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Cellular and Molecular Biology

Invited Review

Recent studies on the biological action of parathyroid hormone (PTH)-related peptide (PTHrP) and PTH/PTHrP receptor in cartilage and bone

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Summary. Mice with a targeted deletion of parathyroid hormone (PTH)-related peptide (PTHrP) develop a form of dyschondroplasia resulting from diminished proliferation and premature maturation of chondrocytes. Abnormal, heterogeneous populations of chondrocytes at different stages of differentiation were seen in the hypertrophic zone of the mutant growth plate. Although the homozygous null animals die within several hours of birth, mice heterozygous for PTHrP gene deletion reach adulthood, at which time they show evidence of osteopenia. Therefore, PTHrP appears to modulate cell proliferation and differentiation in both the pre and post natal period. PTH/PTHrP receptor expression in the mouse is controlled by two promoters. We recently found that, while the downstream promoter controls PTH/PTHrP receptor gene expression in bone and cartilage, it is differentially regulated in the two tissues. 1α,25-dihydroxyvitamin D3 downregulated the activity of the downstream promoter in osteoblasts, but not in chondrocytes, both ***in vivo*** and ***in vitro.*** Most of the biological activity of PTHrP is thought to be mediated by binding of its amino terminus to the PTH/PTHrP receptor. However, recent evidence suggests that amino acids 87-107, outside of the amino terminal binding domain, act as a nucleolar targeting signal. Chondrocytic cell line, CFK2, transfected with wild-type PTHrP cDNA showed PTHrP in the nucleoli as well as in the secretory pathway. Therefore, PTHrP appears to act as a bifunctional modulator of both chondrocyte proliferation and differentiation, through signal transduction linked to the PTH/PTHrP receptor and by its direct action in the nucleolus.

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Introduction

Parathyroid hormone (PTH)-related peptide (PTHrP) was first identified as a tumor derived factor that caused hypercalcemia in patients with a variety of malignancies (Burtis et al., 1987; Strewler et al., 1987; Suva et al., 1987). The high homology within the extreme amino termini of PTH and PTHrP permits them to bind to the common receptor, PTH/PTHrP receptor (Jüppner et al., 1991) on target cells in kidney and bone. Consequently, systemic PTHrP, released by the tumor, mimics the biological activity of PTH (Horiuchi et al., 1987; Kemp et al., 1987; Rabbani et al., 1988; Thompson et al., 1988). However, unlike PTH, which is the major regulator of calcium homeostasis, PTHrP does not circulate in significant quantities under normal circumstances. Rather, in view of the widespread expression of PTHrP and its receptor in a number of fetal and adult tissues (Ureña et al., 1993). It is thought to act as a local regulator of cell growth and differetiation (Merendino et al., 1986; Ikeda et al., 1988; Thiede and Rodan, 1988, Dmcker et al., 1989; Thiede et al., 1990; Moseley et al., 1991; Kaiser et al., 1992, 1994; Deftos et al., 1993). PTHrP is expressed during embryogenesis in mural trophoblast cells, in extra-decidual uterine stroma, in placenta, in the epithelial cells of the dental lamina including ameloblasts, in hair follicles, in keratinocytes, in nasal epithelial cell and in the membranous labyrinth, in epithelial cells in the lung, and in cartilage and bone (Senior et al., 1990, Lee et al., 1994).

Our studies using a mouse with targeted deletion of the PTHrP gene, developed by Karaplis et al. in 1994, demonstrated that PTHrP plays a key role in chondro-osteogenesis (Karaplis et al., 1994). Mice homozygous

for a null mutation of the PTHrP gene die within hours of birth with skeletal abnormalities consistent with osteochondrodysplasia. Our analysis of these homozygous null mutants indicates that the abnormalities are related to perturbations in both proliferation and differentiation of chondrocytes (Amizuka et al., 1994). Adult mice heterozygous for targeted ablation of the PTHrP gene demonstrated reduced expression of PTHrP, but normal levels of PTH. This PTHrP deficient mouse developed a form of osteopenia showing disturbed formation of bony trabecules (Amizuka et al., 1996a), suggesting that PTH could not sub-serve the function of PTHrP in these animals.

