Endocrine, *vol. 12, no. 1, 25–33, February 2000* 0969–711X/00/12/1:25–33/$12.25

Regulation of PTH/PTH-Related Protein Receptor Expression by Endogenous PTH-Related Protein in the Rat Osteosarcoma Cell Line ROS 17/2.8

Pingfeng Du, Patricia K. Seitz, and Cary W. Cooper

Department of Pharmacology and Toxicology, University of Texas Medical Branch, Galveston, TX

We have utilized clonal lines of the rat osteoblastic cell line ROS 17/2.8 stably transfected with full-length parathyroid hormone–related protein (PTHrP) cDNA in a sense or an antisense orientation to examine the effects of alteration in the production of endogenous PTHrP on expression of the PTH/PTHrP receptor. In the stably transfected clonal cell lines, changes in PTH/PTHrP receptor expression were evaluated by Northern blot analysis, whole-cell ligand binding of ^{125}I-[Tyr^{36}] PTHrP (1–36), and exogenous PTHrP (1–34)-stimulated cyclic adenosine monophosphate (cAMP) accumulation. Compared to control (vector-transfected) cells, PTHP-overproducing (sense-transfected) cells exhibited a marked decrease in the expression of PTH/PTHrP receptor mRNA and PTHrP ligand binding, as well as a corresponding decrease in the PTHrP (1–34)-stimulated cAMP response. By contrast, the antisense-transfected cells showed a marked increase in expression of PTH/PTHrP receptor mRNA and PTHrP (1–34) ligand binding, but a significant increase in the PTHrP (1–34)-stimulated cAMP response was not detected. Using antisense-transfected ROS cells, PTH/PTHrP receptor mRNA expression and ^{125}I-[Tyr^{36}] PTHrP (1–36) binding were downregulated by treatment for 24 h with exogenous PTHrP (1–36), forskolin, or dibutyryl cAMP. The findings extend those of earlier studies showing receptor downregulation by exogenous PTH by indicating that endogenous PTHrP, as well as circulating PTH, may help regulate receptor production; and suggesting that even very low concentrations of the peptide may influence receptor production.

Key Words: Parathyroid hormone; PTH-related protein; PTH/PTHrP receptor; PTH1 receptor; osteoblast; ROS cell.

Received September 29, 1999; Revised November 23, 1999; Accepted November 23, 1999.

Author to whom all correspondence and reprint requests should be addressed: Dr. Cary W. Cooper, Office of the Dean, 5.103 Administration Building, Graduate School of Biomedical Sciences, University of Texas Medical Branch, Galveston, TX 77555-0132. E-mail: ccooper@utmb.edu

Introduction

Parathyroid hormone (PTH)–related protein (PTHrP) has high N-terminal amino acid sequence homology with PTH *(1)*. This and other overall structural similarity in the N-terminal region enable both PTH and PTHrP to bind to the same G-protein-linked, plasma membrane receptor *(1,2)*. Activation of these PTH/PTHrP (PTH1) receptors in bone and kidney by PTHrP accounts for the classical PTH-like activity originally described in patients with humoral hypercalcemia of malignancy *(3–6)*. However, unlike PTH, PTHrP is widely expressed in almost all mammalian tissues *(7,8)*. Because the PTH/PTHrP receptor also is widely distributed in these tissues, usually in close proximity to PTHrP, the peptide is thought to function locally in an autocrine/paracrine manner to exert many of its actions *(1,6)*. The cDNA for the PTH/PTHrP receptor has been cloned from rat and human osteosarcoma cell lines, as well as from kidney cells *(9)*. The receptor belongs to the G-protein-coupled receptor superfamily, has seven transmembrane domains, and is linked intracellularly to both the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and Ca^{2+}/protein kinase C second-messenger systems *(10,11)*. Osteoblasts express both PTHrP and the PTH/PTHrP receptor *(12,13)*, and both PTH and PTHrP can regulate the expression of major osteoblast proteins, including Type I collagen, alkaline phosphatase, osteopontin, and osteocalcin *(14–16)*. Also, a critical role for PTHrP during skeletal development has been clearly documented by studies in PTHrP knockout mice; PTH/PTHrP receptor knockout mice; and collagen II promoter-driven, PTHrP-overexpressing mice. All these mice show severe, even lethal, developmental defects associated with abnormal fetal bone development *(17–19)*. These findings clearly show that PTHrP and its cognate receptor play a central role in skeletal development, especially in the regulation of chondrocyte maturation and skeletal mineralization.

Numerous earlier studies have demonstrated desensitization of target tissue responses to PTH on prolonged exposure to high doses of exogenous peptide *(20–23)*. Also, previous studies have shown that the PTH/PTHrP receptor

is downregulated in rats with chronic renal failure and secondary hyperparathyroidism *(24)*. To investigate whether locally produced PTHrP also might help regulate expression of the PTH/PTHrP receptor in osteoblastic cells, we transfected ROS 17/2.8 cells with PTHrP cDNA in a sense or an antisense orientation in order to alter endogenous peptide production, and then we examined PTHrP receptor expression by these same cells.

Results

PTHrP mRNA Expression, Secretion of PTHrP, and Selection of Clones

Northern blot analysis revealed that both parental ROS cells and the control, empty vector-transfected ROS cells expressed only low, barely detectable levels of normal 1.4-kb sense PTHrP mRNA (data not shown). By contrast, Fig. 1 shows that the clonal cell lines selected for study were successfully transfected and expressed the appropriate 1.1-kb sense or antisense mRNA transcript. Figure 1A illustrates that the two sense PTHrP cDNA-transfected clones studied (C5 and C10) expressed relatively high amounts of 1.1-kb sense PTHrP mRNA whereas essentially no sense mRNA was observed in antisense cDNA-transfected clones. Alternatively, 1.1-kb antisense PTHrP mRNA was present in large quantities in the ROS cells transfected with antisense PTHrP cDNA (Fig. 1B) but not in the vector control cells or sense cDNA-transfected ROS cells.

Immunoreactive PTHrP secreted into the culture medium over a 48-h period by clones of stably transfected ROS cells was measured and normalized to cell number. Compared to ROS cells transfected with empty vector, the two clones of ROS cells transfected with PTHrP cDNA in a sense orientation repeatedly showed a highly significant ($p < 0.001$) and markedly increased production of immunoreactive PTHrP (30–50 fmol/10^6 cells) compared to the control clone transfected with vector alone (~3 fmol/10^6 cells). There was no detectable difference in PTHrP production between the clone transfected with antisense PTHrP cDNA (~4 fmol/10^6 cells) selected for study and the vector-transfected control clone. However, since basal concentrations of PTHrP in culture medium from both the vector control (0.36 ± 0.15 p*M*; $n = 3$) and antisense-transfected ROS cells (0.52 ± 0.10 p*M*; $n = 3$) already were near the lower limit of sensitivity of the PTHrP assay, a subtle decrease in PTHrP production from antisense-transfected cells might well go undetected. By contrast, PTHrP concentrations in media from the sense-transfected clones, C5 and C10, were relatively high (2.73 ± 0.31 and 1.45 ± 0.14 p*M*, respectively; $n = 3$) and easily distinguished from the vector control clone ($p < 0.001$).

