J Bone Miner Metab (2001) 19:207–212

Journal of
Bone and
Mineral
Metabolism


*Original articles*

# Parathyroid hormone increases the expression level of matrix metalloproteinase-13 in vivo

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**Abstract** Parathyroid hormone (PTH) increases serum calcium (Ca) by enhancing bone resorption and renal Ca reabsorption. However, detailed mechanisms of enhanced bone resorption by PTH remain to be elucidated. Although PTH has been shown to increase the expression level of osteoblastic matrix metalloproteinase (MMP)-13 in vitro, only limited results are available regarding the in vivo regulation of MMP expression. In the present study, we have examined expression levels of MMPs in PTH-infused rats. Infusion of 1.5 or 2.0 nmol/kg/day rat PTH(1–34) for 3 days resulted in a dose-dependent increase in serum Ca. PTH infusion also decreased serum phosphate levels and increased urinary excretion of Ca and phosphate. Infusion of PTH for 7 days resulted in less severe hypercalcemia and hypophosphatemia. Urinary Ca and phosphate excretion in rats infused for 7 days was less than that in rats infused for 3 days. Northern blot analysis showed that PTH infusion increased the expression level of MMP-13 in calvaria, although it did not affect MMP-2 expression. Furthermore, the time-course and severity of hypercalcemia and hypercalciuria correlated with the expression level of MMP-13. In situ hybridization also showed that PTH infusion increased the expression level of MMP-13 in femora. These results indicate that PTH enhances MMP-13 expression in vivo and suggest that PTH stimulates bone resorption at least partly by enhancing MMP-13 expression.

**Key words** matrix metalloproteinase-13 · parathyroid hormone

## Introduction

Parathyroid hormone (PTH) plays a pivotal role in the regulation of serum calcium (Ca) levels. PTH increases serum Ca by enhancing bone resorption and renal Ca reabsorption. However, detailed mechanisms of the actions of PTH on both bone and kidney remain to be elucidated. For example, although PTH stimulates renal Ca reabsorption, the molecule responsible for Ca reabsorption in PTH-sensitive distal tubules has not been identified. In addition, PTH affects bone metabolism in many ways, and conflicting effects of PTH on osteoblasts and bone formation have been reported, depending on the experimental system used [1]. The mechanisms by which PTH enhances bone resorption also seem variable. Recent identification of osteoclast differentiation factor (ODF)/receptor activator of NF-kappaB ligand (RANKL) indicates that PTH enhances bone resorption at least partly by enhancing the expression of ODF/RANKL, thereby increasing osteoclast formation and stimulating osteoclast activity [2]. In addition, there is much evidence that PTH modulates protease activity produced by osteoblasts. For example, PTH stimulates plasminogen activator activity mainly by suppressing the expression level of plasminogen activator inhibitor-1 [3]. Furthermore, results from in vitro studies indicate that PTH increases the expression of metalloproteinase (MMP)-13 by enhancing the transcription of this gene [4–7]. It has also been shown that binding sites for both activator protein (AP)-1 and corebinding factor alpha 1 (Cbfa1) are involved in the PTH-induced enhanced expression of MMP-13 [6–10]. However, there are only limited data concerning the in vivo effects of PTH on these proteases. In the present study, the effects of PTH on MMPs were evaluated using an in vivo rat model. Our results indicate that infusion of PTH enhances the expression of MMP-13 in vivo, although it has no effect on the expression of MMP-2. In addition, the time-course and severity of hypercalcemia and hypercalciuria correlated with expression levels of MMP-13. These results suggest that the enhancement of bone resorption by PTH is at least partly mediated by its action on MMP-13 expression.

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Received: June 5, 2000 / Accepted: January 12, 2001

## Materials and methods

### *Materials*

Rat PTH(1–34) was obtained from Peninsula Laboratories (Belmont, CA, USA). ISOGEN and 50× Denhardt's solution were purchased from Wako Pure Chemical Industries (Osaka, Japan). Hybond $N^+$ Nylon membrane, the rediprime DNA labeling system, [α-$^{32}$P]dCTP and Hyperfilm were purchased from Amersham (Arlington Heights, IL, USA). Proteinase K, ribonuclease A and the DIG detection kit for in situ hybridization were obtained from Boehringer Mannheim (Mannheim, Germany).

