Gene Expression Profiles and Transcription Factors Involved in Parathyroid Hormone Signaling in Osteoblasts Revealed by Microarray and Bioinformatics*

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Parathyroid hormone (PTH) binds to its receptor PTH1R (parathyroid hormone 1 receptor) in osteoblastic cells to regulate bone remodeling and calcium homeostasis. While prolonged exposure to PTH causes increased bone resorption, intermittent injections of PTH have an anabolic effect on bone. The molecular mechanisms regulating these processes are still largely unknown. Here, we present our results on gene expression profile changes in the PTH-treated osteoblastic cell line, UMR 106-01, using DNA microarray analysis. A total of 125 known genes and 30 unknown expressed sequence tags (ESTs) were found to have at least 2-fold expression changes after PTH treatment at 4, 12, and 24 h. 14 genes were previously known to be PTH-regulated but many were unknown to be regulated by PTH prior to our experiments. Real-time reverse transcriptase-PCR confirmed that 90 and 50% of the genes are regulated more than 2-fold by PTH in UMR 106-01 and rat primary osteoblastic cells, respectively. Most genes belong to the following protein families: hormones, growth factors, and receptors; signal transduction pathway proteins; transcription factors; proteases; metabolic enzymes; structural and matrix proteins; transporters; etc. These results provide a comprehensive and deeper knowledge about PTH regulation of osteoblastic gene expression. Next, we designed a computational method to extract information about transcription factors likely involved in regulating these genes. These factors include those previously known to be involved in PTH signaling (AP-1 and the cAMP response element-binding protein), those that were identified by microarray data (C/EBP), and some novel transcription factors (AP-2, AP-4, SP1, FoxD3, etc.). Our results suggest that a reliable bioinformatics approach can be easily applied for other systems.

Bone is a mineralized tissue that confers multiple mechanical and metabolic functions to the body. The majority of bone consists of extracellular matrix, which contains the primary body storage site for calcium. Cells account for only 2% of the total volume of bone. These cells include osteoblasts, the bone-forming cells, osteocytes, the fully differentiated osteoblasts embedded in the extracellular matrix, and osteoclasts, bone-resorbing cells. Bone remodeling, a physiological process in which bone is destroyed by osteoclasts and then replaced by osteoblasts, is constantly taking place. Imbalance of the two cellular processes leads to either osteoporosis or osteopetrosis (1).

Parathyroid hormone (PTH) is an 84-amino acid polypeptide hormone secreted from parathyroid glands in response to changes in serum calcium levels. A small decrease in serum calcium causes an increase in the secretion of PTH, which initiates a rapid response to raise serum calcium levels by acting directly on bone and kidney and indirectly on intestine (2). In bone, the PTH receptor (PTH1R), a seven transmembrane domain receptor coupled to G-proteins, exists in cells of the osteoblast lineage (3). Upon PTH binding, osteoblasts decrease their own proliferation and recruit osteoclasts to the bone surface, stimulating osteoclastic activity through ligand-receptor interaction (4). During this process, PTH changes the phenotype of the osteoblast from a cell involved in bone formation to one directing bone resorption. Therefore, exposure to high dose PTH leads to increased bone resorption (5). Paradoxically, intermittent administration of PTH stimulates bone formation (6, 7). However, the mechanism underlying this anabolic effect is largely unknown.

The rat osteoblastic cell line, UMR 106-01, derived from an osteosarcoma, is a useful model system for studying the effects of PTH on osteoblasts in vitro (8). Treatment of UMR 106-01 cells with 10^{-8} M PTH inhibits proliferation of these cells by arresting them in G_1 (9) and has a similar effect on gene expression patterns of UMR 106-01 cells as high dose PTH does on osteoblasts in the skeleton (10, 11). Therefore, treatment of UMR 106-01 cells with 10^{-8} M PTH is considered to mimic the effect of high dose PTH in vivo.

It was found that 10^{-8} M PTH strongly activates G_α_s and weakly activates G_α_i and their subsequent signal transduction

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1 The abbreviations used are: PTH, parathyroid hormone; AP-1, activator protein-1; CREB, cAMP response element-binding protein; C/EBP, C/EBF, C/CAAT enhancer-binding protein; MEM, minimum essential medium; ESTs, expressed sequence tags; TGF, tumor growth factor; BMP, bone morphogenetic protein; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; RT, reverse transcriptase; EGF, epidermal growth factor; ET-1, endothelin-1; VEGF, vascular endothelial growth factor; 1,25(OH)_2D_3, 1,25-dihydroxyvitamin D_3.
pathways (5, 12). So far, a number of genes have been found to be regulated by high concentrations of PTH, including genes involved in bone formation, such as type I collagen, alkaline phosphatase, osteocalcin, and osteopontin, and genes involved in bone degradation, such as collagenase-3, tissue inhibitors of metalloproteinases, and RANKL (reviewed in Ref. 11). However, how G protein activation leads to changes in the above genes’ expression, whether there are other genes regulated by high concentrations of PTH, when expression of these genes are changed, and how these genes regulate each other are largely unknown. Thus, it is particularly important to study the global gene expression pattern in osteoblasts treated with PTH. Recently, microarray technology has been used successfully to document changes in osteoblasts in response to a number of factors, such as 1,25-dihydroxyvitamin D$_3$ (13) and bone morphogenetic protein 2 (BMP2, Ref. 14), and during osteoblast maturation (15). In the present report, we used high density oligonucleotide arrays to study the whole genome expression profile changes and found that a large number of novel genes covering multiple biological functions are regulated by PTH.