Expression of Receptor mRNA

Figure 2 shows the expression of PTH/PTHrP receptor mRNA in the clonal lines of stably transfected ROS cells selected for study. PTHrP receptor mRNA was analyzed by densitometry and normalized to cyclophilin mRNA. Compared to cells transfected with vector alone, the two clones transfected with sense PTHrP cDNA showed an approx

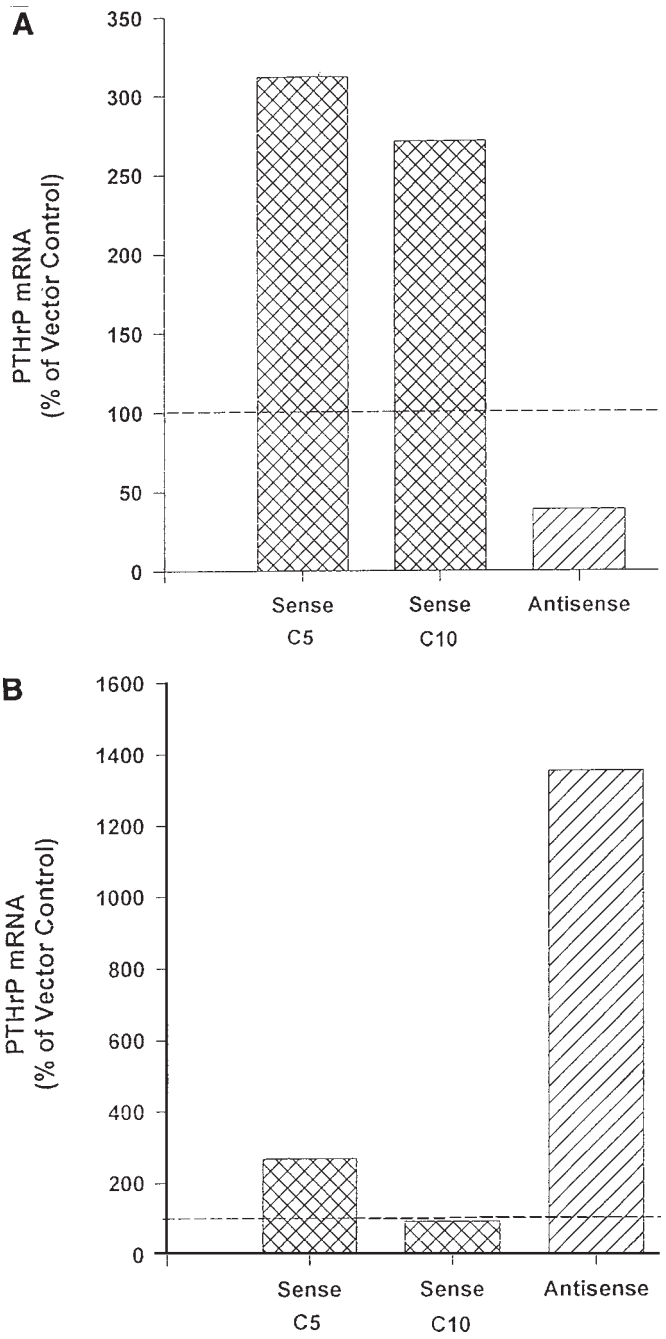

Fig. 1. Northern analysis of 1.1-kb PTHrP mRNA in clonal lines of ROS cells stably transfected with PTHrP cDNA in a sense or an antisense orientation. Control cells were stably transfected with empty vector. Sense PTHrP mRNA **(A)** and antisense PTHrP mRNA **(B)** were detected using probes specific for each, respectively (*see* Materials and Methods). The PTHrP mRNA was quantified densitometrically and normalized to similarly quantified cyclophilin mRNA. Values for each clone then were compared by establishing the vector control as 100%.

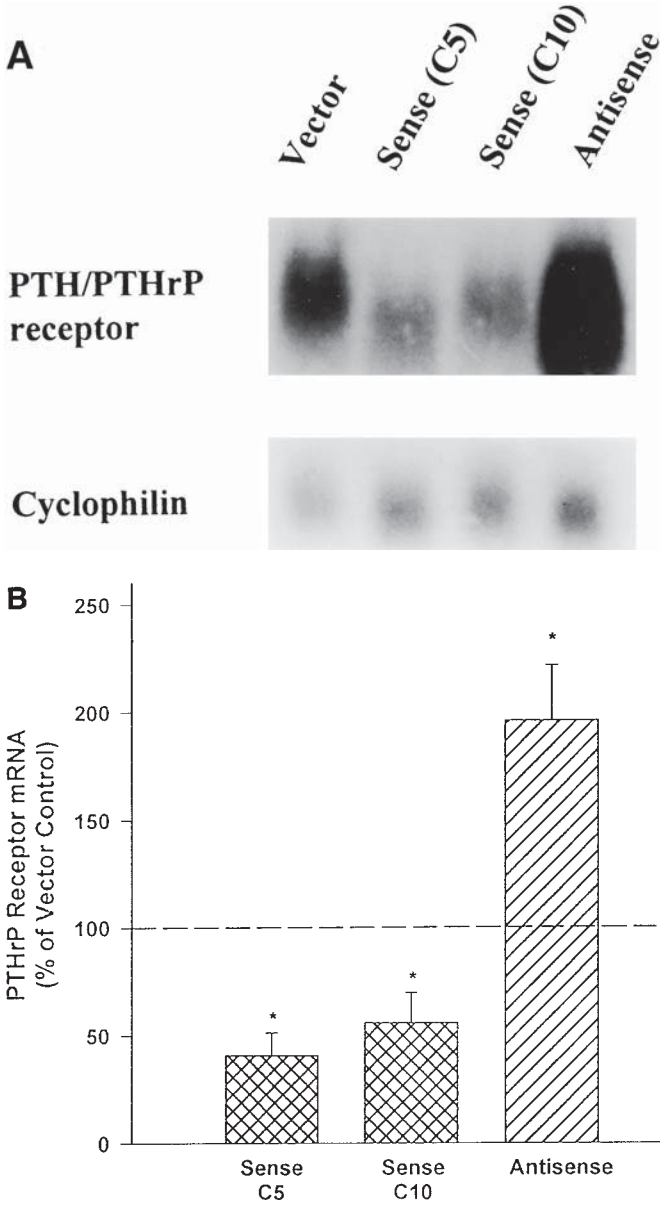

Fig. 2. PTH/PTHrP receptor mRNA in clones of stably transfected ROS cells. **(A)** Representative Northern blots showing mRNA from clonal cells stably transfected with PTHrP cDNA in a sense (C5 and C10) or an antisense orientation. Control cells were stably transfected with vector alone. **(B)** mRNA was normalized to cyclophilin mRNA and quantified by densitometry. The ratio of PTHrP receptor mRNA relative to cyclophilin mRNA in vector-transfected control cells was established as 100%, and the results are presented as mean ± SEM ($n = 4$). $^{*}p < 0.05$ vs vector control.

