimulation of osteocalcin production by 1,25,D_3 and a high level of alkaline phosphatase activity [Eriksen et al., 1988]. In this investigation using normal human osteoblast-like cells, we have shown the presence of high levels of GR mRNA and functional receptor protein in these cells. A significant antagonism between glucocorticoids and 1,25,D_3 at the level of mRNA for the structural genes is also demonstrated. Lastly, it is shown that Dex regulates the expression of certain structural genes coding for bone cell proteins as well as for the nuclear proto-oncogenes (c-myc, c-fos, and c-jun).

METHODS

Cell Culture

Normal human osteoblast-like cells were cultured using a modification of the procedure of Robey and Termine [1985] as described previously [Eriksen et al., 1988]. Briefly, trabecular bone explants obtained during bone grafting procedures were minced in phosphate buffered saline and digested with crude bacterial collage-

nase (Gibco, Grand Island, NY) at 1 mg/ml in Dulbecco's modified Eagles medium (DMEM, Gibco) for two h at 37°C in a shaking water bath. The bone fragments were then cultured in phenol red-free medium approximately equivalent to a Ca^{++} free mixture (1:1) of Ham's F.12 (Gibco) and DMEM plus 10% (v/v) heat inactivated fetal calf serum (FCS). The cells were maintained at 37°C in a 5% (v/v) CO_2 atmosphere. Cells derived from the explants were passaged once and used for experiments at the end of the first passage. Through the duration of the culture period, these cells maintain the many aspects of the mature osteoblast phenotype [Eriksen et al., 1988]. To assess the effect of the glucocorticoids on these hOB cells, the cells were plated at a density of 1×10^6 cells/T75 flask and grown in media (without phenol red) containing 10% (w/w) charcoal stripped fetal calf serum for 48 h. The cells were rendered quiescent by growing them in media containing 1% (w/w) charcoal stripped fetal serum for 48 h. Quiescent cells were exposed to dexamethasone for different time periods and the cells were harvested for RNA isolation. One T75 flask of hOB cells was used for each time point.

Nuclear Binding Assay

Specific nuclear binding of [^{3}H]dexamethasone was assessed using a nuclear binding assay [Spelsberg et al., 1987] modified for tissue culture cells [Eriksen et al., 1988; Colvard et al., 1988]. Near confluent secondary cultures of human osteoblast-like cells were harvested in 1X tyrpsin/EDTA solution and suspended in Eagle's basal medium (Irvine Scientific, Santa Ana, CA). Routinely, aliquots of the cell suspension containing $0.2–1 \times 10^6$ cells were incubated in triplicate at 37°C for 1 h at a final concentration of 10 nM [1,2,4,6,7,^{3}H]dexamethasone ([^{3}H]Dex) (Amersham, Arlington Heights, IL) without or with a 100-fold molar excess of nonradioactive Dex for estimation of total and nonspecific binding, respectively. After the 1 h incubation, an ice-cold solution of 5 mM HEPES, pH 7.5, 0.2 mM EDTA, and 0.1% (w/w) bovine serum albumin (BSA) was added to stop the binding and the cells were immediately collected by low-speed centrifugation. The resultant cell pellet was suspended in 1 ml of homogenization buffer [50 mM Tris, pH 7.5, 10% (v/v) glycerol/10 mM KCl/0.1% (v/v) Triton X-100/0.1% (w/w) BSA] and gently homogenized in a Teflon pestle/glass homogenizer (type A, Thomas Scientific, Swedesboro, NJ). The homogenate was layered over 0.5 ml of 1.4 M sucrose in homogenization buffer and centrifuged for 20 min at 7,000*g*.

Radioactivity was quantitated in ethanol extracts of the nuclei pellets by liquid scintillation spectrometry in an LS-5801 liquid scintillation counter (Beckman Institute, Fullerton, CA). Nuclear binding was normalized to DNA quantity. DNA in the nuclear pellet was estimated using the diphenylamine assay of Burton [1956] with modification [Colvard et al., 1988] using a standard curve generated using calf thymus DNA (Calbiochem, La Jolla, CA). The specific nuclear binding was determined by subtracting the mean of the triplicate assays for the nonspecific binding of [^{3}H]Dex (expressed in disintegrations per minute (DPM) per microgram DNA) from the corresponding mean of the replicate assays for the total binding. For calculation of receptor molecules bound per cell nucleus, the value of 6 μg of DNA per 10^6 human cells was used [Sober, 1970].

Concurrent Osteocalcin and Alkaline Phosphatase Analyses

Human osteoblast-like cells from near confluent first passage cultures were trypsinized and seeded into 24-well plates at densities of 20,000–30,000 cells/cm^2 in 10% (v/v) FBS supplemented medium for 48 h. The cells were then incubated with fresh medium supplemented with 1% dialyzed FBS and 1 mM $CaCl_2$ in the presence or absence of 1 nM 1,25-dihydroxyvitamin D_3 (1,25,D_3), 0.5 or 50 nM Dex, or both 1,25,D_3 and Dex for a total of 120 h (with fresh media changes after 48 and 96 h). Twenty-four hours after the second media change, the media were removed and osteocalcin (OC) was quantitated in the media using a monoclonal antibody against bovine OC (a gift from Dr. K.G. Mann) using radioimmunoassay (RIA) as described elsewhere [Tracy et al., 1987]. The remaining cell layers were assayed directly for alkaline phosphatase (AP) by measuring p-nitro phenylphosphate (PNP) hydrolysis by the method of Puzas and Brand [1985]. Cell counts were taken in wells cultured in parallel with the experimental cultures and the data normalized to represent the activity for 10^4 cells.