The expression of the mouse PTH/PTHrP receptor is regulated by at least two promoters (McCuaing et al., 1994, 1995) which give rise to two transcripts that differ in their 5' untranslated regions but not in their coding sequences. Transcripts from the upstream promoter, P1, contain sequences derived from two 5' untranslated region exons U1 and U2, and are highly and selectively expressed in kidney (Amizuka et al., 1997). P1 functions in all renal cell types expressing the PTH/PTHrP receptor, including tubular epithelial cells, peritubular endothelial cells, glomerular podocytes and vascular smooth muscle (Amizuka et al., 1997). The downstream promoter, P2, gives rise to transcripts that contain 5' untranslated sequences derived from exon U3. P2 function is restricted to tubular epithelial cells in kidney. However, it is active in a wide variety of other tissues, including bone and cartilage, and accounts for the broad expression pattern of the PTH/PTHrP receptor. Recently, we performed *in situ* hybridization studies with probes specific for transcripts expressed from P1 or P2, and found that P2 is the predominant PTH/PTHrP receptor promoter in bone and cartilage (Amizuka et al., 1999). We also found that PTH/PTHrP receptor expression is differentially modulated in bone and cartilage by 1α,25-dihydroxyvitamin D3 [1α,25$(OH)_2D_3$]. 1α,25$(OH)_2D_3$. inhibited expression of the PTH/PTHrP receptor in osteoblasts, but not chondrocytes, both *in vivo* and *in vitro* through downregulation of P2 activity (Amizuka et al., 1997). These studies add another facet to the complex interplay of the calcium regulatory functions of 1α,25$(OH)_2D_3$, PTH and PTHrP.

In addition to its activity mediated through binding of the amino terminus to the PTH/PTHrP receptor, we and others have reported bioactivity mediated through mid-region amino acids 87-107, which represent a nuclear/nucleolar targeting signal (NTS). Although the presence of the PTHrP leader sequence preferentially targets the protein to the secretory pathway, in the absence of the leader sequence, PTHrP is preferentially targeted to the nucleolus in transfected chondrocytic cells (Henderson et al., 1995). In the absence of the NTS, PTHrP localized exclusively to the cytoplasmic compartment.

Thus, this paper will review recent studies, performed by ourselves and others, on PTHrP and the amino terminal PTH/PTHrP receptor. These include the histological abnormalities of the PTHrP deficient mouse, the alternative suppression of PTH/PTHrP receptor transcripts by 1α,25$(OH)_2D_3$, and the nuclear/nucleolar targeting of PTHrP in chondrocytic cells.

Skeletal alterations of the fetal mice homozygous for PTHrP gene deletion

E18-19 day fetal mice homozygous null for the PTHrP gene had shortened limbs, protruding tongues, hypoplastic mandibles and square-shaped calvariae (Karaplis et al., 1994) (Fig. 1A). These dysmorphic characteristics resulted from reduced proliferation and premature differentiation of chondrocytes during endochondral bone formation (Fig. 1B,C). The epiphyseal growth plate of wild-type mice is composed distinct regions refered to as the resting zone, the proliferative zone and the hypertrophic zone. The epiphyses of PTHrP deficient mice had fewer resting and proliferative chondrocytes, although the number of hypertrophic cells did not seem to be altered (Amizuka et al., 1994). In addition, a heterogeneous cell population was observed in the hypertrophic zone of mutant mice, with clusters of non-hypertrophic chondrocytes persisting among the hypertrophic cells (Fig. 2). This was in contrast to the uniform region of hypertrophic cells seen in wild-type mice. The persistance of non-hypertrophic cells up to the metaphyseal side of the epiphyseal plate distorted the longitudinal columns of hypertrophic chondrocytes, resulting in less-developed longitudinal septae of calcified cartilage matrix (Fig. 3). These non-hypertrophic chondrocytes were characterized by expression of the PTH/PTHrP receptor and continued cell proliferation as well as active protein synthesis, evidenced by incorporation of [^{3}H] thymidine and [^{3}H] proline, respectively (data not shown). These cells, therefore, displayed the same properties as resting and proliferative chondrocytes. These studies demonstrated that PTHrP modulates chondrocyte proliferation and differentiation during the fetal period.

