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Molecular Profile of Catabolic Versus Anabolic Treatment Regimens of Parathyroid Hormone (PTH) in Rat Bone: An Analysis by DNA Microarray

Jude E. Onyia,[2] Leah M. Helvering,[1]* Lawrence Gelbert,[2] Tao Wei,[2] Shuguang Huang,[3] Peining Chen,[3] Ernst R. Dow,[2] Avudaiappan Maran,[4] Minzhi Zhang,[4] Sutada Lotinun,[4] Xi Lin,[2] David L. Halladay,[1] Rebecca R. Miles,[1] Nalini H. Kulkarni,[1] Emily M. Ambrose,[1] Yanfei L. Ma,[1] Charles A. Frolik,[1] Masahiko Sato,[1] Henry U. Bryant,[1] and Russell T. Turner[5]

[1]Endocrine Research, Lilly Research Labs, Indianapolis, Indiana
[2]Integrative Biology, Lilly Research Labs, Indianapolis, Indiana
[3]Statistics and Information Science, Lilly Research Labs, Indianapolis, Indiana
[4]Orthopedic Research, Mayo Clinic, Rochester, Minnesota
[5]Nutrition and Exercise Science, Oregon State University, Corvallis, Oregon

Abstract Teriparatide, human PTH (1-34), a new therapy for osteoporosis, elicits markedly different skeletal responses depending on the treatment regimen. In order to understand potential mechanisms for this dichotomy, the present investigation utilized microarrays to delineate the genes and pathways that are regulated by intermittent (subcutaneous injection of 80 μg/kg/day) and continuous (subcutaneous infusion of 40 μg/kg/day by osmotic mini pump) PTH (1–34) for 1 week in 6-month-old female rats. The effect of each PTH regimen was confirmed by histomorphometric analysis of the proximal tibial metaphysis, and mRNA from the distal femoral metaphysis was analyzed using an Affymetrix microarray. Both PTH paradigms co-regulated 22 genes including known bone formation genes (i.e., collagens, osteocalcin, decorin, and osteonectin) and also uniquely modulated additional genes. Intermittent PTH regulated 19 additional genes while continuous treatment regulated 173 additional genes. This investigation details for the first time the broad profiling of the gene and pathway changes that occur in vivo following treatment of intermittent versus continuous PTH (1–34). These results extend previous observations of gene expression changes and reveal the in vivo regulation of BMP3 and multiple neuronal genes by PTH treatment. J. Cell. Biochem. 95: 403–418, 2005.

Key words: PTH; microarray; BMP3; protein tyrosine phosphatase zeta 1; neuron-specific gene 1; rat; bone; anabolic; catabolic

Teriparatide [recombinant human PTH (1–34)] is new treatment for osteoporosis that stimulates new bone formation in humans and experimental animals [Burr et al., 2001; Neer et al., 2001]; however, while increased bone formation is observed with once daily administration, continuous infusion of PTH results in pathological changes similar to hyperparathyroidism [Tam et al., 1982; Hock and Gera, 1992; Dobnig and Turner, 1995]. Intermittent subcutaneous administration of PTH was shown to increase bone mass, improve skeletal architecture, enhance biomechanical strength, and reduce fracture risk for humans [Dempster et al., 2001; Neer et al., 2001; Rubin and Bilezikian, 2002], while continuous exposure to elevated levels of PTH results in a net increase in bone resorption activity and hypercalcemia [Marx, 2000].

The mechanistic basis for these opposing biological effects has been explored at a tissue and cellular level. Current evidence suggests that anabolic PTH results in increased osteoblast activity and number via increased differentiation [Dobnig and Turner, 1995; Onyia et al., 1995] and survival [Jilka et al., 1999]. By contrast, continuous PTH administration through effects on cells of the osteoblast lineage leads to increased osteoclast activity

*Correspondence to: Leah M. Helvering, Lilly Research Labs, Indianapolis, IN 46285.
E-mail: L.Helvering@Lilly.com
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and differentiation [Ma et al., 2001] thus shifting the bone balance to favor resorption and a net local decrease in skeletal mass. At a molecular level, the mechanism for this profound dual effect has been difficult to elucidate primarily because this phenomenon cannot be fully recapitulated in vitro. In vivo, we and others have demonstrated that intermittent PTH modulates the expression of genes such as c-fos, c-jun, c-myc, histone H4 [Onyia et al., 1995], osteocalcin [Dobnig and Turner, 1995; Schmidt et al., 1995], IL-6 [Onyia et al., 1995; Pollock et al., 1996], leukemia inhibitory factor [Pollock et al., 1996], regulator of G-protein signaling 2 [Miles et al., 2000b], a disintegrin and metalloproteinase with thrombospondin motifs 1 [Miles et al., 2000a], MMP9 and creatine kinase [McClelland et al., 1998], type I collagen α 1 [Dobnig and Turner, 1995; McClelland et al., 1998], ubiquitin specific protease 2 [Miles et al., 2002], fos-like antigen 2 [McCauley et al., 2001], and IGF-1 [Watson et al., 1995]. However, the differential expression of genes induced by intermittent and continuous PTH (1–34) treatment in rats has not been described in a comprehensive manner. It is likely that the dual effects of PTH on bone involve a complex interaction of numerous gene products, which to date remain largely unidentified. A broad evaluation of the genes and pathways altered following the two paradigms of PTH, treatment could enhance our understanding of the profound differential skeletal responses to intermittent and continuous PTH. This possibility was evaluated in a rat model using distal femoral RNA from rats treated for 7 days with once daily subcutaneous injections (intermittent) or continuous infusion of PTH (1–34) and oligonucleotide microarrays representing about 8,800 probe sets or ~4,600 known rat genes.

RESULTS

The tibiae and femora of 6-month-old female rats were collected 24 h after the last dose following 7 days of treatment with PTH (1–34) administered intermittently (once daily subcutaneous injection) or continuously (subcutaneous infusion) to stimulate a bone anabolic and catabolic response, respectively. Histomorphometric analysis of the proximal tibial metaphysis confirmed the expected morphological changes that occur with each treatment [Lotinun et al., 2002]. Substantial increases in bone formation rate, as measured by the labeled surface to bone surface ratio, were confirmed in both treatment groups relative to control animals (Table I). However, structural differences in trabecular number, thickness, and volume were not detected within the 7 day treatment period. Bone formation rate indices could not be determined for the continuous group because of the diffuse flourochrome labeling of the osteitis fibrosis that results from this treatment [Lotinun et al., 2002], but intermittent treatment increased the bone formation rate by five-fold.

Gene Expression Profiling by DNA Microarray Analysis

The distal femur metaphysis was chosen for profiling to identify differentially regulated genes by intermittent and continuous PTH (1–34) because this area is highly enriched for trabecular bone and cells of the osteoblast lineage. Both PTH treatments resulted in a common set of 22 genes that were similarly regulated in magnitude and direction (15 genes upregulated and 7 reduced), however each treatment also significantly changed additional subsets of genes (Fig. 1). Intermittent PTH administration regulated an additional 19 unique genes (14 genes increased and 5 decreased) while continuous PTH specifically regulated an additional 173 genes (all of which were elevated except for a reduction in 24 genes).