### *Animals*

Seven-week-old male Wistar rats (Japan Charles River, Yokohama, Japan) were housed in groups on a 12-h light/dark cycle (lights on from 0800 to 2000 h) at 24°C, and were fed with standard laboratory chow (CE-2; Japan Clea, Tokyo, Japan) with free access to water. After 1 week acclimation, rats were housed singly and were divided into six groups of 9 to 12 rats for each experiment. At 8 weeks of age, rat PTH(1–34) or vehicle infusion started. Rat PTH(1–34) in ε-aminocaproic acid or the vehicle alone was administered continuously by an Alzet mini-osmotic pump (model 2002; Alzet, Palo Alto, CA, USA) subcutaneously implanted on the back of the rats. Experimental groups received either 1.5 or 2.0 nmol/kg/day rat PTH(1–34) for 3 or 7 days, whereas control groups were infused with vehicle alone for 3 or 7 days. Although rats were not pair-fed, increases in body weight were not different among the groups during the experiments. After 3 and 7 days infusion, 24-h urine samples were collected in metabolic cages and blood samples were obtained. Rats were anesthetized with diethyl ether and were killed by cervical dislocation. Calvaria and femoral bones were immediately frozen in liquid nitrogen and stored at −80°C until use.

### *Biochemical measurements*

Serum and urinary calcium (Ca), phosphate (Pi) and creatinine were measured by an autoanalyzer (Hitachi 7050; Hitachi, Tokyo, Japan).

### *Northern blotting*

Total RNA was isolated from calvaria using ISOGEN by following the manufacturer's instructions and was stored in ethanol at −70°C. RNA from rats in the same treatment groups was pooled and 20 μg total RNA was separated by electrophoresis on 2.2 M formaldehyde–1.0% agarose gels containing 100 μg/ml ethidium bromide. The RNA was transferred to a Hybond $N^+$ Nylon membrane and fixed by UV cross-linking. Prehybridization was performed in 2× standard sodium citrate phosphate (SSCP) (0.24 M NaCl, 30 mM $Na_3$ citrate, 30 mM $Na_2HPO_4$ and 10 mM $NaH_2PO_4$), 1× Denhardt's solution, 1% sodium dodecyl sulfate (SDS) and 100 μg/ml sheared salmon sperm DNA (ssDNA) at 65°C for at least 4 h. Hybridization was performed in 4× SSCP, 10% dextran sulfate, 1× Denhardt's solution, 1% SDS, 100 μg/ml ssDNA and a $^{32}$P-labeled DNA probe at 65°C overnight. The filter was washed in 1× SSC (0.15 M NaCl, 15 mM $Na_3$ citrate)–0.1% SDS four times for 20 min at 65°C, and then once for 20 min in 0.1× SSC–0.1% SDS at 65°C. Northern blots were analyzed by autoradiography (Hyperfilm; Amersham) and bands were quantified by a Molecular Imager (BioRad, Hercules, CA, USA). Membranes were stripped by washing in 50% formamide-1× SSC at 65°C for 3 h, and then prehybridized and rehybridized as described above. All data were normalized to the expression level of glyceraldehyde phosphate dehydrogenase (GAPDH).

### *DNA probes*

The cDNAs for mouse MMP-2, MMP-13 and GAPDH were obtained by reverse transcription–polymerase chain reaction (RT-PCR) using the total RNA from MC3T3-E1 cells as a template. The cDNA for mouse Cbfa1 was similarly amplified from primary cultured osteoblasts of mouse calvaria. For MMP-2 cDNA, the PCR primers were 5′-AAGGATGGACTCCTGGCA CATGCCTTT-3′ and 5′-ACCTGTGGGCTTGTCAC GTGGTGT-3′. These primers were expected to produce a 963-bp fragment. For MMP-13 cDNA, the PCR primers were 5′-CATTCAGCTATCTGGCCAC CTTC-3′ and 5′-CATCCACATGGTTGGGAAGTT CTG-3′ with an expected 1016-bp fragment. For GAPDH cDNA, the PCR primers were 5′-TGAAGG TCGGTGTGAACGGATTTGGC-3′ and 5′-CATGT AGGCCATGAGGTCCACCAC-3′. For Cbfa1 cDNA, the primers used were 5′-ATGCTTCATTCG CCTCACAAACG-3′ and 5′-GATCGTTGAACCTG GCTACTTGG-3′ with an expected 778-bp fragment. Amplification was performed for 35 cycles (30 s at 95°C, 45 s at 60°C, 2 min at 72°C). The fragments generated were purified by agarose gel electorphoresis, and then ligated to a TA-cloning vector (pCRII vector; Invitrogen, Carlsbad, CA, USA). The DNA sequence of each cloned fragment was confirmed by sequencing. cDNA fragments were radiolabeled with the rediprime DNA labeling system and [α-$^{32}$P]CTP.