Skeletal alterations in adult mice heterozygous for PTHrP gene deletion

Mice heterozygous for targeted disruption of the PTHrP gene reveal no obvious abnormalities at birth. However, a haploinsufficient phenotype was apparent by 3 months of age, which was characterized by short, blunt snouts and generalized osteopenia (Amizuka et al., 1996a) (Fig. 4). PTHrP mRNA was significantly reduced in the heterozygous mice compared with their wild-type littermates. However, plasma PTH and calcium concentrations were normal. Scanning electron microscopic analysis of the metaphysis of 3-month old wild-type mice, revealed uniform bone spicules, which were aligned parallel to the longitudinal axis (Fig. 4B,C). In contrast, the bony spicules in the metaphysis

of heterozygous mice were fewer in number and were not distributed longitudinally. The growth plate of the heterozygotes demonstrated disorganized column of proliferating cells with subsequent distortion of the longitudinal cartilage partitions (Fig. 4D,E). This local failure to form uniform intercolumnar partitions of cartilage matrix most probably accounted for the abnormal distribution of mixed spicules. The

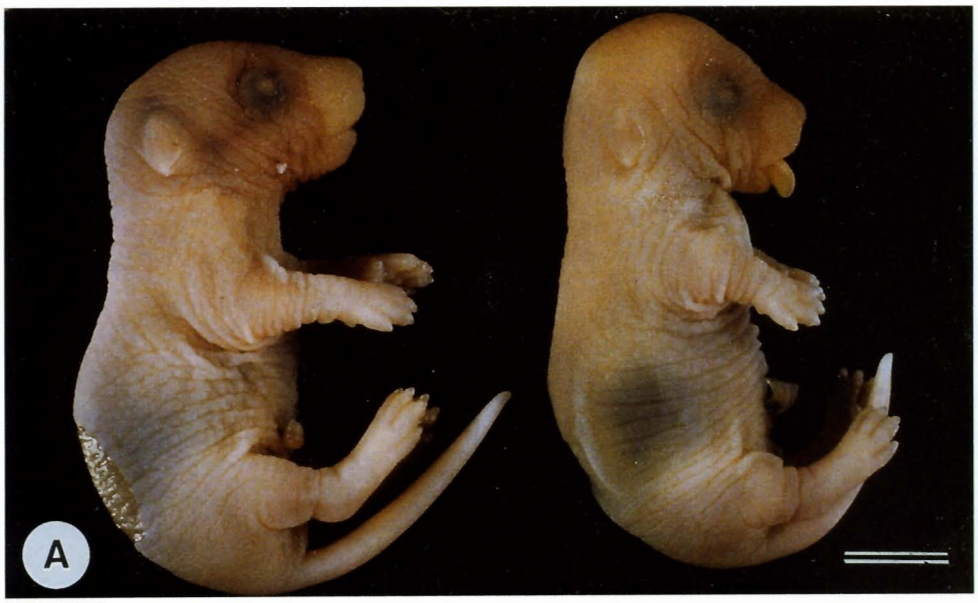
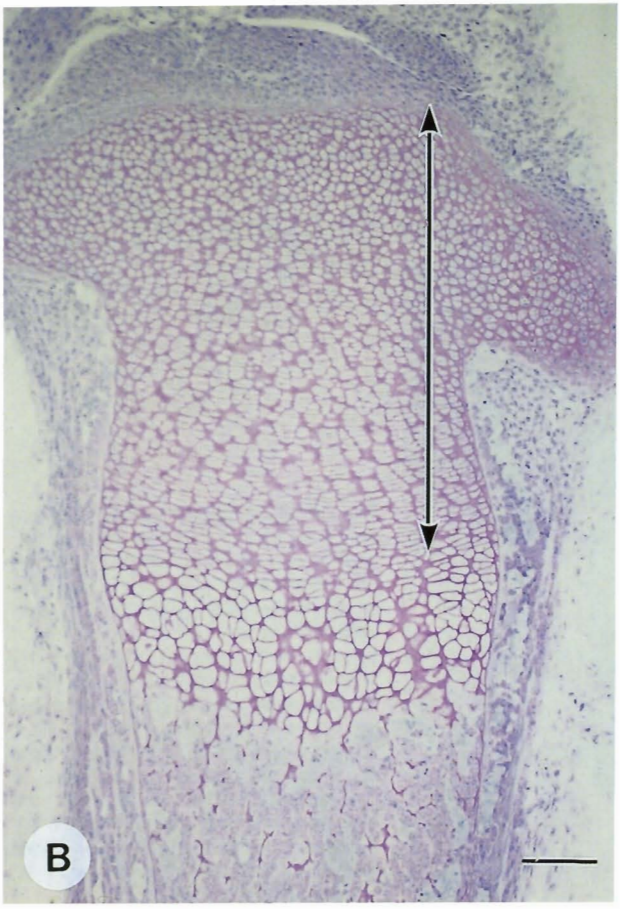
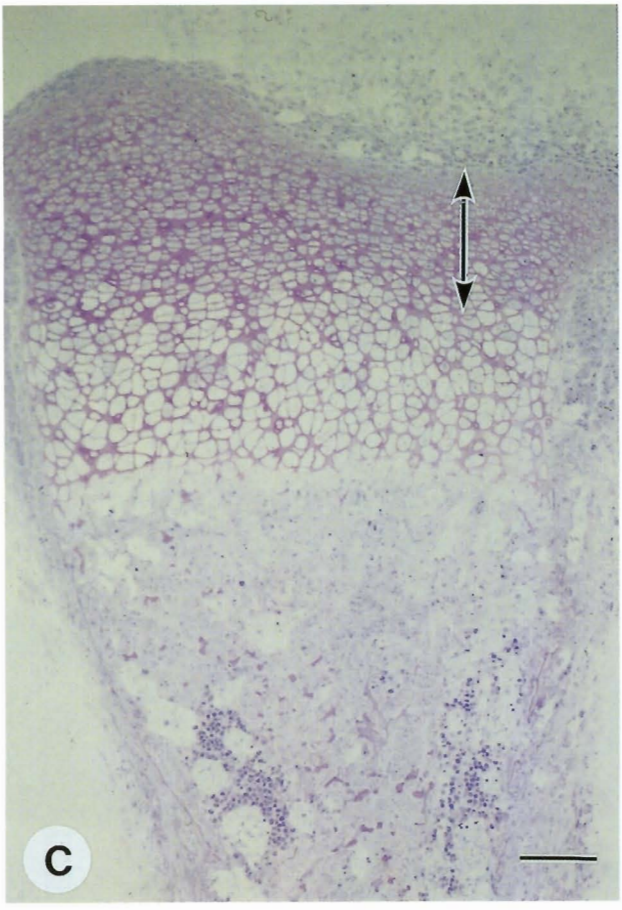


Fig. 1. A. Binocular microscopic observation of an 18.5-day old wild-type fetus (left) and a littermate homozygous (right) for PTHrP gene deletion (quoted from Amizuka et al., 1994; Karaplis et al., 1994). The homozygote shows characteristic abnormalities including a protruding tongue, square-shaped calvaria and short limbs, compared with those of normal litter mates. Bar; 0.5 μm. **B, C.** Low power images of proximal tibiae of PTHrP-normal (B) and PTHrP-deleted (C) fetuses at 18.5 days of gestation. The epiphyseal cartilage of PTHrP-deficient mice (C) is less-developed due to markedly reduced resting and proliferative zones, compared with those of the wild-type littermates (B). Bar: 50 μm.