Pathway Analysis of Gene Changes

The genes regulated by each treatment were evaluated by their gene ontology classification in an attempt to differentiate the biological effects of intermittent versus continuous PTH (1–34). The Gene Ontology (GO) database provides a description of a gene product by its molecular function (or activity) and the biological processes with which it is associated. The GO terms utilized by the GO Consortium are nonoverlapping molecular domains, however, each gene product is annotated in each category in which it has been demonstrated to function [Harris et al., 2004]. Statistical testing of the molecular function and biological processes, potentially affected by genes changed with either PTH treatment relative to the number of genes on the chip, was performed as described in Materials and Methods to determine which categories were more significantly affected and are listed in Tables II and III.

TABLE I. Histomorphometry of Tibiae From Rats Treated for 7 Days With PTH (1–34)

Treatment	Labeled surface/ bone surface	Trabecular thickness	Trabecular spacing	Trabecular number	Mineral apposition rate	Bone formation rate/bone surface	Bone formation rate/bone vol.	Bone formation rate/tissue vol.
Control	2.2	65.4	163.8	4.4	1.26	0.03	0.08	0.02
	0.8	9.2	29.4	0.5	0.13	0.01	0.03	0.01
Intermittent	10.6[a]	70.6	171.2	4.1	1.37	0.15[a]	0.42[a]	0.12[a]
	8.0	12.7	9.9	0.2	0.20	0.12	0.31	0.09
Continuous	13.0[a]	65.9	147.5	4.8	N/A	N/A	N/A	N/A
	3.1	8.9	40.6	0.8	N/A	N/A	N/A	N/A

Values are the mean ± SD.
N/A, not available because treatment-induced osteitis fibrosis resulted in diffuse flourochrome labeling.
[a]$P < 0.05$ from control.

Fig. 1. The number of genes changed by PTH (1–34) treatment. Genes were identified by microarray and were changed by each treatment versus control ($P < 0.05$) by at least 1.5-fold and at an intensity of 400 or more. The number of genes common to both treatments is noted in the overlap of the Venn diagram.

Genes Commonly Regulated by Continuous and Intermittent PTH (1–34) Treatment

As expected, the genes associated with skeletal development, such as those indicative of extracellular matrix production and mineralization, were significantly changed as a result of both PTH treatments (Table IV). Increases in matrix-associated genes, such as Col1αI, Col1α2, osteocalcin (Bglap), Sparc, decorin (Dcn), ColVα2, tissue inhibitor of metalloprotease 1 (Timp1), and serine proteinase inhibitor clade H member 1 (Serpinh1), were observed in both intermittent and continuous administration. For many of the genes, the increase was observed with multiple probe sets on the array (representing different regions of the same gene) and several were confirmed by Northern or real-time PCR analysis in an independent experiment (Fig. 2). These results are consistent with an increase in formation activity as reflected by the substantial increases in bone formation rate found in histomorphometric analyses (Table I). The distal-less homeo box 5 (Dlx5) transcription factor, known to be important in bone formation, and the adhesion protein N-cadherin (Cdh2) were similarly upregulated by both PTH treatments. Additional GO categories, representing genes involved with enzyme inhibitor activity, cell migration, neuronal cell adhesion, wounding and stress responses, anti-apoptosis, and signal transduction, were increased and decreased by both treatments and are listed in Tables II and III.

TABLE II. Significant Gene Ontology Biological Process Terms Altered by PTH (1–34) Treatment Relative to Control

	Biological process						
Treatment	Organogenesis	Response to stimulus	Organismal physiological process	Cellular physiological process	Catabolism	Regulation of apoptosis	Cell communication
Common	Skeletal development#	Response to wounding*, response to stress*		Cell migration		Anti-apoptosis	Neuronal cell adhesion
Continuous PTH	Brain development, lung development, angiogenesis	Wound healing, response to oxidative stress	Bone remodeling*, ossification*	ECM organization and biogenesis, membrane organization and biogenesis, substrate-bound cell migration, cell proliferation	Oxygen and reactive oxygen species metabolism, macromolecule catabolism		Protein kinase C activation, cell-cell adhesion, cell-matrix adhesion
Intermittent PTH		Inflammatory response*	Innate immune response*				

Genes changed by intermittent, continuous, or both PTH (1–34) treatments were evaluated for the Gene Ontology terms potentially affected by the expression changes. All terms were determined to be significant at a confidence level of $P < 0.05$ as described in the Materials and Methods. Terms followed by an *were significant at $P < 0.005$ and # indicates a significance of $P < 0.0005$.

Genes Uniquely Regulated by Intermittent PTH (1–34) Treatment

There were fewer genes changed uniquely by intermittent PTH (1–34) as listed in Table V. Most of the known genes segregated into three GO categories: binding, catalysis, and immune response. It was observed that 2 of the 19 unique genes regulated by intermittent PTH [extracellular matrix protein 2 (Sparcl1) and persephin (Pspn)] are known to be expressed in neuronal tissues and are listed in Table VII [Jaszai et al., 1998; Mothe and Brown, 2001]. To our knowledge, these findings represent the first report of their expression and regulation in bone.

In an independent experiment, two genes annotated with a GO term of catalysis [carboxypeptidase E (Cpe) and signal peptidase complex 18kDa (Spc18)] were evaluated for their regulation by PTH treatment (Fig. 3). The significant elevation of Cpe (twofold) by intermittent PTH was confirmed in the validation study. It should be noted, however, that continuous treatment also significantly enhanced Cpe but to a lesser extent. The suppression of Spc18 did not achieve statistical significance upon validation and thus could not be confirmed as a gene uniquely regulated by intermittent PTH treatment.

Genes Uniquely Regulated by Continuous PTH (1–34) Treatment

Many matrix-associated genes were uniquely induced by continuous PTH treatment but were unchanged with intermittent administration (Table VI). Genes associated with collagen turnover (Timp2, Pcolce, MMP2, MMP13, MMP14, and MMP23), the alpha type I chain of several collagens (Types III, V, VIII, XI, and XII), transcripts encoding molecules associated with collagen crosslinking (lysyl oxidase, lysyl oxidase-like, lumican, procollagen-lysine 2-oxoglutarate 5-dioxygenase, fibronectin 1), and genes associated with biomineralization [alkaline phosphatase, integrin binding sialoprotein (Ibsp), and gamma-glutamyl carboxylase] were all upregulated by continuous PTH administration. Cell adhesion proteins, such as osteomodulin (Omd), thrombospondin 4, and periostin, were robustly increased with continuous PTH treatment.