The recent completion of the draft sequence of the human genome is an invaluable resource for molecular biology research. Taking advantage of this resource and the results from our microarray experiments, we designed a bioinformatics approach to identify the transcription factors that may play an important role in PTH signaling. The results from this approach closely match the current knowledge about PTH-regulated transcription factors and suggest that a set of transcription factors mediates PTH regulation of osteoblast gene expression. In this way, we have established a method to extract more information about transcription factors involved in a certain cellular event from high-throughput gene expression analysis.

EXPERIMENTAL PROCEDURES

Microarray Analysis—UMR 106-01 cells were seeded in 100-mm dishes at 1.2 × 10$^6$ cells/cm$^2$ in Eagle’s minimal essential medium (MEM) supplemented with 5% fetal bovine serum overnight. The cells were then switched to serum-free MEM for 2 days before addition of 10$^{-8}$ M PTH or control medium. Cells were harvested at 4, 12, and 24 h after treatment with or without PTH. Total RNA was isolated using Tri Reagent (Sigma) followed with an RNeasy kit (Qiagen). Double-stranded cDNA was synthesized from 10$^{-8}$ M PTH or control medium, and 8M PTH for 1, 4, and 12 h immediately after changing the medium to BGJb plus 2% fetal calf, and 8M PTH. RNA was isolated in the same way as for UMR 106-01 cells.

Real-time RT-PCR—Two-step real-time RT-PCR was performed to confirm the differential expression results obtained by microarray experiments. TaqMan reverse transcription kit (Applied Biosystems Group) was used for the reverse transcription step. For each gene, two specific PCR primers were designed by PrimerExpress software (Applied Biosystems Group). The PCR reaction was performed on Option (MJ Research) using a SYBR Green PCR Core kit (Applied Biosystems Group). Each RT-PCR experiment was performed three times.

Resources for Data Bases and Computer Programs—GenBankTM release 125 was downloaded from ncbi.nlm.nih.gov. TRANSFAC (17), licensed from BioBase, is used as a base of transcription factors, their genomic binding sites, and DNA binding sites sequence profiles (transfac.gbf.de/TRANSFAC/). One important feature of TRANSFAC is the MATRIX entry representing DNA binding site sequence profiles for individual or groups of transcription factors. Each transcription factor may have more than one matrix in the data base. Match is a computer program that can detect potential sequence matches by automatic searches with a library of precompiled matrices (17). AAT, a sequence alignment software for transcript mapping (18), was licensed from Michigan Technological University. RepeatMasker (ftp.genome.washington.edu/RM/RepeatMasker.html) is a software program screening for DNA interspersed repeats. All non-commercial software used in this study was written in PERL 5.

Identifying Human Orthologs for Rat Genes—A collection of human mRNAs and rat mRNAs were first extracted from GenBankTM flat file (Release 125). The sequences of the 125 PTH-regulated genes revealed by microarray experiments were retrieved from GenBankTM and matched using BLAST against the rat mRNA data base to ensure the longest rat mRNA entries were found. Each of these longest rat mRNAs was matched using BLAST against human mRNA. A reciprocal BLAST analysis against the original rat mRNA data base was performed with human mRNA with the lowest e-value. An ortholog pair was established if the rat mRNA with the lowest e-value was the same as the original rat mRNA. 73 genes were found to have human orthologs in this analysis.

Transcript Mapping—To ensure that the 5’ end of an mRNA is close to the transcription start site, only mRNAs that encode the N terminus of the protein were used for transcript mapping. Furthermore, only sequences in the GenBankTM Refseq data base were used to reduce gene redundancy. A local alignment software package AAT was used for alignment of the 5’ end of the CDNA with the human genome data sequence. To reduce the number of undesirable matches due to interspersed repeats, the DNA sequence was screened for interspersed repeats using RepeatMasker. Only genes having a 5,000-bp continuous region upstream of the first exon start site were kept for further transcription factor site analysis. We term this PTH-regulated gene list as the sample list. It contains 63 genes.

Construction of Promoter Reference Data Base—All human genes in the GenBankTM Refseq section except those that have less than a 5,000-bp continuous region upstream of the first exon start site were used to construct the promoter reference data base. The total gene number is 4,221. For a sample of 150 promoters annotated in GenBankTM (19, 88%) were predicted by the approach. The validation and other applications of this data base have been described previously (19, 20).

Transcription Factor Binding Site Analysis and Statistical Analysis—For each gene in the sample list, 500-, 1000-, and 2000-bp upstream of the first exon start site were retrieved as possible promoter regions. AAT, a sequence alignment software for transcript mapping (18), was licensed from Michigan Technological University. RepeatMasker (ftp.genome.washington.edu/RM/RepeatMasker.html) is a software program screening for DNA interspersed repeats. All non-commercial software used in this study was written in PERL 5.