### *In situ hybridization*

Femoral bones were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, decalcified in 0.5 M ethylene-

diamine tetraacetic acid (EDTA; pH 7.5), and embedded in paraffin. Serial longitudinal sections (4 μm) were cut and mounted on 3-aminopropyltriethoxysilane-coated glass slides. The riboprobe for MMP-13 was prepared using DIG-11-dUTP and in situ hybridization was performed as described previously [11]. Briefly, sections were digested by 5 μg/ml proteinase K solution at 37°C for 10 min and hybridized with approximately 0.5 μg/ml probe at 50°C for 16 h. After washing and ribonuclease A (10 μg/ml) treatment at 37°C for 30 min, signals were detected using the DIG detection kit.

### *Statistical analyses*

Statistical significance between groups was analyzed by one-way analysis of variance followed by Bonferroni's method for comparison of multiple means or Student's *t*-test, as appropriate. Unadjusted *P* values less than 0.05 were considered significant.

## Results

### *Effects of PTH infusion on calcium metabolism*

Infusion of rat PTH(1–34) for 3 days resulted in a dose-dependent increase in serum Ca, as shown in Table 1. PTH infusion also decreased serum phosphate levels. Urinary Ca and phosphate excretion increased with PTH infusion (Table 2), but serum creatinine and urinary creatinine excretion did not change following PTH(1–34) infusion. Although rats were not pair-fed, the increase in body weight was not different among the three groups (data not shown), suggesting that food intake was almost the same in these groups. Thus, these biochemical features are similar to those seen in patients with primary hyperparathyroidism and are explained by enhanced bone resorption and reduced phosphate reabsorption by PTH. Infusion of PTH(1–34) for 7 days also increased serum Ca and decreased serum phosphate levels. However, urinary Ca excretion in the 1.5 mmol/kg/day PTH-treated group was not different from that of control rats. Urinary phosphate excretion of rats infused with PTH for 7 days was not different from that of control rats either. In addition, serum Ca and phosphate, and urinary Ca and phosphate excretion in rats infused with PTH for 7 days were significantly lower than in rats infused with the same doses of PTH for 3 days. These results suggest that enhancement of bone resorption by PTH in rats infused with PTH for 7 days was significantly attenuated compared with rats infused for 3 days.

### *Effects of PTH on MMPs expression levels*

Figure 1a shows representative northern blot analysis for MMP-13 and MMP-2. As shown in Fig. 1a, infusion of rat PTH(1–34) for 3 days increased the expression level of MMP-13 in calvaria. However, the amount of MMP-2 mRNA did not change with PTH infusion. Figure 1b gives quantified results of the expression levels in MMPs. Infusion of rat PTH(1–34) for 3 days resulted in a dose-dependent increase in MMP-13 expression. In contrast, although the expression level of MMP-13 in rats infused with rat PTH for 7 days showed a tendency to increase compared with the control group, this did not reach statistical significance. Infusion of PTH for either 3 or 7 days did not change the expression level of MMP-2. Because Cbfa1 was shown to be involved in the enhancement of expression of MMP-13 by PTH [6,9,10], the expression level of Cbfa1 was also