Two additional sets of genes regulated by continuous treatment were those associated

TABLE III. Significant Gene Ontology Molecular Function Terms Altered by PTH (1–34) Treatment Relative to Control

	Molecular function				
Treatment	Enzyme inhibitor activity	Catalytic activity	Transporter activity	Signal transducer activity	Structural molecule activity
Common	Serine-type endopeptidase inhibitor activity#			MCH class II protein binding*	Extracellular matrix structural constituent*
Continuous PTH		Transferase activity transferring nitrogen groups, metalloendopeptidase activity, nuclease activity, phospholipase activity, transmembrane receptor protein tyrosine kinase activity	Calcium ion binding*		
Intermittent PTH		Exopeptidase activity			

Genes changed by intermittent, continuous, or both PTH (1–34) treatments were evaluated for the Gene Ontology terms potentially affected by the expression changes. All terms were determined to be significant at a confidence level of $P < 0.05$ as described in the Materials and Methods. Terms followed by an *were significant at $P < 0.005$ and # indicates a significance of $P < 0.0005$.

with catalysis and signal transduction. In addition to the catalytic metalloproteases mentioned above, several serine proteases and cathepsin K were upregulated with continuous PTH treatment. Signal transduction mediators for growth factors, cytokines, and transcription factors were also induced with continuous treatment. This includes receptors: FGF receptor 1, syndecan-2, discoidin domain receptor 2, frizzled homolog 2, thyrotropin-releasing hormone receptor, PDGFRa, osteoprotegerin, and PTH receptor; and ligands: PDGFa, growth hormone releasing hormone (Ghrh), TGF-β1, wingless-type MMTV integration site family member 5a, secreted frizzled-related protein 4 (Sfrp4), and bone morphogenetic protein 3

TABLE IV. Genes Commonly Regulated by Intermittent and Continuous PTH (1–34)*

		Fold (intermittent)	Fold (continuous)
Adhesion			
Cdh2	N-cadherin	3.4	5.7
Matrix associated			
Bglap	Osteocalcin	2.3	2.6
Col1a1	Collagen I alpha 1	1.7	1.9
Col1a2	Collagen I alpha 2	1.6	1.8
Col5a2	Collagen V alpha 2	2.4	3.7
Dcn	Decorin	2.1	2.4
Serpinh1	Serine protease inhibitor clade H	2.7	4.9
Sparc	Osteonectin	1.9	2.6
Timp1	Tissue inhibitor of metalloprotease 1	2.5	2.4
Catalysis			
Cpz	Carboxypeptidase Z	1.7	2.1
Psat1	Phosphoserine aminotransferase	−1.6	−1.5
Signal transducer activity			
Ptprz1	Protein tyrosine phosphatase receptor-type zeta polypeptide 1	1.7	1.6
Agt	Angiotensinogen	−1.6	−1.8
Nucleic acid binding			
Eif4ebp1	Eukaryotic translation intiation factor 4E binding protein	−1.7	−1.6
Dlx5	Distal-less homeo box 5	1.7	1.9
Hist4	Germinal histone H4	−1.8	−1.8
Ogg1	8-Oxoguanine DNA glycosylase 1	−1.5	−1.7
Chaperone			
Hspa9a	Heat shock 70 kDa protein 9B	−1.7	−1.6
Motor			
Myh13	Myosin heavy polypeptide 13	1.6	1.7
Unknown			
X62325		1.8	2.3
AI638966		−1.7	−1.9
AA892561		1.6	1.7

*In cases where multiple probe sets for genes were changed on the microarray, only the fold change values for the most specific probe set are reported. Gene identity was determined as described in the Materials and Methods and to eliminate redundancy genes are listed in a single gene ontology category even though they may have additional roles in other biological processes.

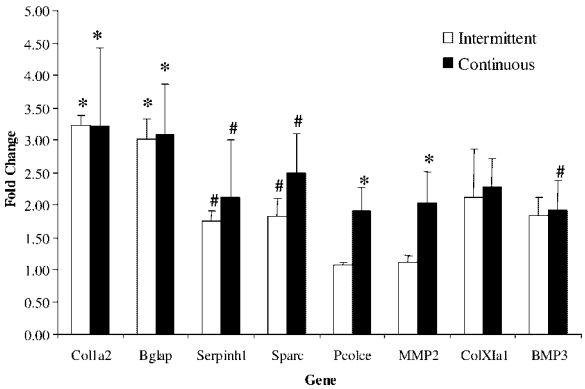

Fig. 2. Validation of genes identified by microarray in a replicated experiment. Five animals were analyzed in triplicate by Northern blot and/or real-time PCR. The group mean ± SEM was calculated and the fold change from control is shown. All genes were normalized to 18S. # indicates group mean is significantly different from control ($P < 0.05$) and * is different from control ($P < .0003$).

(BMP3). BMP-3 mRNA levels were measured in an independent animal study and found to be elevated with both PTH treatments but was only significantly ($P < 0.05$) with continuous PTH (Fig. 2). Two additional transcripts for signal transducing molecules were statistically induced by continuous PTH (1–34) but were not included because they missed the selection criteria; namely TGF-β3 (2.6-fold increase but basal level of 324) and Frizzled homolog 1 (a 1.4-fold increase). A mixture of cell cycle genes that have been associated with increased cell proliferation and with apoptosis were also uniquely regulated with continuous treatment and are listed in Table VI.

Genes that have only previously been described to be regulated in neuronal tissues were regulated in this study by continuous treatment including transcripts encoding for neuropeptides (preprogalanin, Ghrh) and transporters (neuron specific gene 1, orphan transporter v73, and dynamin 1). An additional neuropeptide, prepronociceptin (Pnoc), was expressed at a high basal level in bone (hybridization intensity of 4,000) but was only elevated 1.2-fold, while the neurotransmitter receptor, gamma-aminobutyric acid A receptor alpha 3 was elevated twofold by continuous treatment but had a hybridization intensity below that of our selection criteria (315). See Table VII for a complete listing of neuronal genes regulated by both intermittent and continuous PTH (1–34).

Validation of Neuronal Genes

Real-time PCR was utilized to measure gene expression levels of three neuronal genes, protein tyrosine phosphatase receptor-type zeta polypeptide 1 (Ptprz1), neuron specific gene-1

TABLE V. Genes Uniquely Regulated by Intermittent PTH (1–34)*

		Fold
Immune response		
CD8a	CD8 antigen alpha chain	3.3
CD5	Lymphoocyte antigen CD5	1.7
Icam2	Intracellular adhesion molecule 2	1.6
Catalysis		
Cpe	Carboxypeptidase E	1.9
Capn9	Calpain family of calcium-dependent non-lysosomal protease	1.8
Spc18	Signal peptidase complex 18kDa	−1.6
Binding		
Igfbp6	IGF binding protein 6	1.7
Pspn	Persephin	1.5
Nup54	Nucleoporin p54	−1.7
Nrbp	Nuclear receptor binding protein	−1.5
Pcoln3	Procollagen (type III) N-endopeptidase	−1.5
Structural		
Sparcl1	Extracellular matrix protein 2	1.8
Transport		
Ndufb6	CI-B17 subunit of NADH-ubiquinone oxidoreductase (complex I)	1.7
Hdh	Huntingtin	1.5
Signal transduction		
Olfr353	Olfactory receptor 50	1.6
Unknown		
AI639012		1.9
AI639507		1.5
D84482		1.5
AI639306		−1.5

*In cases where multiple probe sets for genes were changed on the microarray, only the fold change values for the most specific probe set are reported. Gene identity was determined as described in the Materials and Methods and to eliminate redundancy genes are listed in a single gene ontology category even though they may have additional roles in other biological processes.