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Genes Regulated by PTH in Osteoblasts
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MATRIX libraries. The output file from Match was parsed and stored in the Sybase relational data base table. Matrix similarity scores (a value to measure the similarity between the matrix and the actual DNA sequence, complete match has a value of 1) of 0.8, 0.9, and 0.95 were used as cutoff scores to calculate the observed number of each matrix in the sample list. The same analysis was applied on the reference data base to calculate each matrix's expected frequency (total number of matrix occurrences in the reference data base/total promoter number) for three different promoter lengths and three matrix cutoff scores. The expected number of each matrix in the sample list is equal to the expected frequency times the promoter number, that is, 63. As the discrepancies between the observed and expected numbers increase, the value of the statistical variable chi-square ($\chi^2$) becomes larger, and the resulting $p$ value becomes smaller, which describes the probability of randomly selected subjects having this large discrepancy between observed and expected values. With the degree of freedom = 1 in our case, a reasonable significance level would be $p < 0.005$, which corresponds to $\chi^2 = 8$. Chi-square value is calculated by Equation 1.

$$\chi^2 = \frac{\text{observed} - \text{expected}}{0.5 \times \text{expected}}$$  

A matrix and its corresponding transcription factor are considered to have significantly higher occurrence in the promoter regions of the sample list if it meets the following conditions. 1) The transcription factor should belong to the vertebrate category. 2) The observed number should be higher than the expected number. 3) $\chi^2$ should be higher than 8 ($p < 0.005$) in at least one matrix cutoff in 2 of 3 promoter distances.

RESULTS AND DISCUSSION

Microarray Analysis to Identify PTH-regulated Genes—In order to identify genes regulated by PTH, UMR 106-01 cells, a rat osteoblastic osteosarcoma cell line, were treated with $10^{-8}$ M PTH or control medium for different time periods: 4, 12, and 24 h. The total RNAs extracted at these time points were converted to cRNA for hybridization with the Affymetrix rat RG_U34A oligonucleotide array. After filtering analysis (see “Experimental Procedures”), 104 known genes and 58 ESTs were identified to be regulated by PTH. Among those ESTs, 28 have corresponding mRNAs by BLAST search against the GenBank™ nucleotide data base (expectation value = 0). In subsequent studies, we have used the corresponding genes to designate those ESTs. Among those 28 identifiable ESTs, 7 EST-corresponding genes are in the list of 104 known PTH-regulated genes. The remaining 21 ESTs do not have the corresponding genes present on the RG-U34A array probably because of the fact that these genes were identified after the array was designed in 1999. These 21 ESTs and 104 known genes (total 125 genes) were combined together and are listed in Table I, grouped based on their functions.

As shown in Table I, two-thirds of the genes (82 of 125 genes) were stimulated by PTH, suggesting PTH mainly stimulates transcription activators or inhibits transcription repressors. A large number of genes were regulated in the same direction at two contiguous time points, for instance 4 and 12 h or 12 and 24 h. A few genes were regulated at all three time points, such as IL-18, UMRCase (collagenase-3), and interferon-induced mRNA. Only one gene (CCAAT/enhancer-binding protein (C/EBP) δ) was regulated at 4 and 24 h but not at 12 h. However, the raw data at 12 h indicate that there is a significant increase in expression level (the fold change is 1.90 in the first experiment, the absolute calls for PTH-treated samples are Present and for control are Absent in both second and third experiments), albeit this increase is not high enough to pass the filtering conditions. This time-dependent gene distribution feature clearly suggests that PTH has a prolonged impact on the gene expression profile of osteoblastic cells by inducing immediate response genes, middle response genes, and late response genes. In comparison to other publications where microarray has been performed with osteoblastic cells treated with 1,25(OH)$_2$D$_3$ or BMP2 (13, 14), and/or during differentiation (15), very few of the same genes appear to be regulated. This is not surprising since the agents do not act through the same pathways as PTH. It is also revealing that PTH is not stimulating many of the genes identified as changing in differentiation except lumican (14, 15).

Real-time RT-PCR Confirms Microarray Results—Among 125 genes in Table I, 14 genes have been previously demonstrated to be PTH-regulated, implying the microarray result is reliable. To further validate this, we used quantitative real-time RT-PCR to examine the effect of PTH on individual genes. 19 genes from the 4-h PTH-regulated list and 12 genes from the 12-h PTH-regulated list were randomly selected as targets for real-time RT-PCR. Note that 5 genes among them were regulated at both time points. As shown in Fig. 1, only three genes did not achieve more than 2-fold change in mRNA level by real-time RT-PCR upon PTH treatment: interferon-induced mRNA at 4 h (1.6-fold), RGC-32 at 12 h (1.5-fold), and GADD45 at 12 h (1.5-fold). Hence, we conclude that about 90% (28 of 31 genes from the 4 and 12 h treatments) of genes are indeed regulated more than 2-fold by PTH. Our high validation rate could be attributed to the stringent filtering conditions we used to remove false positive genes as much as possible. However, this strategy risks losing real positive genes. For example, c-myc, IL-6, and the fibroblast growth factor receptor (FGFR1) have been demonstrated to be PTH-inducible genes (10, 21), yet none of them passed the filtering conditions in the microarray experiment. Notwithstanding, all of these genes do show a significant increase upon PTH treatment if the raw data are examined. For example, c-myc at 4 h has more than a 2-fold increase in all triplicate experiments. But it has an average difference in one PTH-treated sample of less than the average difference in one of the other control samples, resulting in a failure to pass the filtering conditions. IL-6 has 1.8-, 2.3-, and 2.1-fold increases in three experiments at 12 h while the FGFR1 has 1.9-fold increases in all three experiments at 4 h.