**Table 1.** Serum calcium, phosphate and creatinine in rats infused with rat parathyroid hormone (1–34)

| | [PTH(1–34)] infused (nmol/kg) | | |
|---|---|---|---|
| | 0 | 1.5 | 2.0 |
| Day 3 | | | |
| Ca (mg/dl) | 10.5 ± 0.1 | 13.0 ± 0.9* | 14.4 ± 0.6* |
| Pi (mg/dl) | 9.22 ± 0.39 | 7.88 ± 0.31* | 7.40 ± 0.24* |
| Cr (mg/dl) | 0.49 ± 0.02 | 0.46 ± 0.02 | 0.48 ± 0.03 |
| Day 7 | | | |
| Ca (mg/dl) | 10.4 ± 0.1 | 11.6 ± 0.6*,** | 13.0 ± 0.7*,** |
| Pi (mg/dl) | 8.75 ± 0.45 | 7.11 ± 0.47*,** | 6.54 ± 0.36*,** |
| Cr (mg/dl) | 0.47 ± 0.03 | 0.41 ± 0.03 | 0.47 ± 0.06 |

Data are the mean ± SEM from nine to 12 rats
* Significantly different from the control group of the same treatment length by one-way analysis of variance followed by Bonferroni's method
** Significantly different from the 3-day treatment group administered the same dose of parathyroid hormone (*PTH*) by Student's *t*-test
*Ca*, calcium; *Pi*, phosphate; *Cr*, creatinine

**Table 2.** Urinary Ca, Pi and Cr in rats infused with rat PTH(1–34)

| | [rat PTH(1–34)] infused (nmol/kg) | | |
|---|---|---|---|
| | 0 | 1.5 | 2.0 |
| Day 3 | | | |
| Ca (mg/day) | 0.61 ± 0.13 | 3.71 ± 1.26* | 5.42 ± 1.66* |
| Pi (mg/day) | 33.1 ± 1.2 | 44.8 ± 3.8* | 46.0 ± 4.3* |
| Cr (mg/day) | 8.07 ± 0.24 | 8.30 ± 0.33 | 8.45 ± 0.31 |
| Day 7 | | | |
| Ca (mg/day) | 0.42 ± 0.11 | 0.80 ± 0.22** | 1.82 ± 0.60*,** |
| Pi (mg/day) | 20.3 ± 3.8 | 18.9 ± 3.3** | 17.2 ± 4.0** |
| Cr (mg/day) | 8.66 ± 0.36 | 8.91 ± 0.39 | 9.11 ± 0.82 |

Data are the mean ± SEM from nine to 12 rats
* Significantly different from the control group of the same treatment length by one-way analysis of variance followed by Bonferroni's method
** Significantly different from the 3-day treatment group with the same dose of PTH by Student's *t*-test

examined. However, PTH treatment did not change the expression level of Cbfa1 in vivo (Fig. 1a). Enhancement of the expression level of MMP-13 was also evident by in situ hybridization. As shown in Fig. 2, expression of MMP-13 was detected in the femoral bone from control rats. Cells expressing MMP-13 were mononuclear mesenchymal cells on the bone surface. PTH infusion for 3 days clearly increased the number of cells expressing MMP-13 and the intensity of the signal in these cells.

## Discussion

In the present study, we have shown that PTH infusion for 3 days in our model results in hypercalcemia and hypercalciuria, and that these features are associated with an increased expression of MMP-13. Infusion of PTH for 7 days resulted in less severe hypercalcemia and hypercalciuria, and MMP-13 expression was not significantly increased compared with control rats according to results of northern blot analysis. Thus, although the mechanism of amelioration of hypercalcemia by longer PTH infusion is not evident at the moment, the severity of hypercalcemia and hypercalciuria correlated with the expression level of MMP-13 in calvaria. These results suggest that enhanced expression of MMP-13 contributes to bone resorption stimulated by PTH infusion. There is much evidence that PTH enhances the expression of MMP-13 in osteoblasts in vitro through transcriptional activation of this gene [4–7]. However, direct in vivo evidence of the regulation of MMP-13 expression by PTH is limited. For example, PTH injection into mouse calvaria increases MMP-13 expression [6,12]. These results are compatible with those of the present study showing enhanced expression of MMP-13 in PTH-infused rats by northern blot analysis. In addition, Zhao et al. have reported that enhanced bone resorption following PTH injection in mice with a mutant MMP-13 resistant collagen gene was markedly suppressed compared with wild-type mice [12]. Thus, MMP-13 activity seems to be necessary for the stimulatory effect of exogenous PTH on bone resorption.