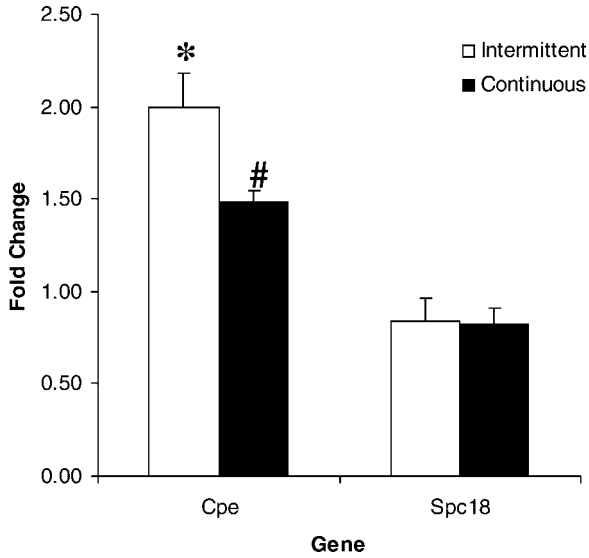

Fig. 3. Validation of genes identified by microarray to be unique to intermittent PTH (1–34) administration in a replicated experiment. Five animals were analyzed in triplicate by real-time PCR. The group mean ± SEM was calculated and the fold change from control is shown. All genes were normalized to 18S in the same PCR run. # indicates group mean is significantly different from control ($P < 0.01$) and * is different from control ($P < .0001$).

(Bsmrb), and preprogalanin (Gal) in an independent animal study. All three genes were found to be expressed in rat femora and Ptpzr1 expression was elevated by both PTH treatments and Bsmrb was induced uniquely by continuous PTH. The increased expression of Gal by continuous PTH in the validation experiment was present but not statistically different from control (Fig. 4).

DISCUSSION

PTH has complex effects on bone cell function depending upon the mode of administration. PTH stimulates bone turnover, but net gain or loss of bone mass is determined by the kinetic profile of systemic PTH [Tam et al., 1982; Hock and Gera, 1992; Dobnig and Turner, 1997; Frolik et al., 2003]. Once daily administration of PTH induces net gain in bone mass in vivo by stimulating osteoblastic activity to a greater extent than osteoclastic activity, while continuous infusion of PTH has variable site specific effects on bone mass and induces peritrabecular marrow fibrosis [Dobnig and Turner, 1997]. Despite the many years of study [Burr et al., 2001; Dempster et al., 2001; Rubin and Bilezikian, 2002], the molecular events responsible for the markedly different skeletal responses to PTH are incompletely understood. Therefore, we attempted to ascertain the molecular networks responsible for the dual effects of PTH by identifying the gene expression patterns and pathways associated with anabolic and catabolic processes in bone. In the present study, microarray analysis and gene ontology classification were used to provide a comprehensive view of the in vivo mRNA changes induced by intermittent and continuous PTH (1–34) treatment in rat bone.

Confidence in the novel molecular changes observed with the array was gained by the observation that the common and unique sets of genes found to be changed in the bone were reflective of the anticipated histomorphometric changes. For example, both PTH treatments caused an increase in bone formation activity, and the microarray detected increases in bone formation genes (Col1α1, Co1α2, Bglap, Dlx5) by both treatment regimes. Continuous PTH results in increased osteoclast number and indices of bone resorption [Tam et al., 1982; Dobnig and Turner, 1997] and was observed to uniquely induce genes on the microarray that were associated with bone turnover and osteoclast-associated genes (i.e., MMPs and cathepsin K). Thus understanding the other unexpected genes that are co-regulated with these known signature genes of bone formation and resorption may yield novel information about the mechanisms of continuous and intermittent PTH (1–34). Additional confidence in these array data was obtained by identifying increased levels of Pdgfa following continuous treatment as previously described in the rat [Lotinun et al., 2002]. Lotinun et al. also observed similar increases in genes encoding bone matrix proteins following treatment with continuous and intermittent PTH (unpublished data). Finally, the confirmation of the same pattern of expression for multiple genes in a second independent experiment further validated these array data (Figs. 2 and 4).

Nearly 40% of the genes commonly regulated by both PTH treatments were matrix-associated transcripts. In addition to the bone formation genes, transcripts encoding molecules, such as Cdh2, carboxypeptidase Z, and Ptprz1, a neuronal proteoglycan, were induced by both PTH treatments (Fig. 4). Only 30% of the genes commonly changed by both treatment regimens were negatively regulated [8-oxoguanine DNA glycosylase 1, germinal histone H4 (Hist4),