Northern Blot Analysis

Total RNA was isolated from single cell strains that had been treated or untreated with dexa-

methasone using a modified method of Chirgwin et al. [1979] as described previously [Lau et al., 1991]. Total RNA preparations were denatured using glyoxal-DMSO and resolved by electrophoresis in a 1% (v/v) agarose gel [Lau et al., 1991]. The RNAs were transferred overnight to a magna 66 nylon membrane (MSI, Fisher Scientific, Pittsburgh, PA) by capillary action in 20 × SSC (3 M NaCl, 0.3 M trisodium citrate, pH 7.0) as described previously [Lau et al., 1991]. The filters were baked for two h at 80°C, hybridized with [^{32}P]-cDNA probes, and the blots washed, as previously described [Lau et al., 1991]. The cDNA probes were labelled with [^{32}P] by random hexanucleotide primer extension using the Multiprime DNA labelling system from Amersham (Arlington Heights, IL). The [α-^{32}P]dCTP with a specific activity of approximately 3,000 Ci/mmol (New England Nuclear Research Products, Boston, MA) was used to radiolabel the cDNA to achieve specific activities approximately -10^9 cpm/mg.

For Northern blot analyses of GR mRNA, first passage human osteoblast-like cells were grown to near confluence in 75 cm^2 flasks as described above without additional steroids added to the medium. For analysis of AP and OC mRNAs, the cells were then cultured for 72 h (with one refeeding after the first 48 h) in medium supplemented with 1% (v/v) dialyzed FBS with various steroids or combinations of steroids as indicated in the figure legends.

cDNA Probes

The cDNA probes were used with permission and represented a human glucocorticoid receptor cDNA, hGR1.2 [Eastman-Reks and Vedeckis, 1986], a rat OC cDNA, pR22-11 [Celeste et al., 1986], and a human bone/liver/kidney AP cDNA, B/L/K-ALP [Weiss et al., 1986]. The probes used for control mRNA to assure equal amounts of RNA loaded in each gel lane were the rat glyceraldehyde-3-phosphate dehydrogenase cDNA [Fort et al., 1985]. For nuclear proto-oncogene analysis, the same methodology as described above for the structural genes was utilized. The human c-myc plasmid containing the third exon of the gene was purchased from American Tissue Type Collection. The human c-fos cDNA was obtained as a gift from Dr. Inder Verma, San Diego, CA. The human c-jun cDNA was a gift from Drs. Tjian and Turner, University of California, Berkeley. Restriction enzymes were used to cut out the proto-oncogene cDNAs from the vectors. Restriction enzyme treated DNA was then resolved by gel electrophoresis and the insert fragment excised from the gel and purified using a gene clean kit (Bio 1-1, La Jolla, CA). For the nuclear proto-oncogene analyses, an rDNA probe containing 18S ribosomal RNA coding sequences was used as reference.

Densitometry of Autoradiographs of the Blots

The relative amounts of the mRNAs were determined by densitometric analysis of the autoradiographs developed from the Northern blots. Autoradiographs were scanned with a transmitting scanning densitometer (Model GS 300, Hoefer Instruments, San Francisco, CA) and the data calculated with the GS-360 data system program (Hoefer Instruments, San Francisco, CA). Each experiment discussed in the Results was performed 3 to 5 times, each with a different strain of cells. The data presented represent example experiments from three or more replicate experiments. In every hOB strain examined, the steroids induced the mRNA steady state levels. The degree of the induction did vary, but only moderately.

RESULTS

Detection of Functional Specific Nuclear Glucocorticoid Receptor Proteins and mRNA in Human Osteoblast-Like Cells

Since the presence of GR in normal human osteoblasts (hOB) had not been reported, a functional receptor assay which measures the amount of specific nuclear binding of [^{3}H]-steroids in nuclei of target cells was applied to the hOB cells [Spelsberg et al., 1987; Colvard et al., 1988]. Specific nuclear binding of [^{3}H]-Dex was found in all 15 strains of human osteoblast-like cells examined. A mean of 11,600 ± 1,300 (mean ± SEM) nuclear bound [^{3}H]-steroid molecules per cell nucleus was measured with a range of 5,800–21,100 receptors per cell. Each bound steroid molecule reflects the presence of one glucocorticoid receptor (GR). No statistically significant difference was observed between the mean concentration of GR in the female strains (11,100 ± 3,700, mean ± SEM) compared to the male strains (12,290 ± 5,010) (data not shown).

To verify that the observed nuclear binding was indicative of active steroid receptors, a steroid specificity (Fig. 1) and saturation analyses (Fig. 2) were performed using several cell strains.

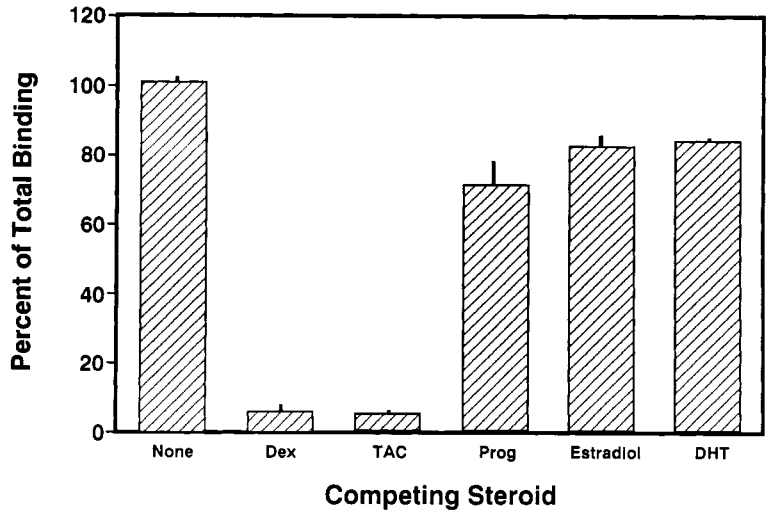


Fig. 1. Steroid specificity of the specific nuclear [^{3}H]dexamethasone binding in human osteoblast-like cells. Human osteoblast-like cells were incubated with 10 nM [^{3}H] dexamethasone alone and with a 100-fold excess of unlabelled dexamethasone (Dex), triamcinolone acetonide (TAC), progesterone (Prog), estradiol, and dihydrotestosterone (DHT) and processed for nuclear binding as described in Methods and elsewhere [Spelsberg et al., 1987; Colvard et al., 1988]. Data presented is expressed as the mean percent of nuclear binding relative to the binding in the absence of competing steroid. The latter represents the average from experiments using six different cell strains. The vertical bars indicate SEM.