In contrast, the physiologic role of MMP-13 in normal bone metabolism without exogenous PTH administration is not fully understood. For example, Lanske et al. have shown that expression of MMP-13 in osteoblasts is reduced in PTH/PTH-related peptide (PTHrP) receptor null mouse fetus compared with wild-type or PTHrP null mice [13]. In these experiments, MMP-13 expression was observed in wild-type mouse fetal tibia by in situ hybridization without exogenous PTH stimulation [13]. We have also shown MMP-13 expression by northern blot analysis in 8-week-old Wistar rat calvaria and by in situ hybridization in femoral bones without any stimulation. In contrast, expression of MMP-13 in calvaria from C57BL/6 control mice has not been observed either by RT-PCR [6] or in situ hybridization [12]. Because stimulation of bone resorption by PTH is indispensable for the maintenance of serum Ca levels, lack of MMP-13 expression should result in hypocalcemia if MMP-13 activity is mandatory for bone resorption by endogenous PTH. However, it has not been reported whether Ca metabolism in mice with mutant MMP-13-resistant collagen is abnormal or not [12]. Further studies using different skeletal tissues and species are necessary in order to clarify the physiologic role of MMP-13 in normal bone metabolism without exogenous PTH stimulation.

It has been reported that binding sites for both AP-1 and Cbfa1 are involved in the enhancement of MMP-13 expression by PTH in vitro [6–10]. PTH has also been shown to induce transactivation of Cbfa1 by a protein kinase A-dependent mechanism without affecting

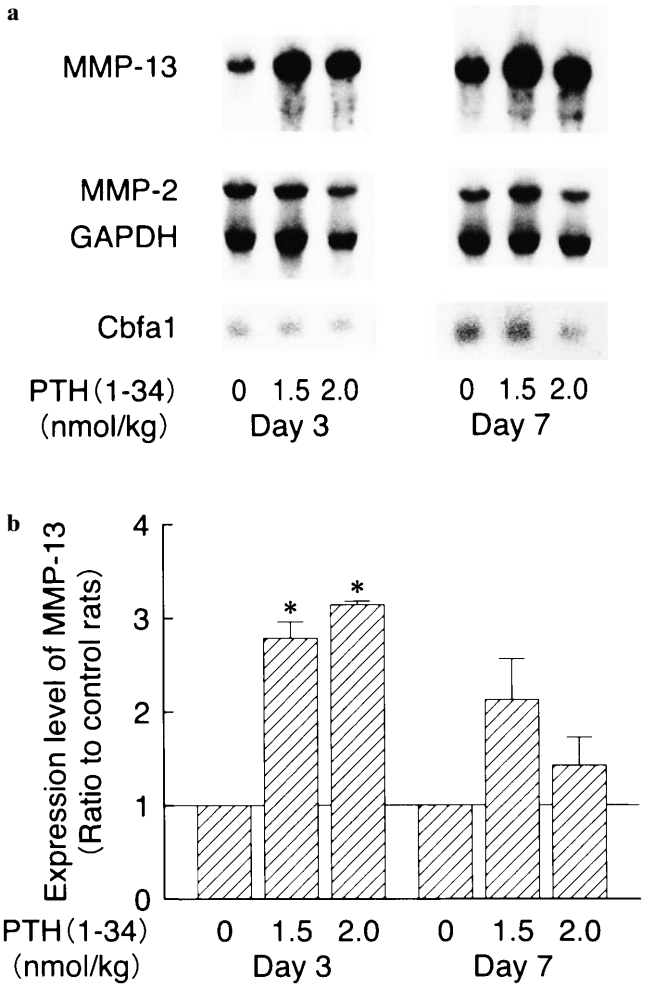


**Fig. 1.** Northern blot analysis for matrix metalloproteinase (*MMP*)-13, MMP-2 and core-binding factor alpha 1 (*Cbfa1*). **a** Total RNA was extracted from calvaria of rats infused with rat parathyroid hormone (1–34) (*PTH(1–34)*) or vehicle (ε-aminocaproic acid) either for 3 or 7 days and subjected to northern blot analysis for MMP-13, MMP-2, Cbfa1 and glyceraldehyde phosphate dehydrogenase (*GAPDH*). This figure shows representative results from three experiments. **b** Hybridized bands by northern blot analysis were quantified by a densitometer and results from three experiments were combined. *Significantly different compared with the control group with vehicle alone by one-way analysis of variance followed by Bonferroni's method for comparison of multiple means