TABLE VI. Genes Uniquely Regulated by Continuous PTH (1–34)*

		Fold
Adhesion		
Osf2	Periostin	7.4
Omd	Osteomodulin	5.2
Thbs4	Thrombospondin 4	4.6
Fn1	Fibronectin	1.6
Esm1	Pineal specific PG25 protein	−1.7
Binding		
Rcn3	Reticulocalbin 1	15
Egfl3	Epidermal growth factor-like domain multiple 3	2.9
Ril	Reversion-induced LIM protein	2.6
N4wbp4	Nedd4 WW-binding protein 4	2.1
Par3	Partitioning defective 3 homolog	2
Fstl	Follastatin-like 1	1.9
A430107P09Rik	RIKEN cDNA A430107P09	1.8
Pls3	t-plastin	1.8
S100A4	S100 calcium-binding protein A4	1.8
Limr	Lipocalin 1-interacting membrane receptor	1.7
Gnao	G protein alpha subunit o1	1.7
Tjp1	Tight junction protein 1	1.7
Rcn	Reticulocalbin	1.6
Crtap	Cartilage associated protein	1.5
Dlgh4	Discs large homolog 4	1.5
Sbp	Serpmine binding protein	1.5
Umod	Uromodulin	1.5
Structural Molecule		
Tagln	Transgelin	4.3
Ibsp	Integrin binding sialoprotein	1.7
Chaperone		
Tra1	Tumor rejection antigen 1	1.8
Motility		
Tpm2	Member of the tropomysoin family	2.5
Enpp2	Autotaxin	2
Tpm1	Tropomyosin 1 alpha	2
Mles	Myosin light chain 1	1.8
Csrp2	Cystein-rich protein 2	1.6
Catalysis		
Gpx7	Glutathion peroxidase 7	5.4
Aldh7a1	Antiquitin	2.4
Dao1	D-amino-acid oxidase	2.4
Cyp1b1	Cytochrome P4501b1	2.3
Alpl	Tissue-non-specific alkaline phosphatase	2.1
Ggcx	Vitamin K dependent gamma glutamyl carboxylase	2
Pla2g5	Phospholipase A2 group V	2
Facl3	Fatty acid coenzyme A ligase long chain 3	1.7
Myh3	Embryonic skeletal muscle myosin heavy polypeptide 3	1.7
Cyp2a1	Cytochrome P450IIA1	1.6
Rnase4	Ribonuclease 4	1.6
Arhc	Ras homolog 9	1.5
Bace	Beta-site APP-cleaving enzyme	1.5
Cask	Ca+/calmodulin-dependent serine protein kinase	1.5
Ctsk	Cathepsin K	1.5
Fut1	Fucosyltransferase 1	1.5
Gale	UDP-galactose-4-epimerase	1.5
Nedd4	Neural cell precursor expressed developmentally downregulated 4	1.5
Oat	Ornithine aminotransferase	1.5
Pgcp	Plasma glutamate carboxypeptidase	1.5
Ptk9	Protein tyrosine kinase 9	1.5
Rnlip	Lingual lipase	1.5
Fmo1	Flavin-containing monooxygenase 1	−1.5
Cdc34	Cell division cycle 34	−1.6
Ddx1	DEAD/H-box polypeptide 1	−1.6
A2M	Alpha-2-macroglobulin	−1.8
Gpt	Glutamic-pyruvate transaminase	−1.9
Ass	Argininosuccinate synthase 1	−2
Sult1a1	Sulfotransferase family 1A phenol-preferring 1	−2.1
Death		
Lgals1	Beta galactoside binding lectin 1	3.4
Aplp2	Amyloid precursor-like protein 2	1.8
Pawr	PRKC apoptosis WT1 regulator	1.8
Btg2	B-cell translocation gene 2	−1.5
Casp2	Caspase 2	−1.5
Development		
Gp38	Podoplanin	2.6
Motor		
Myo1b	Myosin 1b	1.8

TABLE VI. (*Continued*)

		Fold
Stress		
Klrb1b	Killer cell lectin-like receptor subfamily B member 1B	1.6
Defense		
Loc192264	Eosinophil associated ribonuclease 1	1.7
Igtp	Interferon gamma induced GTPase	1.6
Igkab	Immunoglobulin kappa chaining variable region 1–9	1.5
Collagen		
Lox	Lysyl oxidase	14
Col3a1	Collagen 3 alpha 1	5.9
Mmp2	Gelatinase A	4.8
Col5a1	Collagen 5 alpha 1	4.1
Pcolce	Procollagen c-proteinase enhancer	4.1
Col11a1	Collagen 11 alpha 1	3.2
Mmp14	Matrix metalloproteinase 14	2.8
Mmp23	Matrix metalloproteinase 23	2.6
Col12a1	Collagen 12 alpha 1	2.5
Loxl	Lysyl oxidase-like	2.4
Col8a1	Collagen 8 alpha 1	2.2
P4ha1	Proline 4-hydroxylase alpha subunit	2.1
Lum	Lumican	2
Plod2	Procollagen-lysine 2-oxoglutarate 5-dioxygenase	2
Anxa5	Annexin V	1.8
Mmp13	Matrix metalloproteinase 13	1.5
Timp2	Tissue inhibitor of metalloproteinase 2	1.5
Cell cycle		
Cgr11	Cell growth regulatory gene 11	7.1
Rcl	Nuclear protein induced by c-Myc	−1.5
Cdkn1b	Cyclin dependent kinase inhibitor 1B	−1.6
Cytokine		
Pdgfa	PDGF alpha	3
Pdgfra	PDGF receptor alpha	2
Tnfrsf11b	Osteoprotegerin	2.2
Enzyme regulation		
Wfdc1	WAP four-disulfide core domain 1	3.5
Prkcdbp	PCK-delta binding protein	1.9
Serpine1	Plasminogen activator inhibitor 1	1.8
Serpine2	Nexin	1.6
Madd	MAP kinase activating death domain	−1.5
Kng	Kininogen	−1.7
Transcription regulators		
Mad4	Max dimerization protein 4	3
Jundp2	Jun dimerization protein 2	2
Shox2	Short stature homeobox 2	1.8
Pmx1	Paired mesoderm homeobox 1	1.7
Klf4	Kruppel-like factor 4	1.6
Nupr1	Nuclear protein 1	1.6
Stat1	Signal transducer and activator of transcription 1	1.5
Signal transduction		
Plcd1	Phospholipase C delta 1	4.5
Fgfr1	FGF receptor 1	4.4
Sfrp4	Secreted frizzled-related protein 4	2.7
Gal	Preprogalanin	2.3
Gpc1	Glypican 1	2.3
BMP3	Bone morphogenetic protein 3	2.2
Pthr	Parathyroid hormone Receptor	2.1
Trhr	Thyrotropin releasing hormone receptor	2.1
Ddr2	Discoidin domain receptor 2	2
Fhl2	Four and a half LIM domains 2	2
Ptgfrn	Prostaglandin F2 alpha receptor regulatory protein	2
Sdc2	Syndecan-2	2
Crkas	Crk-associated substrate	1.9
Ryk	Receptor-like tyrosine kinase	1.9
Fzd2	Frizzled homolog 2	1.8
Apbb1	Amyloid beta precursor protein-binding family B member 1	1.7
Mox2	OX2 membrane glycoprotein	1.6
Wnt5a	Wingless-type MMTV integration site family member 5a	1.6
App	Amyloid beta precursor protein-binding family B member 1	1.5
Cd9	CD9 antigen	1.5
Edg2	Endothelial differentiation lysophosphatidic acid G protein-coupled receptor 2	1.5
Fyn	Fyn proto-oncogene	1.5
Ghrh	Growth hormone releasing hormone	1.5
Brca1	Breast cancer 1	−1.5
Rara	Retinoic acid receptor alpha	−1.6

(*Continued*)

TABLE VI. (*Continued*)