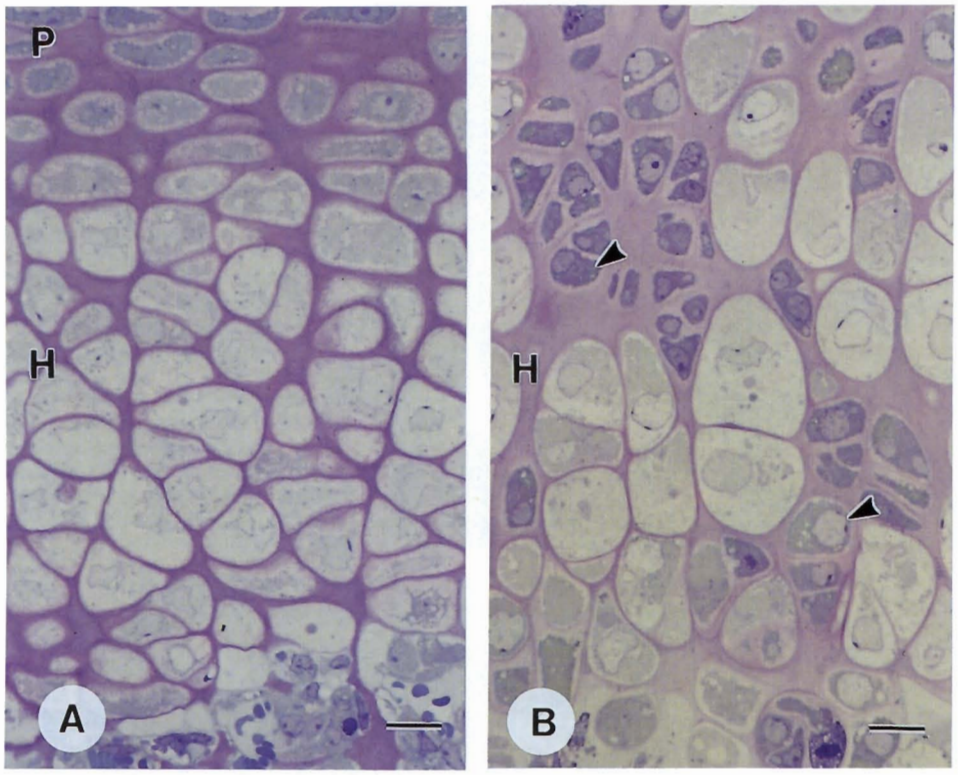




proliferative capacity of growth plate chondrocytes in the heterozygous mutants was also significantly reduced (data not shown). Thus, PTHrP appears to modulate normal chondrocyte proliferation and differentiation in adult, as well as fetal mice.

Localization of PTHrP and PTH/PTHrP receptor in cartilage and bone

As PTHrP is believed to act primarily at the local level, it is important to identify cells that express the PTH/PTHrP receptor, in relation to PTHrP expressing cells, in the cartilage and bone microenvironment.