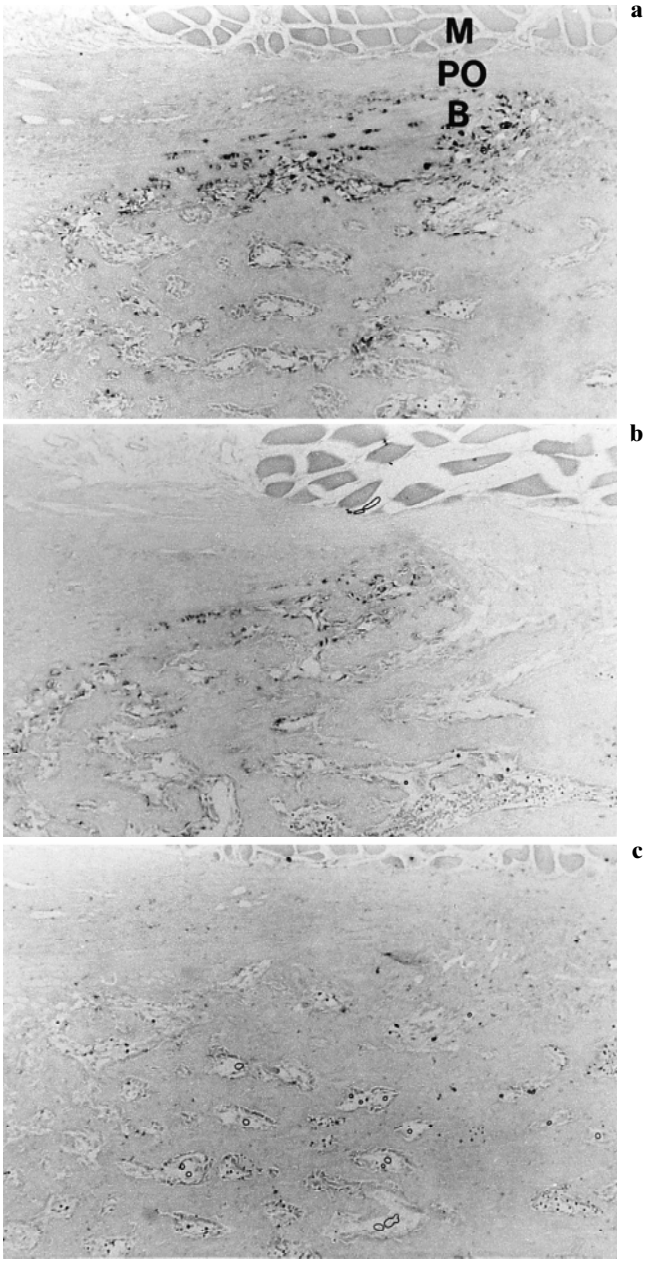


**Fig. 2.** In situ hybridization for MMP-13 in femora. Rats were infused with rat PTH(1–34) (**a**) or vehicle (**b**) for 3 days and femoral bone was subjected to in situ hybridization with an antisense probe for MMP-13, as described in Materials and methods. **c** Femoral bone from a rat infused with rat PTH(1–34) was hybridized with the sense probe for MMP-13. Original magnification ×25. *M*, muscle; *PO*, periosteum; *B*, bone

mRNA and protein levels of Cbfa1 in rat osteoblastic cells [9]. Enhancement of c-fos expression by PTH has already been shown in vivo [14]. In the present study, we have demonstrated that the expression level of Cbfa1 is not altered by PTH treatment (Fig. 1a). Although it is difficult to show transactivation of Cbfa1 by PTH in vivo, expression of MMP-13 is completely absent in mouse embryos lacking Cbfa1 [6], indicating that Cbfa1 is essential for MMP-13 expression. Therefore, our results support the concept that both AP-1 and Cbfa1 are involved in the enhanced expression of MMP-13 by PTH.