		Fold
Transporter activity		
Bsmrb	Neuron specific gene 1	8.4
Copz2	COPI zetz2 subunit	3.5
Ntt73	Orphan transporter v73	2.6
Cacna1g	Calcium channel alpha 1G subunit	1.8
Atp1a1	Alpha 1 subunit of the Na+K+ transporting ATPase	1.6
Slc8a3	Solute carrier family 8 member 3	1.6
Cacnb3	Calcium channel subunit beta 3	1.5
Nup62	Nuclear pore glycoprotein p62	−1.5
Aqp3	Aquaporin 3	−1.6
Slc28a2	Solute carrier family 28 member 2	−2
Transport		
Dnm1	Dynamin 1	2.9
Selel	Golgi apparatus protien 1	2
Vap1	Vesicle-associated protein 1	1.5
Nucleic acid binding		
Sc65	Synaptonemal	3.3
Fkh3	Forkhead homolog 3	2.2
Rbm9	RNA binding motif protein 9	2
Nbl1	Neuroblastoma candidate region suppression tumorigenicity 1	1.7
Slbp	Histone mRNA 3′ stem-loop binding protein	−1.5
Metabolism		
Ckb	Brain creatine kinase	1.5
Unknown		
AA866443		3.2
AA893846		2.6
AA874875		2.5
AA875232		2.3
AA866419		2.2
AI639299		2.2
AI175776		2.1
U48828		2.1
AA859643		2
AA893454		1.9
AA874978		1.9
AA859661		1.8
AA894305		1.8
AA891950		1.7
D00920		1.7
AA894088		1.5
AA800597		1.5
U75921		1.5
AI235631		1.5
AA800188		−1.7
U16359		−1.9
AA874803		−2
AA860014		−2.4

*In cases where multiple probe sets for genes were changed on the microarray, only the fold change values for the most specific probe set are reported. Gene identity was determined as described in the Materials and Methods and to eliminate redundancy genes are listed in a single Gene Ontology category even though they may have additional roles in other biological processes.

phosphoserine aminotransferase, and eukaryotic translation initiation factor 4E binding protein 1, angiotensinogen, and heat shock 70 kDa protein 9B (Hspa9a)] and the remainder of the mRNA changes were found to be elevated from control. The suppression of the genes associated with proliferation (i.e., Hist4 and Hspa9a) further supports the suggestion that intermittent and continuous PTH treatments do not induce proliferation of osteoblasts but modulate the activity of existing bone lining cells [Dobnig and Turner, 1995].

Intermittent PTH had minimal unique changes (Table V) yet these genes are of great interest given that they are associated with an anabolic response in bone. Carboxypeptidase E expression was confirmed to be elevated in response to intermittent PTH treatment in an independent experiment; however, continuous PTH also significantly elevated its expression (to a lesser extent). This highlights the importance of validating changes observed by microarray in independent experiments and the need to determine whether these genes are truly unique to intermittent administration of PTH in future experiments.

Additional molecular changes associated with the intermittent PTH treatment were genes that heretofore had only been described as expressed in nervous tissues in the rat, such

TABLE VII. Neuronal Genes Regulated by PTH (1–34)[a]

		Fold	Intensity[c]
Continuous treatment			
Bsmrb	Neuron specific gene 1	8.4	1,460
Dnm1	Dynamin 1	2.9	1,120
Ntt73	Orphan transporter v73	2.6	400
Gal	Preprogalanin	2.3	760
Gabra3[b]	Gamma-aminobutyric acid A receptor alpha 3	2.0	313
Ryk	Receptor-like tyrosine kinase	1.9	924
Shox2	Short stature homeobox 2	1.8	472
Apbb1	Amyloid beta (A4) pecursor protein-binding family B member 1	1.7	1,030
Argbp2[b]	Arg/abl-interacting protein	1.6	345
Dlgh4	Discs large homolog 4	1.5	1,360
Fyn	Fyn proto-oncogene	1.5	3,420
Ghrh	Growth hormone releasing hormone	1.5	3,140
Ddn[b]	Dendrin	1.4	1,000
Pnoc[b]	Prepronociceptin	1.2	4,000
Intermittent treatment			
Sparcl1	Extracellular matrix protein 2	1.8	994
Pspn	Persephin	1.5	966
Common to both treatments			
Ptprz1	Protein tyrosine phosphatase receptor-type zeta polypeptide 1	1.7	466

[a]As annotated by Human PSD™ (Incyte Genomics).
[b]Additional genes noted as neuronal but did not achieve filtering criteria of fold change or hybridization intensity.
[c]For comparison to intensity of commonly associated bone genes: decorin = 4,000, Col8a1 = 800, and PTH receptor = 2,000 in the same experiment.

as the Sparcl1 glycoprotein and Pspn, a member of the neurotrophic glial cell-derived neurotrophic factor family. Continuous PTH treatment also uniquely induced a subset of neuronal transcripts and pointed to the expression of several neuropeptide precursors (Ghrh, Gal, and Pnoc) in bone and a transcription factor short stature homeobox 2 that was only thought to be expressed in adult brain. The basal expression of these genes was well above background and was similar to the levels of expression of well-known bone transcripts such as Dcn or PTH receptor (PTHr). Sensory and sympathetic nerve fibers in rodent femora are especially concentrated in the periosteum but are also present in mineralized bone and bone marrow [Mach et al., 2002]. Because our sample preparation removed the periosteal layer, we were surprised to see such high expression levels of genes associated with neural tissue. We found these genes to be expressed at similar levels in additional micorarray data from our lab that profiled genes expressed in both intact and ovariectomized rat metaphysis (data not shown), and confirmed the expression of three genes (Gal, Bsmrb, and Ptpzr1) in an independent PTH study. The repeated animal study confirmed that continuous PTH significantly regulates Bsmrb and that both intermittent and continuous administration of PTH significantly increases the expression of Ptpzr1 (Fig. 4).

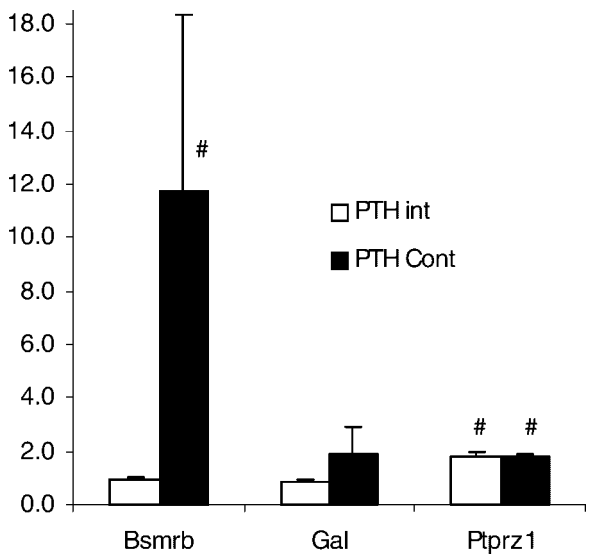

Fig. 4. Validation of neuronal genes identified by microarray in a replicated experiment. Five animals were analyzed in triplicate by real-time PCR. The group mean ± SEM was calculated and fold change from control is shown. All genes were normalized to 18S in the same PCR run. # indicates group mean is significantly different from control ($P < 0.05$).

Further evaluation of the regulation and function of these genes is necessary to clarify their role in bone. Certainly there is a growing body of evidence suggesting a role for various neuronal-associated factors, such as hypothalamic leptin [Takeda et al., 2002], glutamate receptors [Chenu, 2002], serotonin receptors [Bliziotes et al., 2001], proenkephalin [Rosen and Bar-Shavit, 1994; Goss et al., 2002], and

others that suggest brain-bone signaling pathways may be important in bone metabolism.