We also tested whether those genes are PTH-regulatable in other osteoblastic cells. We treated rat primary calvarial osteoblastic cells cultured in the differentiation phase with $10^{-8}$ M PTH for 1, 4, and 12 h and harvested RNA for real-time RT-PCR analyses. Genes selected for this assay were the same as genes in Fig. 1 except for interferon-induced mRNA and GADD45 because the expression level of these two genes did not achieve more than 2-fold change even in UMR 106-01 cells. The total gene number for this analysis in the primary osteoblastic cells is 24. Among those genes, 13 genes were found to have more than 2-fold change in mRNA level in at least one time point (Fig. 2). Therefore, we conclude that about 50% of the genes listed in Table I can be validated in primary osteoblastic cells. By comparing Figs. 1 and 2 we notice that the fold change or the peak time of the same gene may be regulated differently by PTH in UMR 106-01 and primary osteoblastic cells. The reason for this discrepancy and the reason for the 50% validation rate are probably due to the following. 1) UMR 106-01 is a highly PTH-responsive clonal cell line giving a strong signal-to-noise ratio and sensitivity (8, 22, 23). 2) Rat primary osteoblastic cells are a mixture of different osteoblastic cells. Some may not respond to PTH at all (23, 24). 3) Rat primary osteoblastic cells undergo three phases (proliferation, differentiation, and mineralization) in culture. Some genes that are not regulated at the differentiation phase may be regulated at the other two phases. Nevertheless, our results with primary osteoblastic cells clearly demonstrate that the PTH-regulated gene list obtained from the UMR 106-01 cells can be validated in primary cells, and therefore this list has a general impact on PTH studies.

Implication of the Microarray Results—The microarray experiment reveals that $10^{-8}$ M PTH has a profound effect on the
For up-regulated gene: fold change is the average difference in PTH-treated sample/average difference in control sample. If the absolute call for the control sample is Absent, fold change was given as $>/2$. For down-regulated gene, negative change in the average difference in control sample/average difference in PTH-treated sample. If the absolute call for the PTH-treated sample is Absent, fold change was given as $>/2$. The value of fold change shown in this table was the average of three experiments. If in one or two experiments the fold changes are $>/2$ or $>/2$, the fold change shown here was the average of the remaining two or one experiments. Some genes have several sets on the array chip. Only the set with the highest fold change were shown in this table. Genes that are previously known to be regulated by PTH are labeled with #. EST sequences are labeled with &.

| GenBank™ | Gene name | Fold change |
|----------|-----------|-------------|
| Hormone, growth factor | 4 h | 12 h | 24 h |
| AF012891 | Frizzled related protein (frpAP) | $>/2$ | $>/2$ | |
| X55183 | Amphiregulin | 2.5 | 3.6 | |
| M31076 | TGF $\alpha$ | 5.2 | 6.1 | |
| L38483 | Jag1 | 10.2 | 11.9 | 3.6 |
| A16585 | Preprorelaxin | 2.2 | 3.0 | |
| U77777 | IL-18, IGIF | 10.8 | 10.8 | |
| U41183 | Placental pre-pro-growth hormone-releasing hormone | $>/2$ | $>/2$ | $>/2$ |
| S77492 | BMP3 | $>/2$ | $>/2$ | $>/2$ |
| Z22607 | BMP4 | $>/2$ | $>/2$ | $>/2$ |
| D29789 | BMP7 | $>/2$ | $>/2$ | $>/2$ |
| AF022952 | Vascular endothelial growth factor B | $>/2$ | $>/2$ | $>/2$ |
| Receptor | 4 h | 12 h | 24 h |
| X05137 | Fast nerve growth factor receptor | 2.7 | 3.3 | |
| D12524 | c-kit receptor tyrosine kinase | 9.1 | 6.1 | |
| A167710& NOB-1 | 9.1 | 6.1 | |
| A1639318& Ret | 2.7 | 3.3 | |
| S61883 | Syndecan 4 | 3.9 | 2.3 | |
| U68272 | Interferon gamma receptor | 2.3 | 1.4 | |
| X19898 | Urinary plasminogen activator receptor 1 | 3.9 | 2.3 | |
| Z83757 | Growth hormone receptor | 2.3 | 1.4 | |
| S65355 | Noneselective-type endothelin receptor (ET-B) | $>/2$ | $>/2$ | $>/2$ |
| L31394 | PTH receptor | $>/2$ | $>/2$ | $>/2$ |
| M85214 | Trk | $>/2$ | $>/2$ | $>/2$ |
| Signal transduction | 4 h | 12 h | 24 h |
| J04563 | cAMP phosphodiesterase | 2.7 | 4.1 | |
| M25350 | cAMP phosphodiesterase (PDE4) | 2.5 | 2.6 | |
| AA799729& PDE4B | 2.5 | 2.6 | |
| D28560 | Phosphodiesterase I, NPH-type III | 4.0 | 3.0 | |
| U02553 | MKP-1 | 2.9 | 2.3 | |
| L01624 | Interferon gamma receptor | 2.3 | 1.4 | |
| X71898 | Urinary plasminogen activator receptor 1 | 3.9 | 2.3 | |
| Signal transduction | 4 h | 12 h | 24 h |
| X06769# c-fos | 7.0 | 2.5 | |
| X60769 | rNFIL-6, C/EBP $\beta$, silencer factor B | 3.1 | 4.3 | |
| M65114 | C/EBP $\beta$, CELF | 6.1 | 4.3 | |
| AA900750& Lot1 | 3.6 | 4.3 | |
| AA900750& Lot1 | 3.6 | 4.3 | |
| U40835 | CREMedeltaC-G gene | 7.6 | |
| S66024# CREM delta | 7.6 | 4.3 | |
| U17250# Nurr77 | 3.8 | |
| U71293 | Hairless | 4.8 | |
| AA891527& LIM protein, FHL2 | 3.7 | 4.3 | |
| U56241 | Maf1 | 2.4 | 4.3 | |
| U56242 | Maf2 | 2.4 | 4.3 | |
| AF000942 | ID3 | $>/2$ | $>/2$ | |
| L23148 | Inhibitor of DNA-binding, splice variant Id1.25 | $>/2$ | $>/2$ | $>/2$ |
| J03179 | D-binding protein | $>/2$ | $>/2$ | $>/2$ |
| Cell cycle and apoptosis | 4 h | 12 h | 24 h |
| AF036548 | RGC-32 | 8.8 | 4.2 | |
| X96437 | PRG1 | 2.3 | 16.0 | |
| L32591 | GADD45 | 3.2 | |
| D14014 | Cyclin D1 | $>/2$ | $>/2$ | $>/2$ |
| Ligand binding and carrier | 4 h | 12 h | 24 h |
| L32132 | Lipopolysaccharide-binding protein | 3.2 | 4.3 | |
| M21281# | Insulin-like growth factor-binding protein 5, IGBP-5 | 3.2 | 4.3 | |
| A123027& Lbp1 (Latent TGF-β binding protein 1) | 3.2 | 4.3 | |
| M10934# | Retinol-binding protein | 4.2 | 5.2 | |
| J02773 | Low molecular weight fatty acid-binding protein | $>/2$ | $>/2$ | $>/2$ |
| S69874 | Cutaneous fatty acid-binding protein | $>/2$ | $>/2$ | $>/2$ |
| AA875025& Cellular retinoic acid-binding protein | $>/2$ | $>/2$ | $>/2$ |
| Protease and regulator | 4 h | 12 h | 24 h |
| M60016# | UMRCase | $>/2$ | $>/2$ | $>/2$ |
| M23697# | Tissue-type plasminogen activator | 3.7 | 4.3 | |
gene expression profile of osteoblastic cells. More than 100 genes, having a wide range of different biological functions, were either up- or down-regulated by PTH treatment. Several important features about PTH signaling emerged after we carefully reviewed this gene list. These are listed in the following paragraphs.