50% decrease in receptor mRNA expression, whereas the clone transfected with antisense PTHrP cDNA showed about a twofold increase in receptor mRNA.

Receptor Binding

Expression of functional PTHrP membrane receptors in the clonal ROS cells was assessed by examining whole-cell binding of ^{125}I[Tyr^{36}]-PTHrP (1–36). Figure 3 indicates

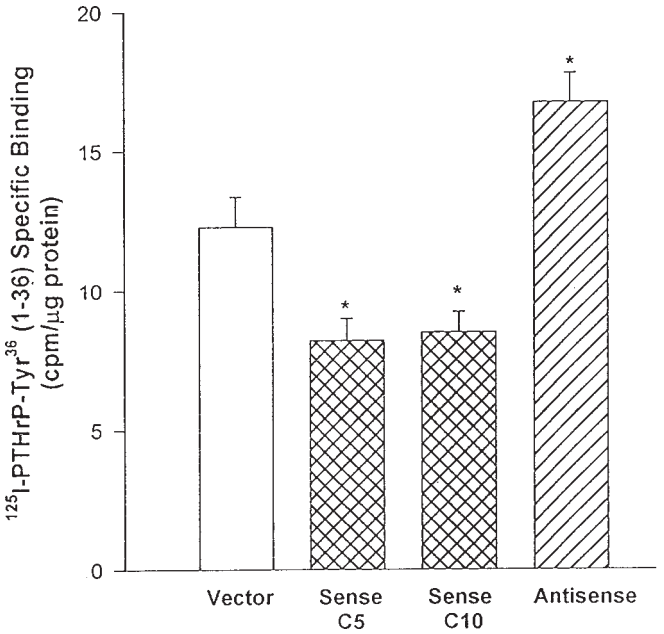

Fig. 3. PTH/PTHrP receptor binding in clones of stably transfected ROS cells. Confluent cells were incubated with ^{125}I-PTHrP (1–36) at room temperature for 2 h. Nonspecific binding was determined in the presence of unlabeled 10^{-6} M PTHrP (1–34). Results were normalized to protein content and are presented as mean ± SEM ($n = 6$). $^{*}p < 0.05$ compared to vector-transfected control cells.

that ROS cell clones overexpressing PTHrP showed significantly decreased specific binding of radiolabeled PTHrP (1–36) by ~30% compared to cells transfected with vector alone, whereas antisense-transfected cells showed significantly increased binding (~45%) compared to vector control cells.

cAMP Response

The PTH/PTHrP receptor is known to be linked primarily to the cAMP/PKA second messenger system in osteoblasts. Therefore, theoretically, cAMP production stimulated by PTH/PTHrP should reflect the functional state of these receptors. Figure 4 shows the changes in cAMP that occurred in response to acute treatment with exogenous 10^{-7} *M* PTHrP (1–34) compared to basal cAMP in three of the clones of stably transfected ROS cells. The basal cAMP levels did not differ significantly among clones of transfected cells. However, the marked ~17-fold increase in cAMP in response to added PTHrP (1–34) evident in the vector-transfected clonal line was reduced by >90% in the PTHrP-overexpressing transfected cells. The antisense-transfected cells showed a larger mean increase than vector controls, but the difference did not reach statistical significance, and this finding was borne out in additional experiments conducted using doses of PTHrP (1–34) ranging from 10^{-9} to 10^{-7}*M* (data not shown).

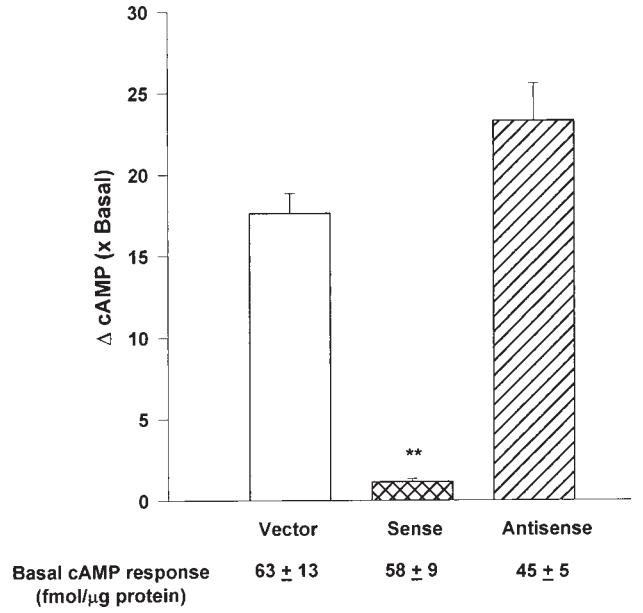

Fig. 4. Acute cAMP response of stably transfected ROS cell clones (sense clone = C5) to 10^{-7} *M* PTHrP (1–34). Accumulation of cAMP in ROS cells was measured 15 min after addition of vehicle (basal) or 10^{-7} *M* PTHrP (1–34) in the presence of 1 m*M* 3-isobutyl-1-methylxanthine (IBMX). Each bar represents the fold increase in cAMP above the respective basal level after PTHrP treatment. Values are mean ± SEM (n = 4). $^{**}p < 0.01$ compared to that of vector control cells.

Receptor mRNA and Receptor Binding in Antisense-Transfected Cells Treated with Exogenous PTHrP (1–34), Forskolin, or Dibutyryl cAMP

To examine potential mechanisms involved in the PTHrP receptor downregulation by endogenous PTHrP, the antisense-transfected clonal line was treated with exogenous PTHrP (1–34), dibutyryl cAMP, or forskolin. Figure 5 shows that a 1-d exposure to 10^{-8} M PTHrP (1–34) reduced the enhanced expression of receptor mRNA by antisense-transfected cells by about 60%. Treatment with either 10^{-4} or 10^{-5} *M* dibutyryl cAMP or 10^{-6} *M* forskolin for 1 d also decreased receptor mRNA expression by about 30–50% (Fig. 6).