Each experiment was repeated several times and the results from example experiments are presented. When nuclear [^{3}H]Dex binding competition assays were performed, the homologous nonradiolabelled glucocorticoids Dex and triamcinolone acetonide (TAC) competed for the nuclear binding of [^{3}H]Dex, and progesterone displayed modest competition for [^{3}H]Dex nuclear binding characteristic of most GR systems. Estradiol (E_2) and dihydrotestosterone (DHT) displayed no competition (Fig. 1).

For the analyses of saturable binding, osteoblast-like cells were incubated with increasing concentrations of [^{3}H]Dex and processed for nuclear binding. In each of five separate strains assayed, saturation of the nuclear binding sites

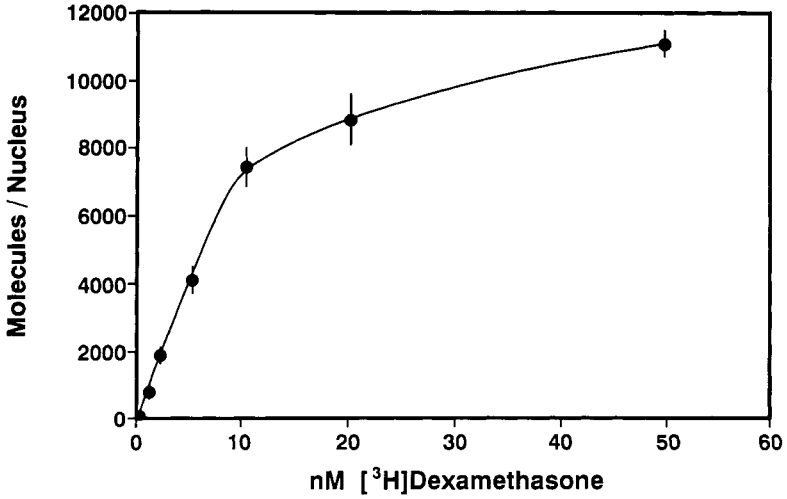


Fig. 2. Saturation analysis of the specific nuclear [^{3}H]dexamethasone binding in human osteoblast-like cells. Normal human osteoblast-like cells were incubated with 1 to 50 nM [^{3}H]dexamethasone alone or with 100-fold excess of unlabelled dexamethasone and processed using the nuclear binding assay as described in Methods. The data represent the means for specific binding from five experiments using separate cell strains. The vertical bars indicate SEM. The molecules of [^{3}H]dexamethasone bound per cell nucleus were calculated using the bound radioactivity, the specific activity of the [^{3}H]dexamethasone, and the 6×10^{-12} g DNA per human cell.

by [^{3}H]Dex was found to occur in the physiologic range of 10 to 50 nM Dex (see Fig. 2 for an example experiment). Therefore, a steroid specific and saturable nuclear binding of the [^{3}H] occurs in these cells and is characteristic of steroid in target tissues as found in vivo [Spelsberg et al., 1987; Colvard et al., 1988].

The presence of GR mRNA in normal human osteoblast-like cells was confirmed by Northern blot analysis (Fig. 3). The GR mRNA was detected in all seven strains of human osteoblast-like cells which were examined. As shown in the example experiment of Figure 3, the predominant band at approximately 7.0 kilobases was detected using total RNA from human osteoblast-like cells when probed with a cDNA for human GR. A similar band is seen when the total RNA from the GR-positive human fibroblast cell line, HT1080, was probed with this cDNA.

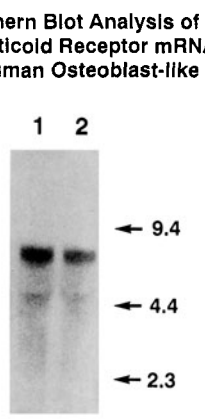


Fig. 3. Northern blot analyses for the mRNA of the glucocorticoid receptor gene in normal human osteoblast-like cells. Total cellular RNA was isolated from two cell strains and processed for Northern blot analysis using a human GR cDNA as described in Methods. **Lane 1** contains 12 μg of RNA from one cell strain and **lane 2** contains 12 μg of RNA from another cell strain. Size markers are indicated in kilobases (kb) on the right of the lanes.

Steroid Effects on Alkaline Phosphatase (AP) Activity and OC Protein Production

The effect of glucocorticoids on AP activity in the osteoblast-like cell strains was then examined at 0.5 nm and 50 nm doses of Dex. Treatment of 10 cell strains with 0.5 nM Dex led to only a 1.5-fold increase in enzyme activity, while treatment with 50 nM Dex for 120 h induced an average 2.1-fold increase in AP activity (Fig. 4A). Both doses demonstrated significant inductions over values from untreated cells ($P < 0.05$, paired t-test) (Fig. 4A). Incubation of hOB cells with 1 nM 1,25,D_3 for 120 h also increased the AP activity approximately 1.8-fold ($P < 0.01$). Interestingly, the combined treatment of hOB cells with both 1,25,D_3 and 0.5 nM Dex further elevated the enzyme activity an average of 3.8-fold ($P < 0.01$) in an apparently additive fashion.