(a) PTH significantly changes the expression levels of many hormones, cytokines, and growth factors produced by osteo-

### Table I—continued

| GenBank™ | Gene name | Fold change |
|----------|-----------|-------------|
| A1189327# & | TIMP-1 | 4.9 | 2.9 |
| X66693 | Granzyne-like protein 1 | 2 | 2 |
| D88250 | Serine protease | 4.9 | 2.3 |
| X03518 | γ-Glutamyl transpeptidase (GGT) | 2 | 2 |
| K02814 | Major acute phase α-1 protein (MAP), Kngt1 | 3.3 | 2.2 |
| L09120# | Calpain II | 2.2 | 2.2 |
| S73583 | Glutamyl aminopeptidase | 2.4 | 2.4 |
| M15944 | Enkephalinase (neutral endopeptidase) | 2.7 | 2.7 |
| A1230712# & | Pace4 (paired basic amino-acid cleaving enzyme 4) | <2 | -3.7 |
| D63886 | MT3-MMP-del | -2.5 | -2.5 |
| U38379 | γ-Glutamyl hydrolase, Ggh | 4.5 | 4.5 |

Metabolic enzyme

| Gene name | Fold change |
|-----------|-------------|
| SS1025 | UDP-galactose-N-acetylglucosamine β-1,4-galactosyltransferase homolog | 3.2 |
| L33869 | Ceruloplasmin | 2.7 | 2.4 |
| M91652 | Glutamine synthetase | 3.0 | 3.0 |
| J03637 | Aldehyde dehydrogenase | 3.5 | 2.5 |
| M77850 | Serine protease | 2.2 | 2.2 |
| M95591 | Hepatic squalene synthetase | <2 | -2.5 |
| M29472 | Mevalonate kinase | -2.5 | -2.5 |
| ABO18049 | GM3 synthase | -2.2 | -2.2 |
| X56228 | Rhodanese, thiosulphate sulphurtransferase | -8.3 | -8.3 |
| U175784& | Liver stearyl-CoA desaturase | -3.0 | -3.0 |
| L22294 | Pyruvate dehydrogenase | -2.3 | -2.3 |
| S73583 | Glutamyl aminopeptidase | -2.5 | -2.5 |
| M21354# | Collagen type III alpha 1 | 6.9 | 6.9 |
| M81025 | transforming growth factor-β, Tgf-β | 2.8 | 2.8 |
| M29472 | Mevalonate kinase | 2.5 | 2.5 |
| J04215 | Cell-binding bone sialoprotein | -6.5 | -6.5 |
| S9158 | Lamin A | 2.5 |
| X05859# | Decorin | 4.1 | 8.4 |
| M21354# | Collagen type III alpha 1 | 6.9 | 6.9 |
| U92612# | Fibronectin, fn-1 | 2.8 | 2.8 |
| M14733 | TRPM-2 | 2.5 | 2.5 |
| X54039 | Lumican | 3.3 | 3.3 |
| J04215 | Cell-binding bone sialoprotein | -6.5 | -6.5 |
| SS1025 | Transporter | 3.2 |
| X62839 | Potassium channel protein | 2 | 2 |
| A1176566& | Cyp1b1, cytochrome P450 | 2.9 |
| U75395 | Furosemide-sensitive K-Cl cotransporter | -2.2 | -2.2 |
| U35420 | Sodium-calcium exchanger form 3, NCX3 | -5.2 | -5.2 |
| Y00826 | Integral membrane glycoprotein gp210 | -4.5 | -4.5 |
| U70476 | Cationic amino acid transporter 1, CAT-1 | -2.6 | -2.6 |
| S86135 | Glucose transporter 1 | -2.5 | -2.5 |
| A1177026& | Atp1a2, Na/K-ATPase α 2 subunit | -4.4 | -4.4 |
| U76714 | Cell adhesion regulator, CAR1 | 2.7 | 2.7 |
| AF104362# | Osteoadherin | 2.1 |
| D83348 | Long type FB-cadherin, cdh22 | -2.7 |
| Others | 4.7 | 4.7 |
| X09261 | Hypertension-regulated vascular factor-1 | >2 | >2 |
| X03547 | FBR-murine osteosarcoma provirus genome | 4.1 |
| M80367 | Isoprenylated 67kDa protein | 5.3 |
| M92962 | IgE-binding protein | 3.5 | 2.2 |
| A1176456& | Metallothionein, Mt1a | 4.5 | 4.5 |
| X96394 | Multidrug resistance protein, mrp | 2.5 |
| U95178 | DOC-2 p59 isofrom | 2.6 |
| AP030089 | Activity and neurotransmitter-induced early gene | 3.6 |
| A1230247& | Sepp1, selenoprotein P | 3.0 | 4.2 |
| D00680 | Plasma glutathione peroxidase | 2 |
| A012275& | TPO1 | -3.5 | -3.4 |
| E12625 | A rat novel protein which is expressed with nerve injury | -3.0 |
| M55534 | α-Crystallin B chain | -3.1 |
| AA891422& | Putative hypoxia induced gene HIG1 | -2.2 |
| AA900516& | Pdi2, peptidylarginine deiminase | -5.5 |
| X08111 | CD2 antigen | -2.5 |
Blastic cells and modifies the way that osteoblastic cells respond to many signaling factors.