Continuous PTH had a much larger effect on gene expression and was most evident in those groups of genes associated with catalysis (serine proteases and inhibitors, metalloproteases and cathepsin K), signal transduction (increases in growth factors and cytokines), transport, and binding. Continuous PTH also regulated genes that could result in cell proliferation changes. Some of the gene alterations, such as increased nuclear protein 1 and decreased B-cell translocation gene 2, could be indicative of cell proliferation. However, anti-proliferative cell responses would also be expected to result from decreases of other genes (cell division cycle 34, and the increases of cell growth regulatory gene 11, beta galactoside binding lectin1, PRKC apoptosis WT1 regulator, neuroblastoma candidate region suppression tumorigenicity 1, and reversion-induced LIM protein). This mixed profile of cell cycle effectors could be a function of the effects of continuous PTH on multiple cell types (osteoblasts, osteoclasts, and fibroblasts) in the bone. Thus, the gene expression data reflect the composite of these effects on different cell populations in the distal femur metaphysis. The marked enhancement of gene expression associated with matrix degradation and catalysis reflects the strong effects of continuous PTH on bone turnover. The data suggest that the increased production of matrix metalloproteases (MMP2, MMP13, MMP14, and MMP 23) and many serine proteases in the metaphysis found in continuously treated rats versus that of intermittent treatment may contribute to the catabolic outcome of sustained PTH treatment.

BMP3 was uniquely regulated by continuous PTH in the microarray and the validation studies. This is the first report of BMP3 regulation by PTH in vivo. The significance of BMP3 upregulation during PTH treatment is difficult to ascertain, as the role of BMP3 in bone formation is still controversial. Increases in BMP3 mRNA in cultured human osteoblasts and bone marrow stromal cells have been noted with $1,25(OH)_2D_3$ treatment [Faucheux et al., 1999] while an additional in vitro report noted a decrease with PTH treatment [Qin et al., 2003] that is discussed in more detail below. It has been proposed that BMP3 is a negative regulator of bone because knockout mice have increased trabecular metaphyseal bone [Daluiski et al., 2001], however, BMP3 fusion proteins embedded in collagen matrices were osteoinductive in two rat models [Han et al., 2002] and its protein is intensely expressed during human fracture healing [Kloen et al., 2003]. While our study shows significant increases only with continuous PTH, it appeared that intermittent PTH treatment also enhanced BMP3 expression (Fig. 2). BMP3 is structurally distinct from its other osteoinductive family members and is the BMP most closely related to TGFβ [Centrella et al., 1994]. It may be that BMP3 induction in bone may exhibit the same dichotomous responses as that of TFGβ, both osteoinductive [Marcelli et al., 1087; Joyce et al., 1990; Beck et al., 1991] and inhibitory to formation [Erlebacher and Derynck, 1996].

While previous studies have identified individual genes to be regulated by PTH with a variety of methods, only one in vitro study in a rat osteosarcoma cell line (UMR106) utilized a microarray approach [Qin et al., 2003]. When our in vivo rat data were compared to that of in vitro PTH treatment, only two genes were found to be similarly regulated by intermittent PTH (Dcn and TIMP1). Our continuous treatment however, had 12 genes regulated in common with the in vitro analysis of Qin et al. (Dcn, TIMP1, fibronectin, lumican, Col3α1, MMP13, Ghrh, cytochrome P450 1b1, Omd, ectonucleotide pyrophosphatase-phosphodiesterase 2, Sfrp4, and kininogen). Recapitulating the anabolic effects of PTH in vitro has been difficult to achieve, and our results demonstrate that the gene changes observed with UMR106 cells resemble more closely those of a PTH catabolic profile in vivo. Furthermore, it is noted that four genes that had been shown to be suppressed by PTH in vitro were actually increased in the current in vivo study (Ibsp, Pthr, solute carrier family 8 member 3, and BMP3). An additional factor possibly contributing to the differences observed between the in vitro data of Qin et al. and this study (which was conducted in normal aged rats) is that UMR106 is an osteosarcoma cell, which by definition, may display aberrant cell regulation.

It should be noted that the present study is far from a complete representation of all PTH regulated genes, but merely a snapshot of the mRNA changes observed 24 h after 7 days of intermittent and after 7 days of continuous PTH treatment in the rat femoral metaphysis. These changes in gene expression may stem from the true induction/suppression of specific gene

transcription or from stabilization/destabilization of mRNA in the cell population. Alternatively, changes in gene expression could stem from enrichment/recruitment of different cell populations in PTH-treated groups relative to control animals [Calvi et al., 2003] particularly in the case of continuous PTH treatment that results in increased numbers of fibroblasts and osteoclasts [Lotinun et al., 2002]. These genes would reflect the expression fingerprint of the changing cellular residents. These mechanisms cannot be distinguished since all have been shown to contribute to PTH action in bone. Given the differences in PTH exposure/pharmacokinetics between intermittent and continuous treatments [Tam et al., 1982; Hock and Gera, 1992; Dobnig and Turner, 1997; Frolik et al., 2003], extended studies evaluating both dose- and time-dependent changes in gene expression are needed to determine which changes occur due to differences in kinetics of regulation.

In summary, a comprehensive analysis of the genes that are differentially regulated in vivo by intermittent and continuous PTH using a gene array approach was conducted. These data support earlier histomorphometric observations and highlight important similarities and differences between intermittent and continuous PTH. Many genes such as Ptprz1, Gal, Bsmrb that were previously not considered to be expressed in bone or to be regulated by either PTH treatment regimen were identified and validated (Fig. 4) and the upregulation of BMP3 by PTH treatment was also described (Fig. 2). The function of these newly identified genes in bone and their precise role, if any, in the anabolic and catabolic bone state deserves further investigation. Finally, when these in vivo data were compared to a previously reported profiling of PTH-induced gene changes of an osteosarcoma cell line, it was observed that the in vitro gene changes are most similar to the catabolic continuous PTH (1–34) treatment of the current study. These findings provide a basis for further molecular studies aimed to understand more fully the complex dual actions of PTH in bone metabolism.

MATERIALS AND METHODS

Animals and Study Drug

Synthetic human PTH (1–34), (Bachem, Torrance, CA) was prepared in a vehicle of acidified saline containing 2% heat-inactivated rat serum. For the array experiment, five virus-antibody-free, 6 month-old virgin female Sprague-Dawley rats (Harlan, Madison, WI) were housed with a 12 h light-dark cycle. Animals were fed Purina chow [(calcium 1%, phosphate 0.61%): PMI Feeds, Inc., St. Louis, MO] and water ad libitum. At the end of the experiment, tibiae were removed for histomorphometric analysis and femora collected for array analysis. For the repeat validation study, eight, 6-month-old female Sprague-Dawley rats (Harlan, Indianapolis, IN) were fed Teklad chow (TD 89222 with 0.5% calcium and 0.4% phosphate, Madison, WI) and were housed as above. For intermittent treatment, animals were injected sc with 80 μg/kg/day for 7 days and were sacrificed 24 h after the last dose. For continuous treatment animals, PTH (40 μg/kg/day) was administered via an osmotic mini-pump continuously for 7 days up to the time of sacrifice. Vehicle controls were administrated saline once daily for 7 days and in the validation study, a second control group was administered a continuous dose of saline via mini-pump. All animal protocols were approved by the appropriate institutional animal care and use committee to ensure compliance with NIH guidelines.