Figure 7 shows specific binding of ^{125}I-PTHrP (1–36) in antisense- transfected cells treated with exogenous PTHrP (1–34), dibutyryl cAMP, or forskolin for 1 d. Specific binding of the PTHrP ligand was reduced in a dose-dependent manner by PTHrP (1–34) treatment; the reduction was ~82% at the higher dose. Binding also was significantly reduced by treatment with forskolin and dibutyryl cAMP. Interestingly, however, the reduction was only ~20% in response to 10^{-4} *M* dibutyryl cAMP and ~40% in response to 10^{-6} *M* forskolin.

Discussion

Numerous studies have reported previously that prolonged exposure of bone and kidney target cells to high

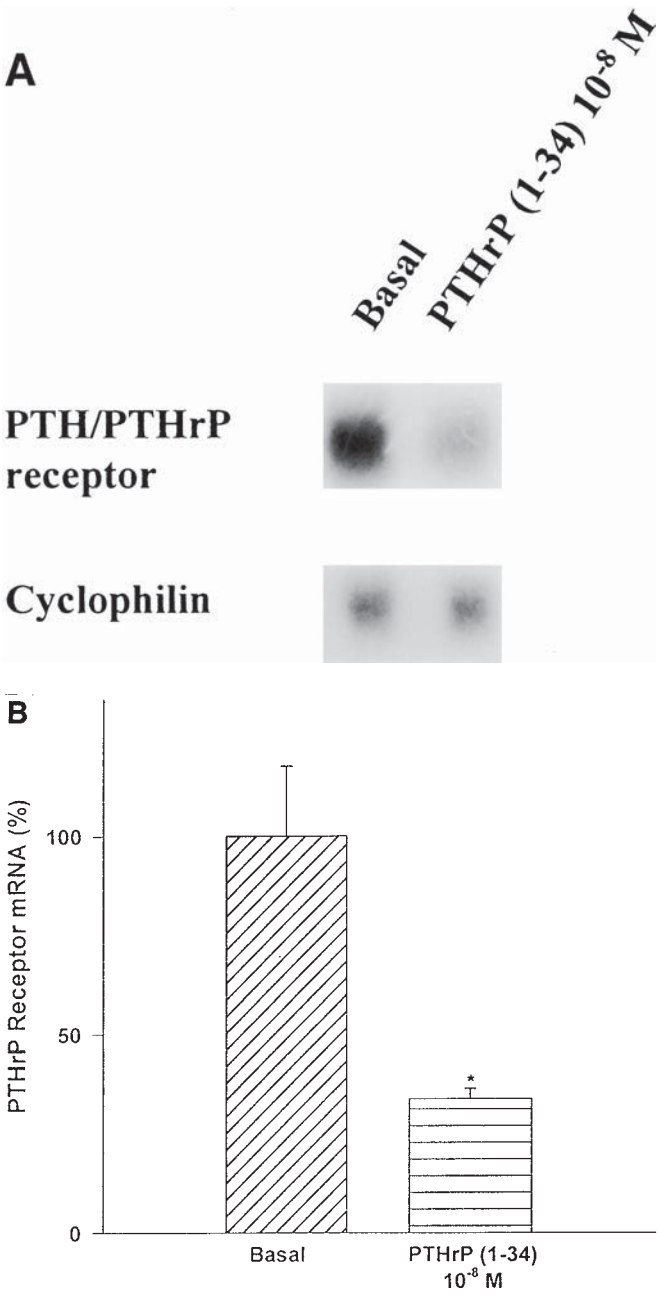

Fig. 5. PTH/PTHrP receptor mRNA in ROS cells stably transfected with antisense PTHrP cDNA and treated with 10^{-8} *M* PTHrP (1–34) for 24 h. **(A)** Representative Northern blot showing PTH/PTHrP receptor mRNA and cyclophilin mRNA. **(B)** PTHrP receptor mRNA was normalized to cyclophilin mRNA and quantified by densitometry. The ratio of PTHrP receptor mRNA relative to cyclophilin mRNA in vehicle-treated cells was established as 100%. Results are presented as mean ± SEM (n = 3). $^{*}p < 0.05$.

levels of PTH results in a decreased response (downregulation/desensitization) to subsequent acute challenge with the hormone *(19–25)*. The mechanisms involved in the reduced response to PTH likely involve alterations at the level of the PTH/PTHrP receptor, its second messengers, or its effector systems, both PKA and PKC *(6,18,26–28)*. Because PTHrP is coexpressed with the PTH/PTHrP receptor in osteoblasts,