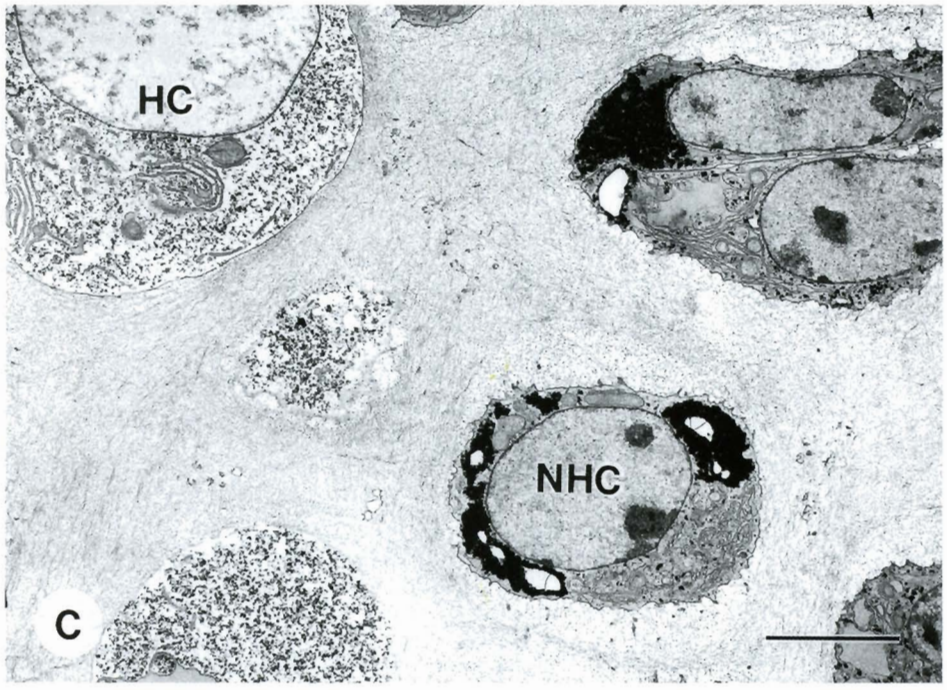


Fig. 2A. ,B . Light microscopic image of the hypertrophic zone of the wild-type (A) and littermates homozygous (B) for PTHrP-deficiency (quoted from Amizuka et al., 1994). The hypertrophic zone of wild-type littermates is composed of hypertrophic cells (A). However, the homozygote shows non-hypertrophic chondrocytes (arrowheads) interspersed among normal hypertrophic cells in the hypertrophic zone (B). Bar: 10μm **C.** Electron microscopic image of hypertrophic chondrocytes (HC) and non-hypertrophic chondrocytes (NHC) in the hypertrophic zone of the PTHrP-deficient animal. P: proliferative zone; H: hypertrophic zone. Bar: 5μm

[^{125}I]-PTH radioautography previously performed by Goltzman and his colleagues identified mature osteoblasts and preosteoblasts as PTH target cells (Rouleau et al., 1986, 1988, 1990). The preosteoblastic cell type, refered to as a "PT-cell", displayed extended cytoplasmic processes, and formed cell-to-cell contacts