1) The osteoblast expresses several TGF-β family members, including TGF-β and BMPs, that have a strong growth and differentiation effect on bone (25). PTH stimulates the expression of Ltbp1, a latent TGF-β-binding protein that is important for modifying the activity of TGF-β (26). PTH also inhibits the production of three BMPs (BMP3, 4, 7), the most potent factors to stimulate bone formation. Therefore, it seems that PTH executes its catabolic roles at least partially through modifying the signaling pathway of TGF-β family members.

2) PTH increases the expression of two members of the epidermal growth factor (EGF) family: amphiregulin and TGF-α, which bind and act through the EGF receptor ErbB1. Previous studies have shown that EGF is a mitogenic factor for osteoblastic cells, and it works cooperatively with PTH (27, 28). However, the origin of EGF in the skeletal system is unknown. It is possible that, in fact, amphiregulin and TGF-α are the critical agents for binding to the EGF receptor in bone and exert part of PTH’s anabolic effects.

3) Recent studies have identified glutamate as a signaling molecule in bone. Osteoblastic cells constantly release glutamate in the same way as presynaptic neurons do (29) and contain functional glutamate receptors (30, 31). Our microarray results and real-time RT-PCR experiments demonstrate that PTH stimulates the expression of a glutamate transporter, required for glutamate uptake and glutamine synthetase, an enzyme converting glutamate into glutamine, thereby, recycling the glutamate. It appears PTH may increase glutamate uptake, decrease the extracellular glutamate concentration, and thus, regulate glutamate abundance.

4) Bone is rich in vascular tissues. Endothelial cells lining the bone are in close proximity with osteoblasts and osteoprogenitor cells. Extensive cross-talk exists between endothelial cells and osteoblasts since endothelial cells stimulate the differentiation of osteoblasts by secreting endothelin-1 (ET-1), and osteoblasts stimulate proliferation of endothelial cells by secreting vascular endothelial growth factor (VEGF) (32, 33). The microarray data provide a deeper understanding of this cross-talk. ET-1 has a strong anabolic effect on bone in vivo, stimulating the proliferation and differentiation of osteoblasts (32). Recent studies demonstrated that PTH decreases the mRNA levels 27–50% of two major ET-1 receptors (ET$_{A}$ and ET$_{B}$) in UMR 106 cells using semiquantitative RT-PCR (34). Our microarray experiments also identified that ET$_{B}$ expression was inhibited by PTH (more than 2-fold decrease at 4 h
and 2.3-fold decrease at 12 h). Further quantitative RT-PCR revealed that PTH decreases E\textsubscript{T\_a} expression by more than 80% at 4 h (Fig. 1A). The expression of E\textsubscript{T\_a} is undetectable on our arrays. Enkaphalinase, a protease controlling turnover of ET-1 in renal tissues (35), was stimulated by PTH (2.9-fold) at 24 h as revealed by our microarray experiment. Furthermore, BMP-7, which is down-regulated by PTH at 24 h in our microarray experiment has been shown to stimulate ET-1 synthesis in fetal rat calvarial cells (36). These results imply that PTH treatment inhibits the osteoblast cell's response to ET-1 by receptor down-regulation, ET-1 clearance, and inhibition of ET-1 synthesis. VEGF is a potent endothelial-specific mitogen. It is well-documented that VEGF-A production by osteoblast-like cells is further enhanced by stimulators of osteoblast differentiation (IGF-I and 1,25(OH)\textsubscript{2}D\textsubscript{3}) and suppressed by inhibitors of osteoblast differentiation (dexamethasone and PThrP) (37, 38). The microarray experiment reveals that VEGF-B, a close homologue of VEGF-A, is down-regulated by PTH at 4 h, implying the inhibition of angiogenesis accompanying bone resorption.