The effects of Dex and 1,25,D_3 on the secretion of OC protein by hOB cells clearly contrast with the effects on AP activity. Treatment of the cells with 0.5 nM or 50 nM Dex alone did not result in any measurable changes in the baseline production of OC (120 ± 60 ng/ml, mean ± SEM) (Fig. 4B). In fact, OC production by hOB cells was often below the RIA assay limits of detectability. However, when cells were incubated with 1 nM 1,25,D_3 for 120 h, a significant stimulation of OC release was observed resulting in a mean (± SEM) of 380 ± 150 pg/ml ($P < 0.05$). The addition of 50 nM Dex with 1,25,D_3 completely eliminated the stimulation of OC secretion by 1,25,D_3 ($P < 0.05$), while 0.5 nM Dex attenuated the production of OC ($P < 0.05$). The mechanism of this antagonism has not been determined.

Steroid Hormone Effects on the mRNA Steady State Levels of the Bone Matrix Protein

It was of interest to determine whether the additive increase in AP activity observed with Dex and 1,25,D_3 treatment occurred at the level of mRNA. When total RNA was probed with a cDNA for B/L/K AP, both the 50 nM Dex (Fig. 5, lane 3) and 1 nM 1,25,D_3 (lane 2) were found individually to increase AP mRNA levels over untreated cells (lane 1). Interestingly, treatment with both Dex and 1,25,D_3 caused the greatest induction in the mRNA steady state levels (Fig. 5, lane 4). In order to verify that the changes were specific to AP mRNA, the RNA blots were simultaneously probed with a cDNA for a constituently expressed gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). No changes in the GAPDH mRNA levels were observed, supporting that the changes in AP mRNA levels induced by Dex and 1,25,D_3 appear to be specific. These results closely parallel those obtained from the measurement of the AP activity at the protein level. These experiments were repeated several times with different strains of cells and the results from one example experiment are presented.

Similar results were obtained with the OC gene expression. Using the cDNA for rat OC as a

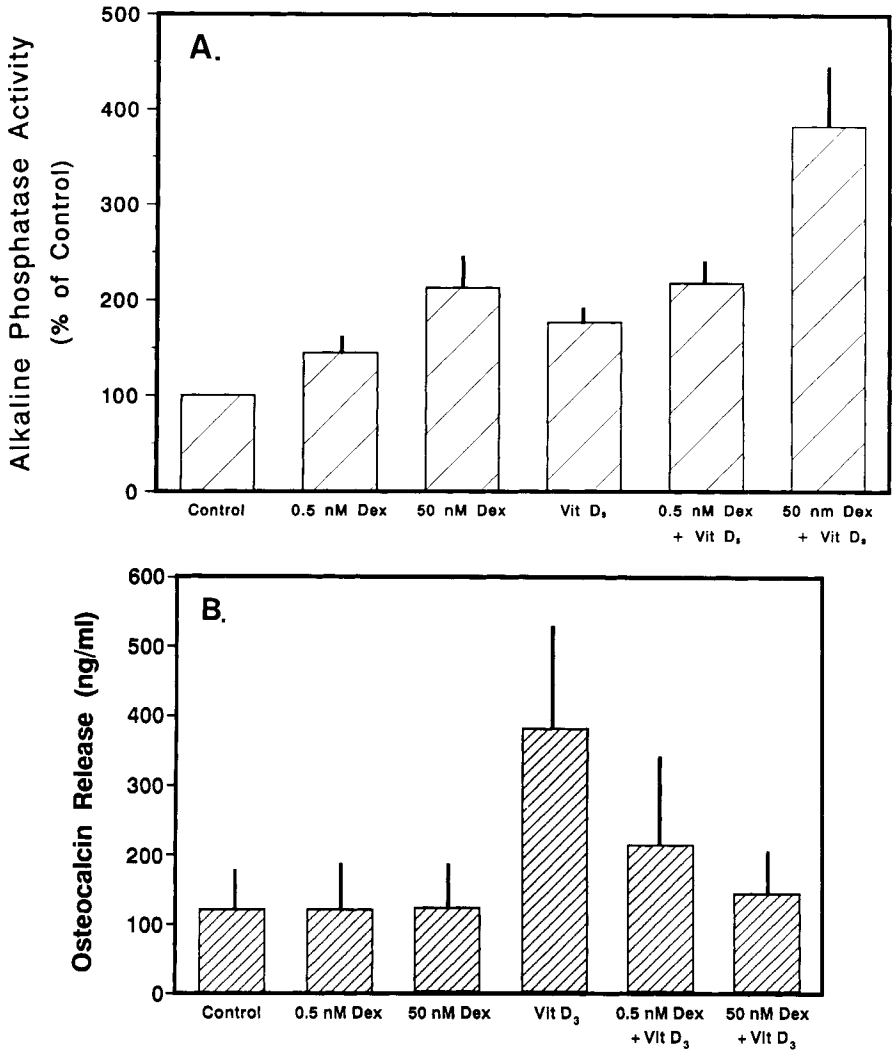


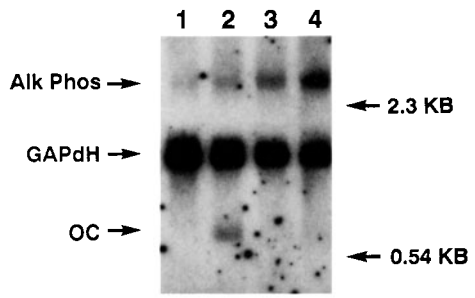
Fig. 4. Steroid regulation of alkaline phosphatase activity secreted from human osteoblast-like cells. Normal human osteoblast-like cells were incubated in 1% FBS for 5 days with 1 nM $1,25,D_3$, 0.5 or 50 nM dexamethasone, or both $1,25,D_3$ and dexamethasone as described for concurrent measurement of OC and alkaline phosphatase. **A:** Alkaline phosphatase activity was measured in the cell layer. **B:** OC was measured in the conditioned medium by RIA. Data values are from seven experiments. Data values are expressed as percent of control from each of 10 experiments. The control value of alkaline phosphatase was 111 ± 59 (mean ± SEM) pmol PNP per h per 10^4 cells.