Histomorphometry

Histomorphometric procedures were carried out using an imaging system that has been described in detail previously[Dobnig and Turner, 1995, 1997].

Validation of Gene Changes

RNA was isolated from the distal femoral metaphysis of rats used in the original and validation study with 30 ml of Ultraspec^{TM} RNA isolation reagent as described by the manufacturer (Biotecx, Houston TX). Thirty micrograms of purified RNA were run on 1% formaldehyde/agarose gels and subsequently transferred to Nytran membranes (Schleicher and Schuelle, Keene, NH) following electrophoretic separation. The RNA was then crosslinked to the membrane in a UV Stratalinker 1800 (Stratagene, La Jolla, CA) and hybridized as described [Church and Gilbert, 1984]. Probes used in Northern analysis were generated by random priming cDNA fragments with a High-Prime DNA Labeling Kit (Roche Indianapolis, IN) and [33P] dCTP (3000 Ci/mMol, Amersham) for MMP2 (accession NM_031054) cDNA from bp 518 to 1044, procollagen C-proteinase enhancer

protein (Pcolce, accession AF016503) from bp 57 to 441, collagen type XI α 1 (ColXIα1, accession XM_124165) bp 4415–5034, and osteonectin (Sparc, accession J03040) bp134–437. Washed membranes were exposed to phosphor imaging plates, and relative mRNA levels were quantified on a Molecular Dynamics Storm (Sunnyvale, CA). All genes were normalized to the expression of 18s RNA following stripping and reprobing of the membrane.

For real-time quantitative RT-PCR, an ABI Prism Sequence Detection System 5700 was used and the primer-probe sets for the genes described were obtained from Applied Biosystems as Assay-on Demand™ reagents (Foster City, CA). Before cDNA synthesis, 5 μg of total RNA were DNase-treated for 30 min at 37°C (DNA-free kit, Ambion, Austin, TX). RNA was reverse-transcribed from random hexamer primers using the SUPERSCRIPT II RT kit. (Invitrogen, Carlsbad, CA). Specific amplification reactions from the cDNAs were carried out via a two-step real time PCR, and relative quantities were obtained by generating a standard curve for each gene. For normalization, amplification of 18S ribosomal RNA was performed for each sample in the same PCR run.

Microarray

Detailed methods for labeling, the samples and subsequent hybridizations to the arrays are available from Affymetrix (Santa Clara, CA). Briefly, total RNA was cleaned using Rneasy Mini kit (Qiagen, Valencia, CA) and quality was examined by using ratio of 28s to 18s rRNA. Ten micrograms of high quality total RNA was converted to double-stranded cDNA (SuperScript cDNA synthesis kit, Invitrogen) priming the first strand synthesis with an T7-(dT)24 primer containing a T7 polymerase promoter. The cDNA was purified by phenol/chloroform/isoamyl alcohol extraction (25:24:1, Invitrogen), ethanol precipitation, and dissolved in 12μl of RNAse-free water. Five microliters of cDNA was used as a template to generate biotinylated cRNA using the incorporated T7 promoter sequence in an in vitro transcription system (Bioarray High Yield RNA Transcript Labeling Kit (Enzo, Farmingdale, NY). The cRNA was cleaned with RNeasy Mini Kit (Qiagen) and fragmented at 94°C for 35 min. Control B2 oligonucleotide and bioB, bioC, bioD, and cre spikes were added to 10 μg of cRNA,, which were then hybridized to duplicate rat U34A oligonucleotide arrays (Affymetrix) for 16 h at 45°C with constant rotation at 60 rpm. After sample hybridization, arrays were washed, stained and scanned as described in the standard protocol in the Gene Chip Expression Analysis Technical Manual (Affymetrix). An absolute analysis was performed using the Microarray Analysis Suite v5.0 software and with a scaling factor of 1,500.

Statistical Analysis

Statistical analysis was done in the SAS system using PROC MIXED (Cary, NC). The Affymetrix's average difference intensity (ADI) was used as the measure of transcript expression. The duplicate chip values of ADI were averaged for each rat. The five average ADI values for each treatment group were the data used in the analysis of variance (ANOVA) for each probe set. ANOVA P-values were used for the individual treatment comparisons. Because of the biological replication, the ANOVA could be computed for each probe set separately. This method allows a separate measure of biological variation to be computed for each probe set using the $15-3=12$ degrees of freedom for biological error, where 15 is the total number of observations and 3 is the number of treatment groups. The P-values are then ranked and the $P<0.05$ threshhold was used to select probe sets for additional bioinformatic evaluation. Genes were reported if their intensity was greater than or equal to 400 and if they were altered 1.5-fold or more from control values at a significance of $P<0.05$.

Bioinformatics Analysis

Target sequences for each chip were downloaded from the Affymetrix website and then compared to the NCBI genome builds, to UniGene, and to RefSeq transcripts with BLAT [Kent, 2002]. Annotations from these sources were used to map the probesets both to LocusLink IDs and to full-length sequence IDs. The LocusLink IDs were mapped into the HumanPSD™ database [Hodges et al., 2002] via indices provided by that database. Note that this database aspires to contain the full protein complement of mouse and rat as well as human. For probesets without a LocusLink based mapping, the full-length sequences were compared to the protein sequences in HumanPSD™. Alignments with at least 100 amino acids of 100% identity and/or a BLAST e value of better than 1e-20 were recorded. Multiple identifications

at the same reliability level were suppressed as potential conflicts. Functional information including Gene Ontology [Harris et al., 2004] classification, gene names, and descriptions were retrieved from HumanPSD™.

To determine the statistical significance of the numbers of genes changed by either PTH (1–34) treatment, the proportion of genes in a given Gene Ontology [Ashburner et al., 2000] term was calculated. For each treatment, the proportion of genes in a given term relative to the total number of genes in that group was calculated. Significance of over-representation, when k representors are present in sample of size n, was calculated based on the hypergeometric cumulative distribution function:

$$p = 1 - \sum_{i=0}^{k-1} \frac{\binom{M}{i}\binom{N-M}{n-i}}{\binom{N}{i}}$$

where N is the number of genes on the chip, M is the number of genes on the chip in the gene ontology term of interest, n is the number of genes that changed in the treatment group, and k is the number of genes in the treatment group, which are in the gene ontology term of interest. N, M, n, and k are integers such that $0 \le M \le N$, $0 < n \le N$. These calculations were done on software written by Eli Lilly & Co., however, comparable software is available [Boyle et al., 2004].

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