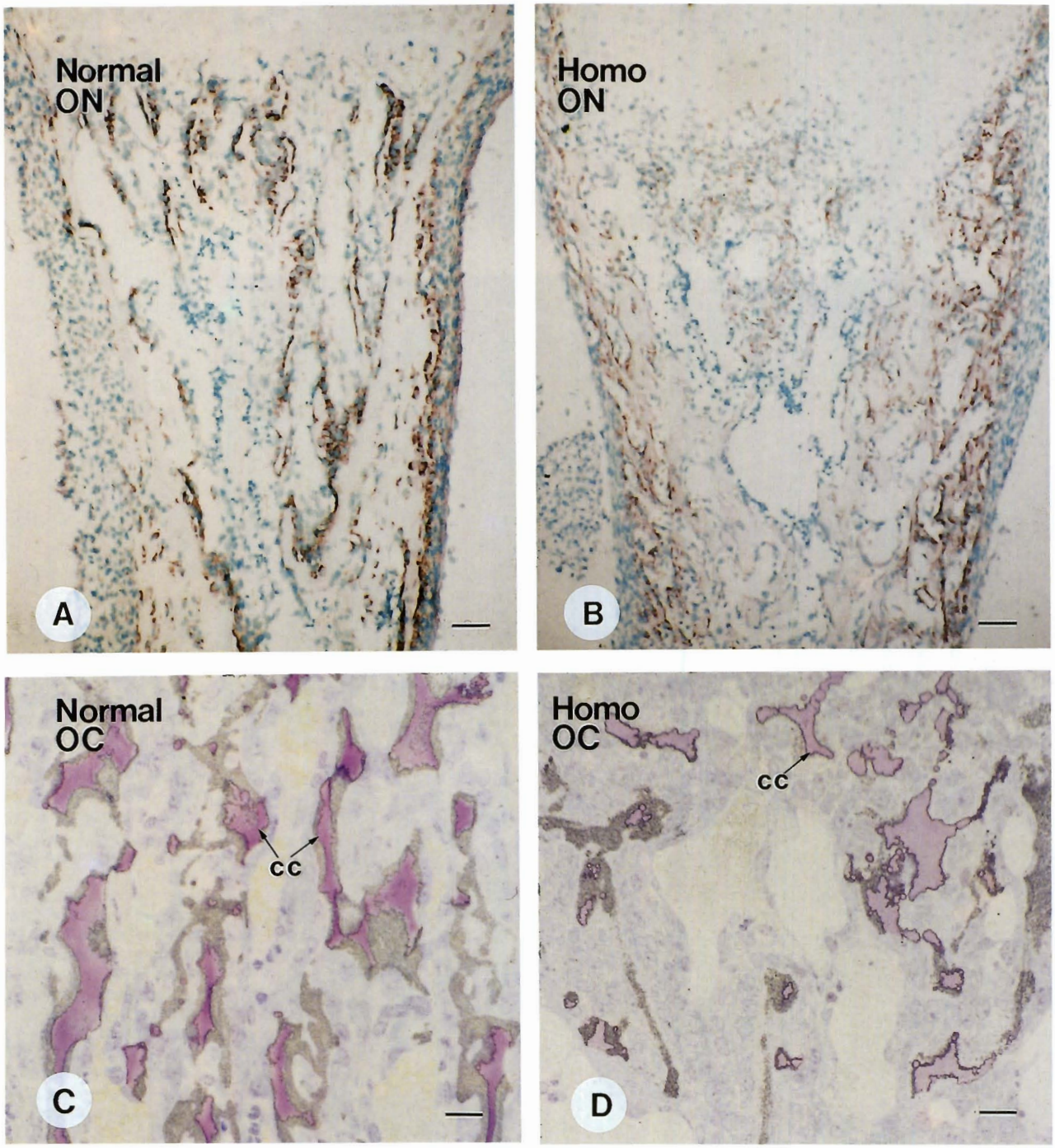


Fig. 3. A, B. *In situ* hybridization for expression of osteonectin in the tibiae of the wild-type (Normal, A) and the homozygote for PTHrP-deletion (Homo, B). The wild-type animal has extended longitudinal trabecules where osteoblasts express the osteonectin gene as indicated in brown (A), whereas osteoblasts in the cortical bone but not trabecular bones show the expression of osteonectin in the homozygote (B). Bar: 50μm. **C, D.** Osteocalcin immunoreactivity on the trabecular bones of the wild-type (C) and homozygote (D). Osteocalcin immunoreactivity seen in black is located in the periphery of the longitudinal cartilage cores (cc) (C), while the homozygote shows irregularly-shaped cartilage cones (cc), resulting in the disorganized accumulation of osteocalcin (D). Bar: 20μm