5) In addition, our microarray experiments suggest that PTH may modify several other signaling pathways whose importance for bone are known or have not been discovered so far. For example, retinoic acid plays a key role in the regulation of bone cell proliferation, differentiation, and functions (39). PTH may affect its signaling pathway by changing the level of retinoid-binding protein and cellular retinoic acid-binding protein. Recently, IL-18 has been found to inhibit osteoclast formation (40). The dramatic induction of IL-18 at 4 and 12 h by PTH (Fig. 1) implies that the stimulation of osteoclastic activity by PTH is finely controlled by an inhibition pathway. PTH also regulates the level of preprorelaxin, Jag1 (ligand for Notch), fast nerve growth factor receptor, and c-kit, etc. suggesting that these signaling pathways need further study in bone.

(b) The PTH effect on cellular metabolism has not been previously well studied. The microarray results reveal that PTH has strong inhibitory effects on glycolysis, sterol biosynthesis, and other metabolic pathways. After PTH treatment, mRNAs for both phosphofructokinase, the key regulatory enzyme in glycolysis that catalyzes the rate-limiting committed step, and hexokinase, the enzyme catalyzing the first step of glycolysis, were decreased at 12 and 24 h, respectively. In addition, the glucose transporter-1 mRNA was down-regulated at 12 h, implying a reduction in glucose uptake. Two important enzymes in sterol synthesis, squalene synthetase and mevalonate kinase, were down-regulated by PTH at 4 h. It would be reasonable to assume that the cholesterol content of the osteoblast may decrease after PTH treatment. In addition, the expression of transcripts for GM3 synthase, an enzyme involved in ganglioside synthesis, and 3-phosphoglycerate dehydrogenase, an enzyme involved in serine synthesis, were also decreased by PTH.

(c) Previous studies have already identified that several proteases are regulated by PTH, such as UMRCase (collagenase 3), plasminogen activator, and calpain. These proteases are important for breakdown of extracellular matrix, bone cell migration, and cell proliferation. Interestingly, the microarray analysis reveals many more proteases and peptidases (nine newly identified) are involved in PTH signaling as shown in Table I. Among them, PACE4 is a proprotein convertase that plays a significant role in tumor progression (41). It is not clear so far how these genes could be involved in bone turnover and calcium homeostasis mediated by PTH since no study on these proteins in osteoblastic cells has been done. However, it would be very interesting to identify their physiological substrates in bone and to further deduce their roles in PTH signaling.

(d) Osteoclasts produce reactive oxygen species such as superoxide and hydrogen peroxide and release them in the interface between the osteoclast membrane and bone surface for bone resorption (42). This process is stimulated by PTH (43). Since the osteoblast is in close proximity with the osteoclast, it is not surprising to observe that the expression of transcripts for antioxidant proteins, such as plasma glutathione peroxidase, selenoprotein P and ceruloplasmin, in the osteoblast were greatly increased by PTH treatment as revealed by our microarray results. These proteins are all extracellular proteins and presumably protect the osteoblast from free radical damages.

(e) PTH is a strong stimulator of the protein kinase A pathway through cAMP generation. The microarray results reveal a feedback mechanism that may function at several levels to quickly eliminate PTH's influence through this pathway. First, the mRNA level of the PTH receptor (PTH1R) was down-regulated at 4 and 12 h (Table I). Second, the levels of mRNA for several cAMP phosphodiesterases were up-regulated, which would increase the breakdown of cAMP (Table I). Third, the level of mRNA for adenylyl cyclase, the enzyme that is activated by PTH to produce cAMP, was significantly decreased at 4 h (in two experiments the fold changes were higher than 2 and in the third experiment the fold change was 1.9). This feedback mechanism may ensure that PTH action on osteoblastic cells is temporary but not prolonged.

(f) MAP kinases are protein serine and threonine kinases that play an important role in the regulation of cell growth and differentiation. Activation of MAP kinase (ERK1/2) by phosphorylation usually leads to cell proliferation. While one report suggested that high concentrations of PTH inhibit ERK1/2 activation in UMR 106 cells (44), other reports demonstrated that high dose PTH transiently activates ERK1/2 with a peak less than 30 min in opossum kidney cell and CHO cells and then the ERK1/2 is quickly dephosphorylated (45, 46). The dephosphorylation mechanism was unknown at that time. Our microarray experiments revealed that the expression level of MKP-1, a dual specificity phosphatase that can efficiently de-
phosphorylate ERK1/2 in vitro and in vivo, was greatly stimulated by PTH. Real-time RT-PCR further proved the mRNA level of MKP-1 increased 13-fold at 1 h and 5-fold at 4 h (Fig. 1). Thus MKP-1 seems to be the missing link between PTH and the inactivation of ERK1/2. Since overexpression of MKP-1 blocks entry into S phase (47), the stimulation of MKP-1 by PTH may be responsible for the growth arrest of osteoblastic cells at high doses of PTH. Recent findings in our laboratory demonstrate that low concentrations of PTH (10−11 M) stimulate the proliferation of UMR 106-01 cells through activation of ERK1/2 (48). Here, we propose a hypothesis that whether PTH has catabolic or anabolic effects on osteoblasts is determined by the relative strength of two PTH-regulated processes: ERK1/2 activation and MKP-1 induction. At a high concentration of PTH, MKP-1 induction is a dominant process and leads to growth arrest. At a low concentration of PTH, ERK1/2 phosphorylation is much stronger than the induction of MKP-1 and therefore stimulates cell proliferation.