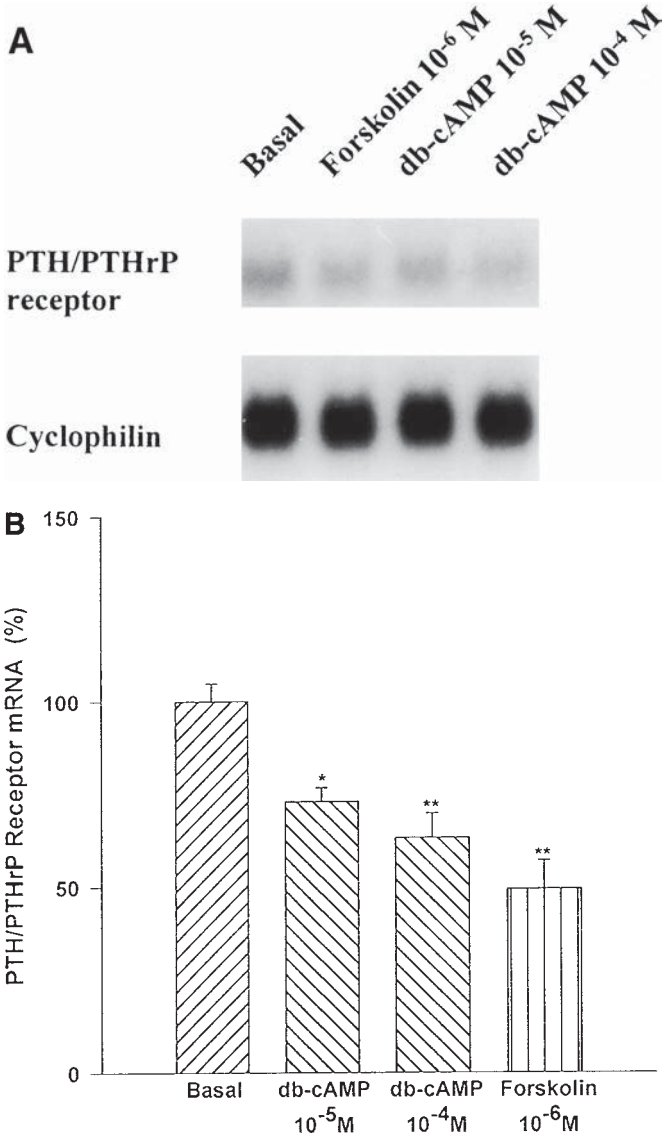

Fig. 6. PTH/PTHrP receptor mRNA in ROS cells stably transfected with antisense PTHrP cDNA and treated with forskolin or dibutyryl cAMP (db-CAMP) for 24 h. **(A)** Representative Northern blot showing PTH/PTHrP receptor mRNA and cyclophilin mRNA. **(B)** PTHrP receptor mRNA was normalized to cyclophilin mRNA and quantified by densitometry. The ratio of PTHrP receptor mRNA relative to cyclophilin mRNA in vehicle-treated control cells was established as 100%. Results are represented as mean ± SEM $n = 3$). $^{*}p < 0.05$; $^{**}p < 0.01$ vs basal.

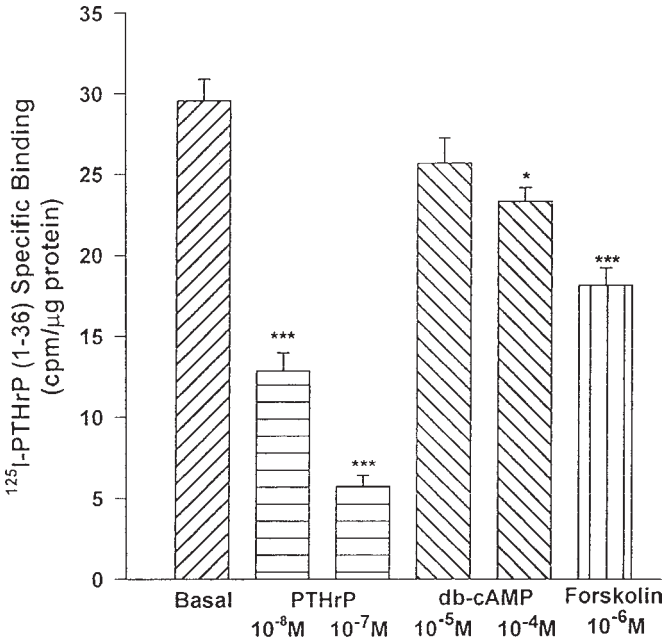

Fig. 7. PTHrP receptor binding in ROS cells stably transfected with antisense PTHrP cDNA and treated with exogenous PTHrP (1–34), forskolin, or dibutyryl cAMP (db-CAMP). Cells were treated for 24 h with PTHrP (1–34), forskolin, or dibutyryl cAMP at various concentrations, and whole-cell ligand binding was measured (*see* Fig. 2). Results were normalized to protein content and presented as mean ± SEM ($n = 6$). $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with vehicle-treated control cells (basal).

we hypothesized that, in addition to circulating PTH, endogenous PTHrP might act in an autocrine/paracrine, or even intracrine, manner to help regulate receptor expression by these bone cells.

The present results clearly demonstrate that endogenously produced PTHrP, secreted into the culture medium by the ROS osteoblastic cells, can alter PTH/PTHrP receptor expression in the same cells. Enhanced production of PTHrP in sense-transfected clones decreased receptor mRNA, receptor binding, and receptor-mediated cAMP production in response to treatment with exogenous PTHrP (1–34). These findings mirror those reported earlier by other investigators using exogenous treatment with nanomolar–micromolar concentrations of PTH or PTHrP *(23)*. However, our present results extend earlier findings by showing that endogenously produced PTHrP can downregulate the receptor and suggesting that picomolar concentrations of peptide may effectively regulate receptor expression. This is borne out by the fact that the clones transfected with sense PTHrP cDNA showed levels of PTHrP in medium that were ~1.5–3.0 p*M* vs ~0.4 p*M* in medium from the vector-transfected control cells. However, since the immunoassay employed here detects either full-length PTHrP or PTHrP fragments longer than 74 residues, our studies do not rule out the potential influence of N-terminal PTHrP fragments (e.g., 1–34) that would go undetected by the Nichols immunoradiometric assay. Additional studies using high-performance liquid chromatography (HPLC) separation of medium and antisera to PTHrP (1–34) will be needed to more fully address this issue.

One of the most interesting findings to emerge from the present study was that receptor mRNA levels and binding of radiolabeled N-terminal PTHrP (1–36) were increased in antisense-transfected ROS cells compared to vector-transfected controls. The simplest explanation for this effect is that PTHrP production was reduced in the antisense-transfected cells. If so, this finding further emphasizes the potential ability of very low levels of PTH or PTHrP to suppress

receptor expression and implies an inverse relationship between extracellular peptide concentration and receptor production. However, we could not detect lower levels of PTHrP in culture medium from antisense-transfected cells compared to medium from the vector control cells. Therefore, we cannot be sure whether relative assay insensitivity at these low levels hampers such detection, nor, for reasons already mentioned, can we rule out the involvement of undetected N-terminal PTHrP fragments in the observed effects.

Regardless of the mechanisms involved, the findings clearly demonstrate an upregulation of PTH/PTHrP receptors in ROS cells stably transfected with antisense PTHrP cDNA. Interestingly, the upregulation of receptor mRNA and ligand binding was not accompanied by a demonstrable increase in the acute cAMP response to exogenous PTHrP (1–34). The reason for this is unclear. It is possible that "spare" receptors, unable, or insufficiently mature, to couple to G-protein and activate second messengers are involved. In theory, receptor expression in the vector control ROS cells already could be sufficiently high to mediate a maximal cAMP response to a maximally effective dose of PTH (1–34). In this case, however, we might have expected to see a higher basal level of cAMP in the antisense-transfected cells and to elicit a greater cAMP response to submaximal ($<10^{-7}M$) doses of PTHrP (1–34). However, neither was observed. Therefore, the upregulation of PTHrP receptor mRNA and binding sites observed in antisense-transfected ROS cells may involve so-called spare receptors. Resolution of these possibilities will require additional studies.