probe, the OC mRNA was detectable only after incubation of the hOB cells with $1,25,D_3$ (Fig. 5, lane 2). No OC hybridizing band was discernable at basal conditions (Fig. 5, lane 1), or after the cells were incubated with Dex alone (Fig. 5, lane 3), or after incubation with Dex plus $1,25,D_3$ (Fig. 5, lane 4). Again, results obtained by steady state mRNA analysis correlated well with those obtained for production of the protein and indicate a regulation by an antagonism between Dex and $1,25,D_3$ occurs at the level of the mRNA. The same results were obtained in repeat experiments using other strains of hOB cells.

Steroid Regulation of the Nuclear Proto-Oncogenes

Dex has been reported to regulate the late responding structural genes via the nuclear proto-oncogene of c-fos/c-jun complexes [Jonat et al., 1990]. Further, the regulation of the expression of the nuclear proto-oncogenes by Dex in reproductive and connective tissue cells has been reported [Eastman-Reks and Vedeckis, 1986; Forsthoefel and Thompson, 1987; Rories et al., 1989]. We therefore initiated studies to determine whether Dex has any effect on the nuclear

Steroid Hormone Regulation of the mRNA for Bone Specific Proteins in Osteoblast-like Cells



1. Control
2. + 1 nM 1,25-Vit D_3
3. + 50 nM Dex
4. + Dex and 1,25-Vit D_3

Fig. 5. Regulation of the steady state mRNA levels of alkaline phosphatase and OC in normal human osteoblast-like cells. Human osteoblast-like cells were incubated in media supplemented with 1% FBS for 120 h either alone or with various steroids and RNA was isolated as described. Northern blot analyses were then performed as described in Methods. Incubation conditions were: control (**lane 1**), 1 nM 1,25,D_3 (**lane2**), 50 nM dexamethasone (**lane 3**), or both 1,25,D_3 and dexamethasone (**lane 4**). Total RNA was isolated from treated cells; Northern analyses were performed with 8 μg of RNA and the blots were probed with a rat OC cDNA, a human B/L/K alkaline phosphatase cDNA [Weiss et al., 1986], and a rat GAPdH cDNA [Celeste et al., 1986].

proto-oncogenes c-myc, c-fos, and c-jun in the quiescent hOB cells. When quiescent hOB cells were treated with Dex and the chronological effects examined (Fig. 6A), a rapid but transient increase in the steady state c-myc mRNA levels was observed within 1 to 4 h after treatment. Using densitometric analysis, the maximum induction of the c-myc mRNA occurred at four h post-Dex treatment with an increase of approximately 3.5-fold over control value (zero time or time matched controls from untreated cells) (Fig. 6B). The same Northern blots probed for the control 18S rRNA levels demonstrated equivalent amounts of RNA loading. These analyses were repeated with additional strains of hOB cells and the same results obtained. This induction was also found to be steroid dose-dependent (data not shown). In other experiments, the Dex treatment of proliferating cells grown in media containing 10% (v/v) FCS also showed an increase in c-myc mRNA steady state levels at 1–4 h of treatment (data not shown).

Effects of Dexamethasone on c-myc mRNA levels in hOB cells

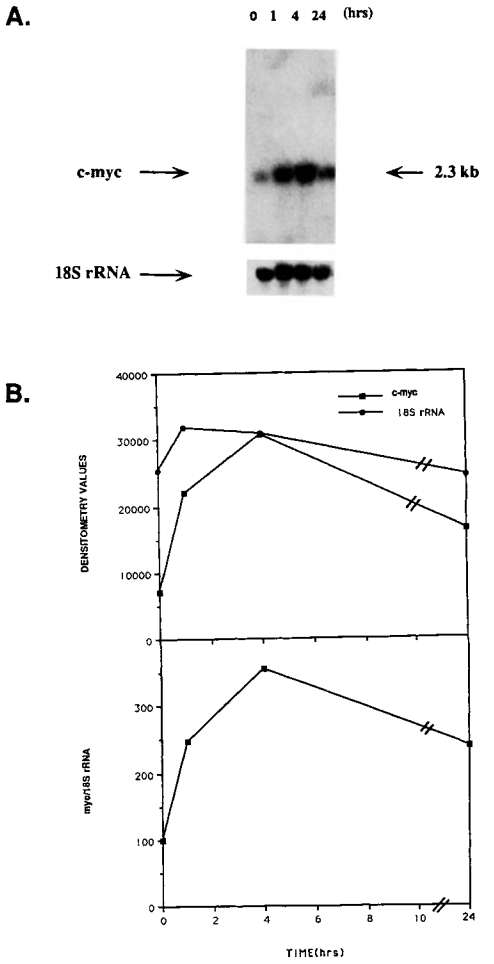


Fig. 6. Northern blot analyses of c-myc mRNA levels in hOB cells after various time periods of dexamethasone treatment. Quiescent hOB cells were treated with dexamethasone (50 nM) for various time periods. Total RNA was isolated and 12 μg of RNA was loaded to each lane in the gel and processed for Northern analysis and probed with [^{32}P]-labelled human c-myc exon-3 DNA probe (**Panel A**). The bottom panel shows the same blot as shown above, striped and reprobed with [^{32}P]-labelled rDNA probe containing 18S coding sequence. The numbers on top of each panel indicate the time of dexamethasone treatment. The zero value represents the control treated with the vehicle (ethanol). **Panel B** shows the densitometric values of blots shown in Panel A. The upper graph shows the values of c-myc mRNA and 18S rRNA levels, the bottom graph shows the percent changes as the ratio of c-myc to 18S rRNA levels.