Promoter Analysis of PTH-regulated Genes—PTH is known to regulate both the expression and transactivation of a number of transcription factors (11). The abundance of transcription factor AP-1 is dramatically up-regulated by PTH through the PKA-dependent pathway (49). In the microarray experiment, more than 10 transcription factors were found to be regulated at the mRNA level. The majority of them are immediate-response genes, because their expression levels change at 4 h. Some of them, such as hairless, C/EBP α and δ, were further confirmed by real-time RT-PCR (Fig. 1). PTH also regulates the activity of transcription factors at the post-translational level, such as phosphorylation of CREB (cAMP response element-binding protein) or Runx2, which cannot be identified by microarray experiments or any other mRNA study (11). Since PTH has profound effects on the gene expression profile in UMR 106-01 cells, we took advantage of the list of 125 PTH-regulated genes and designed a bioinformatics approach to identify additional transcription factors that may mediate effects of PTH.

The rationale of this bioinformatics approach is that a transcription factor might play a role in PTH signaling if the frequency of occurrence of its consensus binding sequence in the promoter regions of PTH-regulated genes is significantly higher than the promoter regions of a random pool of genes. The detailed approach is described under “Experimental Procedures.” The transcription factors whose matrix shows significantly more occurrences in the promoter regions of 63 analyzed PTH-regulated genes are summarized in Table II. It is important to note that one transcription factor’s matrix may tolerate another matrix. For example, the Bach1 matrix (SAT-GAGTCATGNY) is a stringent version of the AP-1 matrix (TGAGTCAC). Therefore, a Bach1 binding site must be also an AP-1 binding site. But an AP-1 binding site may not necessarily be a Bach1 binding site. In this case, the significance of a Bach1 binding site occurring in a promoter region may be a consequence of Bach1 playing a role in PTH signaling or may just reflect an AP-1 binding site. To avoid confusion, we grouped these factors according to whether their matrices can tolerate each other.

Transcription factors AP-1, CREB, and two TATA box elements are identified in Table II, suggesting our approach does indeed correctly identify transcription factors that may mediate PTH signaling. In addition, this approach identified C/EBP and Myc/Max transcription factors. Very little is known about the role of C/EBP in osteogenesis. However, recent studies have identified that C/EBP regulates important bone-related genes, such as insulin-like growth factor-1 and osteocalcin, in osteoblasts and that C/EBP itself is regulated by hormones such as 1,25(OH)2D3 (50, 51). Our microarray experiments demonstrated that two forms of C/EBP, β and δ, were up-regulated by PTH. Further promoter analysis indicated that this transcription factor may be important for expression of many PTH-regulated genes. Previous studies showed that the expression level of c-Myc increases after PTH treatment (10). Our microarray experiments also revealed that c-Myc was substantially increased at 4 h (2.6-fold increase in PTH-treated samples in the first experiment, 2.3-fold increase in the second experiment, and in the third experiment the absolute call for PTH-treated sample is Present and for control is Absent. However, the average difference for the PTH-treated sample in the third experiment is lower than that for the control in the second experiment. Therefore, it does not pass the stringent filtering. Taking these results into account, it appears that Myc may play a role in PTH signaling. This bioinformatics approach also identified some novel factors, such as AP-2, AP-4, SP1, FoxD3, MEF2, etc. whose roles in PTH signaling require further studies. AP-2 (a basic-helix-span-helix transcription factor) has been shown to play an important role in embryonic development (52); AP-4 is a ubiquitously expressed bHLH-Zip transcription factor (53); SP1 is a ubiquitous transcription factor that has been thought to maintain basal constitutive expression of many genes; FoxD3 (also known as Hnf2) is a member of the forkhead family of transcription factors and may be involved with driving neural tube cells to neural crest (54); MEF2 (myocyte enhancer factor-2) transcription factors have been shown to be involved in muscle and neural differentiation, cardiac morphogenesis, and blood vessel formation and are structurally related to serum response factor (SRF) (55).

The reason we chose to use the human orthologs instead of rat genes to undertake this analysis is that the complete human genome project provides the maximum possibility to obtain sufficient length of promoter regions for the analysis. In an effort to retrieve the promoter regions of rat genes, only 16 of 125 genes have the first exon start site annotated and sufficient length (500 bp) of promoter region that can be retrieved from the data base. This small pool is impossible to perform statistically significant analyses. Nevertheless, the conclusions from the human orthologs match our current knowledge well, indicating such a method is feasible and strong conservations exist between human and rat.

In summary, our microarray analysis presents a list of 125 genes that are regulated by PTH in osteoblastic cells. These genes belong to more than ten protein families based on their function and are involved in many signal transduction pathways. These results broaden our knowledge about PTH signaling and provide some important hints for future study of PTH action. In addition, we established a powerful and reliable computation approach using microarray results to identify the transcription factors that may play a role under a certain biological condition. One major limitation of this approach is that only a portion of the known transcription factors have matrix available in the database. This will definitely improve in the future.

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REFERENCES
1. Bruder, J. M., Guise, T. A., and Mundy, G. R. (2001) in Endoendo in Metabolism (Felig, P., and Frohman, L. A., eds) 4th edition, pp. 1089–1093, McGraw-Hill, New York.
2. Feldman, D. (1999) Am. J. Med. 107, 637–639
3. Juppner, H., Abo-Samra, A., Freeman, M., Kong, X. F., Schipani, E., Richards, J., Kolakowski, L. F. J., Hock, J., Potts, J. T. J., Kronenberg, H. M., and Segre, G. V. (1991) Science 254, 1024–1025
4. Teitelbaum, S. L. (2000) Science 289, 1504–1508
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