Theoretically, receptor activity can be regulated at the transcriptional, translational, or posttranslational levels. Previous work has shown that dexamethasone can upregulate PTH/PTHrP receptor mRNA in ROS cells and that the enhanced effect of dexamethasone is owing to increased transcription *(29)*. In the present study, the increase in receptor mRNA observed in antisense-transfected cells is consistent with either increased transcription or an increase in mRNA stability.

Previous studies have implicated both the PKA and PKC second-messenger systems in the downregulation of the PTH/PTHrP receptor by PTH in vivo and in vitro *(20–22,26–28,30)*. Our findings that dibutyryl cAMP, a cAMP analogue, and forskolin, an adenylate cyclase agonist, can mimic the effect of exogenous PTHrP in the downregulation of PTHrP receptor mRNA and radioligand binding support the notion that the cAMP/PKA signaling pathway plays a prominent role in the ability of PTH or PTHrP to regulate receptor expression in these clonal lines of ROS cells.

Interestingly, although forskolin and dibutyryl cAMP mimicked the general effect of exogenous PTHrP in downregulating PTH/PTHrP receptor mRNA and ligand binding, they did not produce downregulation to the same degree as a high dose (10^{-8} *M*) of PTHrP (1–34) (25% of basal receptor level). Dibuturyl cAMP is notoriously poor at penetrating the cell membrane, and it is possible that a higher dose, e.g. 10^{-3} *M*, would have elicited a greater response. In our hands, 1 μM forskolin, which activates adenylate cyclase directly and therefore bypasses the receptor, typically evokes an increase in cAMP in ROS cells equal to or greater than achieved with a maximal dose of PTH (1–34) or PTHrP (1–34). However, we did not measure cAMP in the experiment shown in Fig. 7, so it remains possible that equivalent cAMP responses were not achieved in these experiments. If forskolin, as expected, produced a cAMP response equal or greater in magnitude to that of PTHrP in the experiment shown in Fig. 7, the results could imply that the cAMP/PKA cascade may not be the only second-messenger system through which the endogenous PTHrP regulates its cognate receptor in osteoblasts. Since PTHrP can also activate phosphoinositol turnover and is linked to the PKC system, and since Guo et al. *(31)* have reported downregulation of this receptor by a PKC agonist in the kidney cell line LLC-PK1, our studies do not exclude participation of the PKC system in the regulation of PTH/PTHrP receptor expression.

The results with dibutyryl cAMP and forskolin also suggest that heterologous downregulation of the PTH/PTHrP receptor may occur via activation of the cAMP/PKA second-messenger system. In bone, osteoblast function is under the control of a number of systemic hormones and local factors *(32)*. Among them, prostaglandin E (PGE) is secreted by osteoblasts and can affect osteoblast function, e.g., by stimulating collagen and noncollagen protein synthesis. Therefore, PGE and other agents that stimulate cAMP in bone cells also might activate the cAMP/PKA system and thus downregulate PTH/PTHrP receptor expression. This would be in concert with previous findings that PGE can regulate PTH/PTHrP receptor activity in an osteoblastic cell line *(22)*.

Whether PTHrP production by osteoblasts normally plays a role in osteoblast differentiation and receptor expression remains to be clarified. McCauley et al. *(33)*, using the nontransformed osteoblast cell line MC3T3-E1, which differentiates in a predictable way experimentally under defined culture conditions, found that PTH/PTHrP receptor expression was dependent temporally on the extent of cell differentiation. Maximal expression required several days of differentiation induced by ascorbic acid. Because PTHrP can induce cell proliferation and concurrently suppress differentiation in our clonal ROS cells that overexpress PTHrP *(34,35)*, it would be interesting to know whether the differentiating MC3T3 cells of McCauley et al. *(33)* upregulated PTH/PTHrP receptor expression in response to a decrease in PTHrP production by the cells, which might occur as they cease proliferation and become differentiated.

In conclusion, our present studies in stably transfected ROS cells clearly demonstrate that alterations in endogenously secreted PTHrP can regulate the level of PTH/PTHrP receptor expression by acting on PTH/PTHrP membrane receptors and activating the intracellular cAMP/PKA second-messenger

system. The results provide evidence to support the conclusion that both up- and downregulation of receptor expression occurs, at least in part, by altered cellular production of receptors. The findings imply that endogenous, locally acting PTHrP, as well as the classical circulating hormone PTH, may play an important, receptor-mediated role in regulating osteoblast function.

Materials and Methods

Cell Culture

ROS 17/2.8 cells were cultured in Coon's high-zinc modification of F-12 medium (Irvine, Santa Ana, CA) containing 10% NuSerum (Collaborative Research, Bedford, MA) and 0.3 mg/mL of L-glutamine. Cultures were maintained at 37°C in an atmosphere of humidified air and 5% CO_2, and medium was changed three times a week. After selection of stably transfected clones for study, the clonal cell lines were cultured in the same medium containing 100 μg/mL of G418 (Gibco BRL, Gaithersburg, MD).

Transfection

The cDNA encoding human PTHrP –5-139 (provided by Dr. William Wood, Genentech, South San Francisco, CA) was subcloned in a sense or an antisense orientation into the vector pcDNA 3.1(+) or pcDNA 3.1(–), respectively (Invitrogen, San Diego, CA), according to the manufacturer's directions. The PTHrP cDNA plasmid constructs were transfected into ROS 17/2.8 cells by calcium phosphate coprecipitation using a kit from Promega (Madison, WI). Transfected cells were selected in medium containing 300 μg/mL of G418, and single clones of stably transfected cells were isolated in a standard fashion by limiting dilution.

Northern Blot Analysis

General Procedures

Total cellular RNA was isolated from near confluent cells with RNA STAT-60™ (Tel-Test "B", The Woodlands, TX) according to the protocol provided by the manufacturer. Isolated RNA was dissolved in diethylpyrocarbonate-treated H_2O, and the concentration of RNA was determined by absorbance at 260 nm. For mRNA detection, 20 μg of total RNA was electrophoresed on a formaldehyde-1.2% agarose gel, transferred to a Nytran nylon membrane (Schleicher & Schuell, Keene, NH), and crosslinked using ultraviolet light. After hybridization with radiolabeled probe, blots were autoradiographed at –80°C using Kodak X-OMAT 5 X-ray film (Eastman Kodak, Rochester, NY). Assessment of loading of total RNA was achieved by reprobing the blots with a 380-bp cDNA probe for cyclophilin prepared by random primer labeling using an Amersham Multiprime kit (Amersham, Arlington Heights, IL). Blots were quantified by densitometry using standard methods (Scion Image; Scion, Frederick MD).