Similar experiments were also performed with the Dex regulation of the c-fos gene expression in quiescent hOB cells. The results for these studies are shown in Figure 7. Dex treatment increased c-fos mRNA steady state levels in the hOB cells by 10-fold within 30 min. This in-

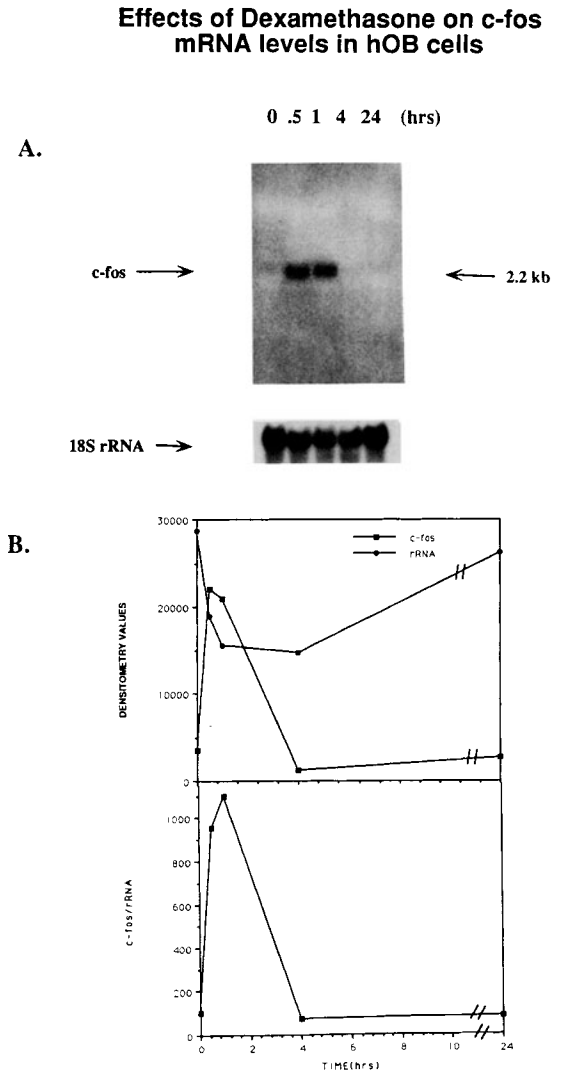


Fig. 7. Northern blot analysis of c-fos mRNA levels in hOB cells after various time periods of dexamethasone treatment. Quiescent hOB cells were treated with dexamethasone (50 nM) for various time periods. Total RNA was isolated, 12 μg of RNA was loaded to each lane in the gel and processed for Northern analyses, and the blots were probed with [^{32}P]-labelled human c-fos cDNA probe (**Panel A**). The bottom panel shows the same blot as shown above, striped and reprobed with [^{32}P]-labelled 18S rDNA probe. The numbers on top of each lane indicate the time of dexamethasone treatment. **Panel B** shows the densitometric values of blots shown in Panel A.

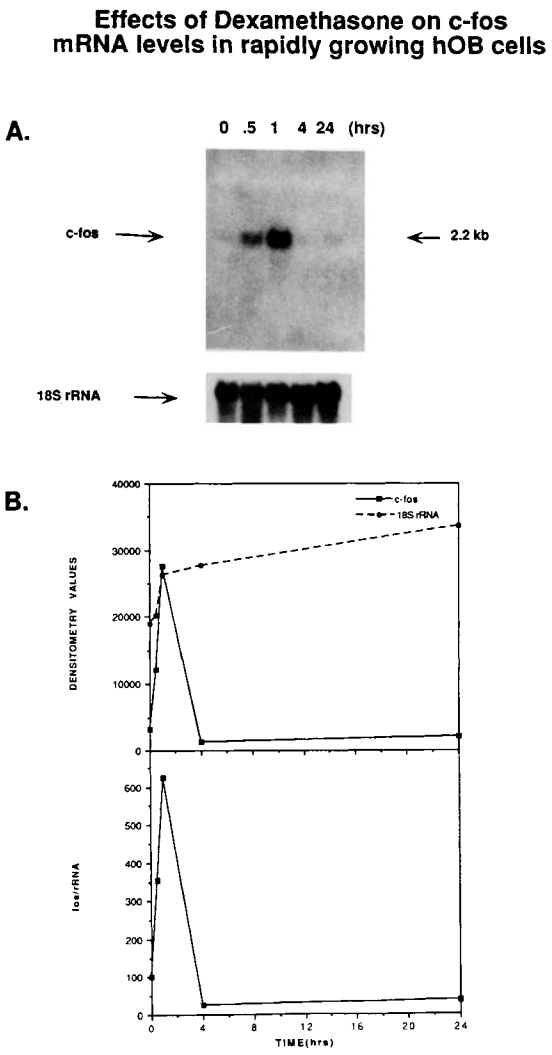


Fig. 8. Northern blot analysis of c-fos mRNA levels in rapidly growing hOB cells. hOB cells grown in media containing 10% FCS were treated with dexamethasone (50 nM) for various time periods. Total RNA was isolated, 12 μg of RNA was loaded to each lane in the gel, and processed for Northern analysis. The blot was probed with [^{32}P]-labelled human c-fos cDNA (**Panel A**). The bottom panel shows the same blot as shown above, striped and reprobed with [^{32}P]-labelled 18S rDNA probe. **Panel B** shows the densitometric values of blots shown in Panel A.

crease in c-fos mRNA levels was also transient with a significant disappearance of the c-fos mRNA after one h post-treatment. The levels of c-fos mRNA levels decrease precipitously to almost undetectable levels by 4 h post-treatment. The same blot probed for the control 18S rRNA to affirm equal loading of RNA per gel is shown in Figure 7B. Again, the effect of Dex on nuclear proto-oncogenes was found not to be unique to quiescent cells. Proliferating hOB cells [grown in 10% (v/v) FCS] treated with Dex showed a similar increase but the response was delayed from 30 min to about one h post-treatment (Fig. 8). Densitometry analysis shows that a 6-fold increase occurs at one h of treatment. The 18S rRNA levels again support an equal loading of the gel.