In conclusion, we have shown that PTH infusion results in hypercalcemia, hypercalciuria and enhanced expression of MMP-13 in calvaria and femoral bones, and that the severity of hypercalcemia and hypercal-

ciuria correlates with the expression level of MMP-13 in bone. These results suggest that the stimulatory effect of PTH on bone resorption is mediated, at least in part, by its action on MMP-13 expression in this rat model.

*Acknowledgments.* This work was supported in part by a Grant-in-Aid for Scientific research from the Ministry of Education, Science, Sports and Culture, Japan.

## References

1. Ishizuya T, Yokose S, Hori M, Noda T, Suda T, Yoshiki S, Yamaguchi A (1997) Parathyroid hormone exerts disparate effects on osteoblast differentiation depending on exposure time in rat osteoblastic cells. J Clin Invest 99:2961–2970
2. Yasuda H, Shima N, Nakagawa N, Yamaguchi K, Kinosaki M, Mochizuki S, Tomoyasu A, Yano K, Goto M, Murakami A, Tsuda E, Morinaga T, Higashio K, Udagawa N, Takahashi N, Suda T (1998) Osteoclast differentiation factor is a ligand for osteoprotegrin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. Proc Natl Acad Sci USA 95:3597–3602
3. Fukumoto S, Allan EH, Yee JA, Gelehrter TD, Martin TJ (1992) Plasminogen activator regulation in osteoblasts: parathyroid hormone inhibition of type-1 plasminogen activator inhibitor and its mRNA. J Cell Physiol 152:346–355
4. Partridge NC, Walling HW, Bloch SR, Omura TH, Chan PT, Pearman AT, Chou WY (1996) The regulation and regulatory role of collagenase in bone. Crit Rev Eukaryot Gene Expr 6:15–27
5. Peeters-Joris C, Hammani K, Singer CF (1998) Differential regulation of MMP-13 (collagenase-3) and MMP-3 (stromelysin-1) in mouse calvariae. Biochim Biophys Acta 1405:14–28
6. Porte D, Tuckermann J, Becker M, Baumann B, Teurich S, Higgins T, Owen MJ, Schorpp-Kistner M, Angel P (1999) Both AP-1 and Cbfa1-like factors are required for the induction of interstitial collagenase by parathyroid hormone. Oncogene 18: 667–678
7. Rajakumar RA, Quinn CO (1996) Parathyroid hormone induction of rat interstitial collagenase mRNA in osteosarcoma cells is mediated through an AP-1-binding site. Mol Endocrinol 10:867–878
8. Selvamurugan N, Chou WY, Pearman AT, Pulumati MR, Partridge NC (1998) Parathyroid hormone regulates the rat collagenase-3 promoter in osteoblastic cells through the cooperative interaction of the activator protein-1 site and the runt domain binding sequence. J Biol Chem 273:10647–10657
9. Selvamurugan N, Pulumati MR, Tyson DR, Partridge NC (2000) Parathyroid hormone regulation of the rat collagenase-3 promoter by protein kinase A-dependent transactivation of core binding factor alpha1. J Biol Chem 275:5037–5042
10. Jimenez MJ, Balbin M, Lopez JM, Alvarez J, Komori T, Lopez-Otin C (1999) Collagenase 3 is a target of Cbfa1, a transcription factor of the runt gene family involved in bone formation. Mol Cell Biol 19:4431–4442
11. Yamagiwa H, Tokunaga K, Hayami T, Hatano H, Uchida M, Endo N, Takahashi HE (1999) Expression of metalloproteinase-13 (collagenase-3) is induced during fracture healing in mice. Bone 25:197–203
12. Zhao W, Byrne MH, Boyce BF, Krane SM (1999) Bone resorption induced by parathyroid hormone is strikingly diminished in collagenase-resistant mutant mice. J Clin Invest 103:517–524
13. Lanske B, Divieti P, Kovacs CS, Pirro A, Landis WJ, Krane SM, Bringhurst FR, Kronenberg HM (1998) The parathyroid hormone (PTH)/PTH-related peptide receptor mediates actions of both ligands in murine bone. Endocrinology 139:5194–5204
14. Lee K, Deeds JD, Chiba S, Un-No M, Bond AT, Segre GV (1994) Parathyroid hormone induces sequential c-fos expression in bone cells in vivo: in situ localization of its receptor and c-fos messenger ribonucleic acids. Endocrinology 134:441–450