Detection of Sense and Antisense PTHrP mRNA

Single-stranded DNA probes for sense and antisense PTHrP mRNA were synthesized from a 231-bp cDNA *(36)* by asymmetric polymerase chain reaction (PCR) using ^{32}P-dCTP *(37)*. To detect antisense mRNA, the upstream primer 5'-CTGGTTCAGCAGTGGAGCGTC was used; to detect sense mRNA, the downstream primer 5'-GTTAGGGGACACCTCCGAGGT was used. Total reaction volume was 50 ml and contained 100–200 ng of DNA, 0.4 μg of primer, 0.14 mCi of ^{32}P-dCTP (Amersham), and 10U of *Taq* polymerase (Gibco, Grand Island, NY). DNA was denatured at 95°C for 30 s, reannealed at 58°C for 30 s, and extended at 72°C for 2 min using 35 cycles in a Perkin-Elmer (Norwalk, CT) GeneAmp 9600. Probes were purified using G-50 minispin columns (Worthington, Lakewood, NJ). Northern analysis was performed in ExpressHyb hybridization solution (Clontech, Palo Alto, CA) at 68°C for 2h, with approx 10^6 cpm/mL of denatured probe. Then membranes were rinsed two times for 20 min each in 2X saline sodium citrate (SSC) (1X = 150 m*M* sodium chloride, 15 m*M* sodium citrate, pH 7.0) and 0.05% sodium dodecyl sulfate (SDS) at room temperature, two times for 30 min each in 0.1XSSC and 0.1% SDS at 65°C, and then autoradiographed for 1–5 d.

Detection of PTH/PTHrP Receptor mRNA

A 483-bp PTH/PTHrP receptor cDNA was amplified by asymmetric PCR using ^{32}P-dCTP to prepare the radioactive probe *(36)*. For analysis, membranes were prehybridized at 42°C for 3 h in a solution of 50% formamide, 6X SSC, 5X Denhardt's solution (50X = 0.1% polyvinypyrrolidone, 0.1% Ficoll, and 0.1% bovine serum albumin [BSA]), 0.1% SDS, and 100 μg/mL of denatured salmon sperm DNA. Hybridization was conducted overnight at 42°C. Filter membranes were washed twice in 2X SSC and 0.1% SDS at 65°C for 30 min, once in 0.2% SSC and 0.1% SDS at 65°C, and then autoradiographed for 7–10 d.

PTHrP Immunoreactivity

Conditioned medium from cultured ROS cells was assayed for PTHrP as previously described *(38,39)* using an immunoradiometric PTHrP assay kit purchased from Nichols Institute (San Juan Capistrano, CA). The standard provided was human PTHrP (1–86). Immunoreactivity was measured in samples of culture medium that had been concentrated fivefold as previously described *(38)*.

Receptor Ligand Binding

[Tyr^{36}]-hPTHrP (1–36) was radiolabeled with ^{125}I using test tubes precoated with Iodogen (Pierce, Rockford, IL) and following the manufacturer's instructions. Labeled peptide was purified for use by reverse-phase HPLC chromatography as described previously *(40)*. Radioligand binding was assessed on confluent monolayers of cells grown in 24-well plates. Cells were rinsed with phosphate-buffered saline (PBS) (0.15*M*, pH 7.4) and incubated with 40,000 cpm/well

of ^{125}I-PTHrP (1–36) for 2 h at room temperature. The incubation was stopped by removal of the binding medium, and the cells were rinsed three times with ice-cold PBS. Bound radioactivity was recovered by solubilizing the cells with 1 mL of 1 *N* NaOH. Radioactivity and protein content were determined in aliquots of the solubilized cells. Nonspecific binding was determined in the presence of 1 μ*M* unlabeled hPTHrP (1–34) (Bachem, Torrance, CA). Specific binding was obtained by subtracting nonspecific binding from total binding, and was normalized to total cell protein *(41)*.

cAMP Radioimmunoassay

Cells were cultured in 24-well plates until confluent. Each well was washed twice with 1 mL of PBS, and then incubated at room temperature for 15 min with 200 μL of test solution (PBS containing 15% NuSerum, 1 m*M* $MgCl_2$, 20 m*M* HEPES, 85 U/mL of aprotinin [Trasylol; FBA Pharmaceuticals, New York, NY], 0.2% BSA, and 0.1% glucose) containing 1 m*M* IBMX and varying concentrations of PTHrP (1–34). Medium was removed, cells were rinsed three times with 1 mL of ice-cold PBS, and 0.5 mL of 10% trichloroacetic acid was added to each well to precipitate the protein. The supernatant was neutralized with calcium carbonate, and cAMP was measured by radioimmunoassay (RIA) as previously reported *(38)*. The precipitated protein was dissolved in 1 mL of 1 *N* sodium hydroxide for measurement of protein.

Statistical Analysis

For PTHrP immunoreactivity, receptor binding studies, and cAMP RIAs, representative results from two to three related experiments are shown (Figs. 2, 5, and 6). Data are presented as mean ± SEM and were analyzed by analysis of variance (ANOVA) followed by a Bonferroni multicomparison posttest *(42)*. For PTH/PTHrP receptor mRNA analysis, data from separate experiments were combined and analyzed nonparametrically using a Kruskal-Wallis ANOVA *(43)*. All statistical analyses were performed using Instat software (GraphPad, San Diego, CA). For establishing differences, $p < 0.05$ was considered significant.

Acknowledgment

This work was supported by NIH Grant PO1-DK-35608.