The effect of 50 nM of Dex on c-jun mRNA levels in quiescent hOB cells was then examined. Figure 9A shows a rapid but transient

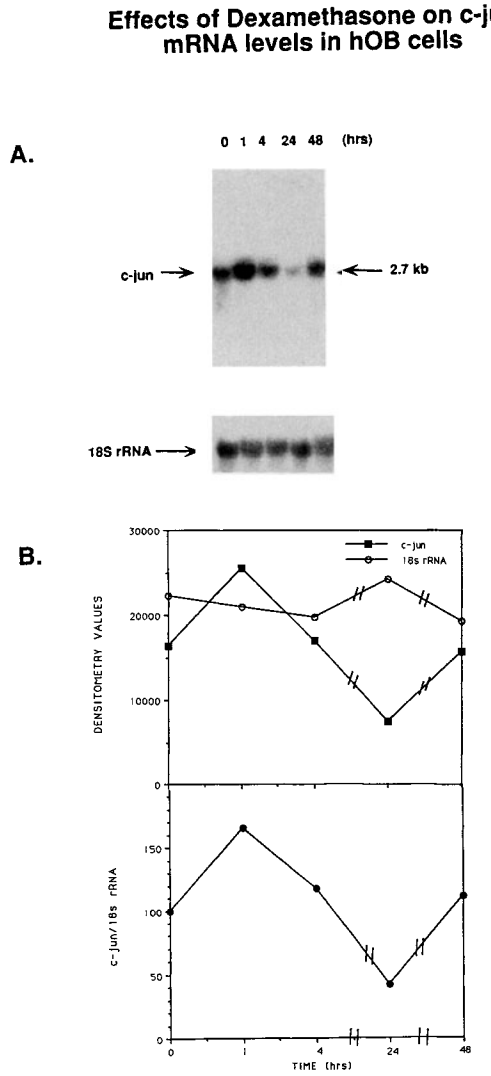


Fig. 9. Northern blot analysis of c-jun mRNA levels in hOB cells after various time periods of dexamethasone treatment. Quiescent hOB cells were treated with dexamethasone (50 nM) for various time periods. Total RNA was isolated, 12 μg of RNA was loaded to each lane in the gel, processed for Northern analysis, and probed with [^{32}P]-labelled human c-jun cDNA probe (**Panel A**). The bottom panel shows the same blot, striped and reprobed with [^{32}P]-labelled 18S rDNA probe. The numbers on top of each lane indicate the time of dexamethasone treatment. **Panel B** shows the densitometric values of blots shown in Panel A.

increase in c-jun mRNA steady state levels as shown above for c-fos and c-myc in RNAs. However, the maximum increase at one h post-Dex treatment was less than twofold. The increase was followed by a decrease to values below controls at 24 h Dex treatment and by a return to control values at 48 h post-Dex treatment. As shown in Figure 9B, the 18S rRNA levels affirm equal amounts of RNA loaded on the gel. In short, these results support a direct action of Dex on the nuclear proto-oncogene expression supporting that the normal hOB cells are target cells for the glucocorticoids.

DISCUSSION

Since the presence of GR has been demonstrated only in transformed human or rodent osteoblast cell lines, comparative analyses in normal human osteoblast cell lines are important to assure the physiologic role of this steroid on human bone physiology. This study identifies the presence of functionally active GR protein and its mRNA in normal hOB cells and further documents Dex regulation of rapidly responding nuclear proto-oncogene and the late responding structural gene expression in these cells, in a steroid dose-dependent, gene specific fashion. These properties are characteristic of a glucocorticoid target cell. These results complement other studies which have shown that GRs are present in fetal rat calvariae and cultured bone cells [Chen and Feldman, 1979; Chen et al., 1977, 1986; Manolagas and Anderson, 1978] and rat [Haussler et al., 1980] and human osteogenic sarcoma cell lines.

Recently, Godschalk et al. [1992] reported that Dex decreases 1,25,D_3 receptor number and mRNA levels in human osteosarcoma (MG-63) cells. Glucocorticoids have been reported to influence the synthesis and secretion of a specific osteoblast product, Osteocalcin (OC), and secretion after 1,25,D_3 treatment has been demonstrated in rat [Chen et al., 1986; Tracy et al., 1987; Price and Baukol, 1980] and human osteoblast-like cell systems [Beresfold et al., 1984]. The 1,25,2D_3 induction of OC gene expression has been observed recently in rat osteosarcoma cells as well [Theofan and Price, 1989]. In previous studies involving normal or transformed human osteoblast-like cells, Gallagher and coworkers reported that glucocorticoids decreased (antagonized) OC secretion after 1,25,D_3 stimulation of these levels [Gallagher et al., 1982; Beresfold et al., 1984]. These reports support the results presented in this paper where the OC protein and mRNA levels in normal hOB cell strains were low or undetectable in the absence of any hormone. The treatment of 1,25,D_3 induced OC protein and mRNA levels in these cells while Dex completely blocked the 1,25-$(OH)_2D_3$ induction. The Dex repression of 1,25,D_3 induced OC gene is known to occur at the OC promoter region between −196 and +34

[Morrison et al., 1989]. Similarly, as demonstrated in the Introduction, Schepmoes et al. [1991] have shown that Dex inhibits the $1,25,D_3$ induced increase in OC gene expression in ROS 17/2-8 cells. This group has shown that the Dex and $1,25,D_3$ regulation of the OC promoter is complex, with variable action of Dex depending on overall promoter activity, as the regulation occurs both transcriptionally and post-transcriptionally with the former involving at least two promoter elements.