References

1. Wysolmerski, J. J. and Stewart, A. F. (1998). *Ann. Rev. Physiol.* **60,** 431–460.
2. Abou-Samra, A.-B., Juppner, H., Force, T., Freeman, M., Kong, X.-F., Schipani, E., Urena, P., Richards, J., Bonventer, J. V., Potts, J. T. Jr., Kronenberg, H. M., and Segre, G. V. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 2732–2736.
3. Moseley, J. M., Kubota, M., Diefenbach-Jagger, H., Wettenhall, R. E. H., Kemp, B. E., Suva, L. J., Rodda, C. P., Ebeling, P. R., Hudson, P. J., Zajac, J. D., and Martin, T. J. (1987). *Proc. Natl. Acad. Sci. USA* **84,** 5048–5052.
4. Suva, L. J., Winslow, G. A., Wettenhall, R. E. H., Hammonds, R. G., Moseley, J. M., Diefenbach-Jagger, H., Rodda, C. P., Kemp, B. E., Rodriguez, H., Chen, E. Y., Hudson, P. J., Martin, T. J., and Wood, W.I. (1987). *Science* **237,** 893–896.
5. Broadus, A. E., Mangin, M., Ikeda, K., Insogna, K. L., Weir, E. C., Burtis, W. J., and Stewart, A. F. (1988*). N. Engl. J. Med.* **391,** 556–563.
6. Orloff, J. J., Reddy, D., dePapp, A. E., Yang,K. H., Soifer, N. E., and Stewart, A. F. (1994). *Endocr. Rev.* **15,** 40–60.
7. Selvanayagam, P., Graves, K., Cooper, C. W., and Rajaraman, S. (1991). *Lab. Invest.* **64,** 713–717.
8. Kramer, S., Reynolds, F. H. Jr., Castillo, M., Valenzuela, D. M., Thorikay, M., and Sorvillo, J. M. (1991). *Endocrinology* **128,** 1927–1937.
9. Juppner, H. (1995). *Bone* **17,** 395–425.
10. Bringhurst, F. R., Juppner, H., Guo, J., Urena, P., Potts, J. T. Jr., Kronenberg, H. M., Abou-Samra, A. B., and Segre, G. V. (1993). *Endocrinology* **132,** 2090–2098.
11. Maeda, S., Wu, S., Juppner, H., Green, J., Aragay, A. M., Fagin, J. A., and Clemens, T. L. (1996). *Endocrinology* **137,** 3154–3162.
12. Lee, K., Deeds, J. D., Bond, A. T., Juppner, H., Abou-Samra, A. B., and Segre, G. V. (1993). *Bone* **14,** 41–45.
13. Lee, K., Deeds, J. D., and Segre, G. V. (1995). *Endocrinology* **136,** 453–463.
14. Torres, R., De La Piedara, C., and Rapado, A. (1993). *Miner. Electrolyte Metab.* **19,** 64–70.
15. Ishizuka, I., Yokose, S., Hori, M. M., Noda, T., Suda, T., Yoshiki, S., and Yamaguchi, A. (1997). *J. Clin. Invest.* **99,** 2961–2970.
16. Yu, X. P. and Chandrasekhar, S. (1997). *Endocrinology* **138,** 3085–3092.
17. Karaplis, A. C., Luz, A., Glowacki, J., Bronson, R. T., Tybulewicz, V. L. J., Kronenberg, H. M., and Mulligan, R. C. (1994). *Genes Devel* **8,** 277–289.
18. Lanske, B., Karaplis, A. C., Lee, K., Luz, A., Vortkamp, A., Pirro, A., Karperien, M., Defize, L. H. K., Ho, C., Mulligan, R. C., Abou-Samra, A. B., Juppner, H., Segre, G. V., and Kronenberg, H. M. (1996). *Science* **273,** 663–666.
19. Weir, E. C., Philbrick, W. M., Amling, M., Neff, L. A., Baron, R. E., and Broadus, A. E. (1996). *Proc. Natl. Acad. Sci. USA* **93,**10,240–10,245.
20. Heersche, J. N. M., Hetboer, M. P. M., and Ng, B. (1978). *Endocrinology* **103,** 333–340.
21. Kent, G. N., Jilka, R. I., and Cohn, D. V. (1980). *Endocrinology* **107,** 1474–1481.
22. Mitchell, J. and Goltzman, D. (1990). *Endocrinology* **126,** 2650–2660.
23. Gonzales, E. A. and Martin, K. J. (1996). *Kidney Int.* **50,** 63–70.
24. Urena, P., Iida-Klein, A., Kong, X. F., Juppner, H., Kronenberg, H. M., Abou-Samra, A. - B., and Segre, G. V. (1994). *Endocrinology* **134,** 451–456.
25. Urena, P., Ferreira, A., Moriex, C., Drueke, T., and de Vernejoul, M. C. (1996). *Nephrol. Dial. Transplant* **11,** 2008–2016.
26. Ikeda, K., Sugimoto, T., Fukase, M., and Fujita, T. (1991). *Endocrinology* **128,** 2901–2906.
27. Fujimori, A., Miyauchi, A., Hruska, K. A., Martin, K. J., Avioli, L. V., and Civitelli, R. (1993). *Am. J. Physiol.* **264,** E918–E924.
28. Cole, J. A. (1997). *J. Bone Miner. Res.* **12,** 1223–1230.
29. Yaghoobian, J. and Drueke, T. B. (1998). *Nephrol. Dial. Transplant* **13,** 580–586.
30. Jongen, J. W. J. M., Willemstein-Van Der Meer, J. M., Bos, M. P., Juppner, H., Segre, G. V., Abou-Samra, A. B., Feyen, J., and Hermann-Erlee, M. P. (1996). *J. Bone Miner. Res.* **11,** 1218–1225.
31. Guo, J., Liu, B., and Bringhurst, F. R. (1997). *Am. J. Physiol.* **273,** E383–E393.
32. Alsina, M., Guise, T. A., and Roodman, G. D. (1996). *Vitam. Horm.* **52,** 63–98.

33. McCauley, L. K., Koh, A. J., Beecher, C. A., Cui, Y., Rosol, T. J., and Francheschi, R. T. (1996). *J. Cell Biochem.* **61,** 638–647.
34. Du, P. F., Ye, Y. M., Wang, C., Seitz, P. K., Bi, L. X., Li, H., Simmons, D. J., and Cooper, C. W. (1998). Bone **23,** S251 (abstract).
35. Bi, L. X., Du, P. F., Seitz, P. K., Li, H., Ye, Y. M., Simmons, D. J., Mainous, E., and Cooper, C. W. (1998). *Bone* **23,** S533 (abstract).
36. Li, H., Seitz, P. K., Thomas, M. L., Selvanayagam, P., Rajaraman, S., and Cooper, C. W. (1995). Lab. Invest. **73,** 864–870.
37. Scully, S. P., Joyce, M. E., Abide, N., and Blander, M. E. (1990). *Mol. Cell Probes* **4,** 485–495.
38. Seitz, P. K., Cooper, K. M., Ives, K. L., Ishizuka, J., and Cooper, C. W. (1993). *Endocrinology* **133,** 1116–1124.
39. Li, H., Seitz, P. K., Selvanayagam, P., Rajaraman, S., and Cooper, C. W. (1996). *Endocrinology* **137,** 2367–2374.
40. Yu, D., Seitz, P. K., Selvanayagam, P., Rajaraman, S., Townsend, C. M. Jr., and Cooper, C. W. (1992). *Endocrinology* **131,** 1188–1194.
41. Bradford, M. M. (1976). *Anal. Biochem.* **72,** 248–253.
42. Morrison, D. F. (1983). *Applied linear statistical methods*. Prentice Hall: Englewood Cliffs, NJ.
43. Mendenhall, W., Wackerly, D. D., and Scheaffer, R. L. (1990). *Mathematical statistics with applications*. PWS-Kent: Boston.