The hOB cells are shown in this study to respond directly to Dex by increasing another osteoblast protein, alkaline phosphatase (AP), activity and steady state levels of the mRNA for AP. These results also support studies from other laboratories wherein the glucocorticoid stimulated the AP enzyme activity in rat [Gallagher et al., 1982; Manolagas et al., 1981; Majeska et al., 1985] and human osteosarcoma cell lines [Majeska et al., 1985]. Interestingly, Dex treatment decreased AP activity in primary cultures of rat osteoblast-like cells in a dose-dependent manner [Chen and Feldman, 1979], while in the studies reported in this paper the opposite responses to Dex are presented. These contrasting data demonstrate that glucocorticoideffects vary in different cell cultures, animal species systems, or under different experimental conditions.

The secosteroid hormone, Vitamin D metabolite, $1,25,D_3$ was also shown to increase AP activity and AP mRNA steady state levels in cultured hOB cells. Increases in AP activity in response to $1,25,D_3$ and to Dex treatments have been reported in rat [Manolagas et al., 1981] and human osteosarcoma cell lines [Mulkins et al., 1983] and in a normal human osteoblast-like cell (hOB) culture system [Gallagher et al., 1982]. The latter group also found that Dex and $1,25,D_3$ treatment acted synergistically to increase AP activity in their hOB cells, which supports the results of this paper.

Interestingly, the results presented in this paper also demonstrate that glucocorticoids rapidly but transiently increase the expression of the c-myc, c-fos, and c-jun proto-oncogenes to significant levels in human osteoblasts in vitro. Glucocorticoids also have a dramatic effect on proto-oncogenes in other cell types [Eastman-Reks and Vedeckis, 1986]. This rapid, transient increase of these nuclear proto-oncogenes by Dex in hOB cells resembles the significant Dex-induced increases in c-myc mRNA levels in avian liver but is opposite to that found in reproductive and other tissues [Rories et al., 1989]. Treatment of mouse lymphoma cell line with glucocorticoids brings about a decrease in c-myc, c-myb, and c-ki-ras mRNA steady state levels [Eastman-Reks and Vedeckis, 1986]. This decrease has been attributed to the mechanism by which glucocorticoids inhibit cell cycle progression in lymphoid cell lines. In avian oviduct systems, the glucocorticoid has been shown to rapidly inhibit c-myc mRNA levels within 30 min of administration [Rories et al., 1989]. In contrast to the glucocorticoid induced decrease in proto-oncogene expression in many target tissues, this paper reports an activation of the nuclear proto-oncogene expression in bone cells.

The exact biological role of the nuclear proto-oncogenes c-myc, c-fos, and c-jun in hOB cells is not known. Glucocorticoid excess in humans resulting from either pharmacologic or pathologic processes leads to profound and often deleterious changes in the metabolism of bone [Tudfud et al., 1988; Peck, 1982]. The net result of chronic glucocorticoid excess in humans is significant bone loss. This is most likely due to long-term interactions with other bone regulators. The negative impact of dexamethasone on $1,25\text{-}(OH)_2D_3$ stimulation is clearly seen even in this in vitro system. Recent studies by Candeliere et al. [1991] have shown evidence that calcitriol ($1,25,D_3$) treatment of osteoblastic cell line (MC3T3-E1) and primary cultures of osteoblasts can transiently induce Fos and Jun families of proto-oncogenes. It is known that the Jun and Fos oncoproteins complex into a heterodimer to form the AP-1 transcription factor complex [Ransone and Verma, 1990; Vogt and Bos, 1990]. This heterodimer binds the AP-1 DNA element with high affinity resulting in the regulation of transcription of genes including the gene coding for the TGF-β [Kim et al., 1990], which is thought to play a major role in bone cell activities [Bonewald and Mundy, 1990]. Therefore, the glucocorticoid regulation of the c-jun and c-fos gene expression may have the important function of regulating the expression of the TGF-β and the other growth factor gene(s).

Recent findings have shown that a glucocorticoid response element (GRE), a 25 base pair element that resides upstream of the mouse proliferin promoter, is sufficient for GR and AP-1 factors to bind and is also responsible for positive and negative regulation by glucocorticoids [Diamond et al., 1990]. Recent studies by Jonat et al. [1990] and Yang-Yen et al. [1990]

have shown that glucocorticoid hormones inhibit the basal and induced transcription of collagenase gene by interfering with the DNA binding of the AP-1 transcription factor complex, the major enhancer factor of the collagenase promoter. Interestingly, studies by Schüle et al. [1990] have shown evidence that the glucocorticoid receptor and transcription factor jun/AP-1 can reciprocally repress one another's transcriptional activation, i.e., c-jun represses GR-mediated induction of gene expression and conversely the GR can repress the c-jun expression.

Although the mechanism by which steroid hormones stimulate or inhibit specific gene transcription in any cell is only lately becoming clear, it is obvious that a specific hormone can effect the expression of various genes in cells in different ways. The rapidity of the observed regulation of Dex on the nuclear proto-oncogene expression in hOB cells indicates that glucocorticoid action on these genes is direct. These nuclear proto-oncogenes can serve not only as excellent marker genes for steroid action in bone cells, but also as candidates for regulatory genes in the cascade model of steroid action [Landers and Spelsberg, 1992].

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