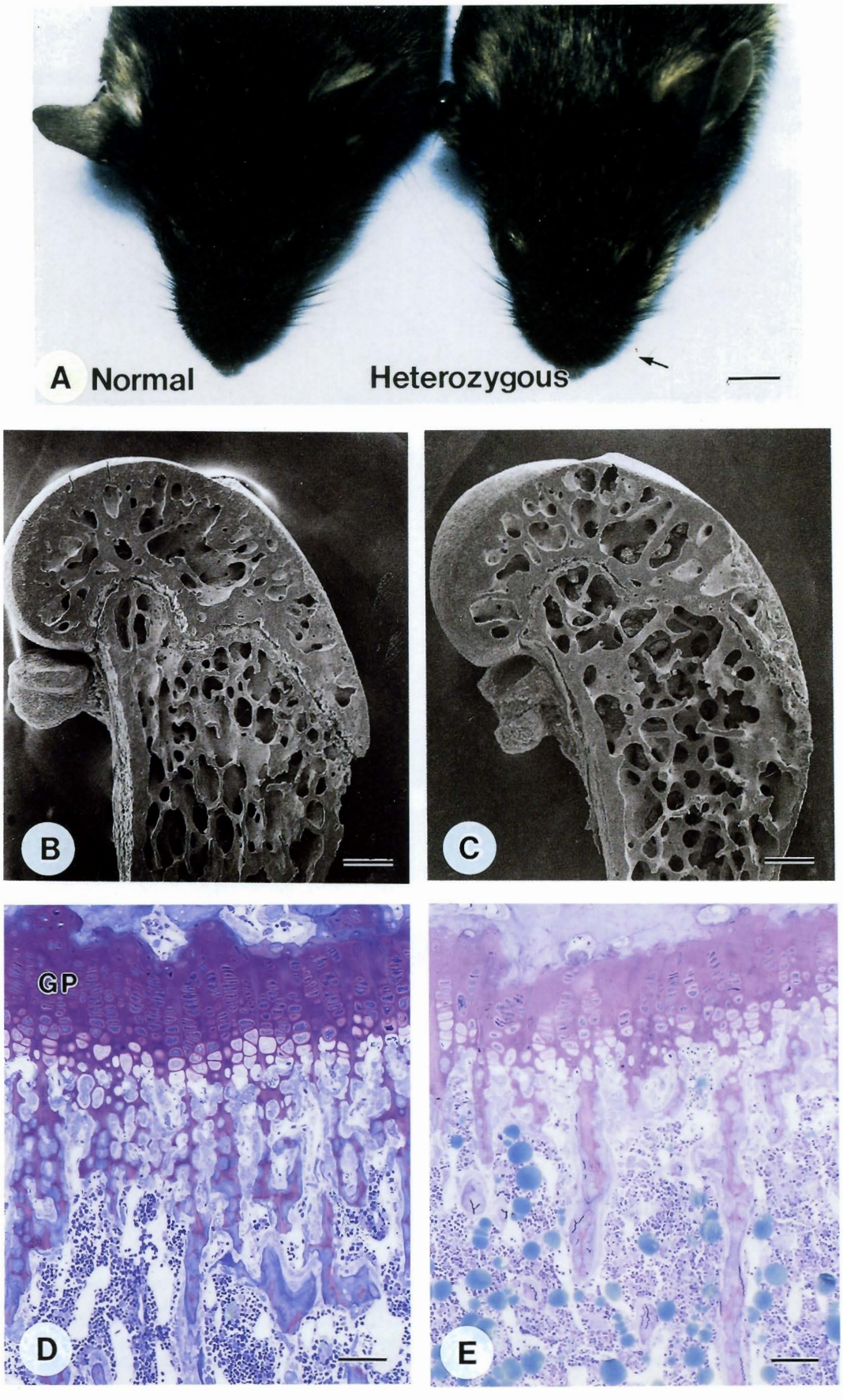


Fig. 4. A. Left and right show a 3-month old wild-type (Normal) and a littermate heterozygous for PTHrP gene deletion (Heterozygous), respectively. Note the less-protruded snout of the heterozygote (arrow). Bar: 0.3 μm. **B, C.** Scanning electron micoscopy displayed the epiphyseal and metaphyseal trabecular bones of femura of the wild-type and heterozygote. The heterozygous animal (C) reveals irregularly distributed trabeculaes compared to the wild-type littermate (B). Bar: 0.5μm. **D, E.** The histological sections of the epiphyseal growth plate (GP) of the wild-type mouse and a littermate heterozygous for PTHrP gene deletion. In the wild-type, longitudinal cartilaginous columns are well formed, while the heterozygote displays poorly-developed columns (quoted from Amizuka et al., 1996a). Bar: 50μm

with neighbouring osteoblasts. Our later studies, using *in situ* hybridization and immunocytochemistry verified the presence of PTH/PTHrP receptor mRNA and protein in osteoblasts and preosteoblasts (Amizuka et al., 1996a-c; Irie and Ozawa, 1996) (Fig. 5). These observations have also been corroborated using Northern blot and chemical analyses of osteoblastic cells in culture. These later studies revealed an upregulation of PTH/PTHrP receptor during osteoblast differentiation (Abou-Samura et al., 1992; Suda et al., 1996). In the developing murine growth plate, the PTH/PTHrP receptor is broadly distributed throughout the resting and proliferative zones, up to the proximal region of the hypertrophic zone (Lee et al., 1994; Amizuka et al., 1996a-c, Irie and Ozawa, 1996). This localization of the PTH/PTHrP receptor almost overlapped with that of the proliferating cells identified by [^{3}H] thymidine radioautography and immunocytochemical localization of the proliferating

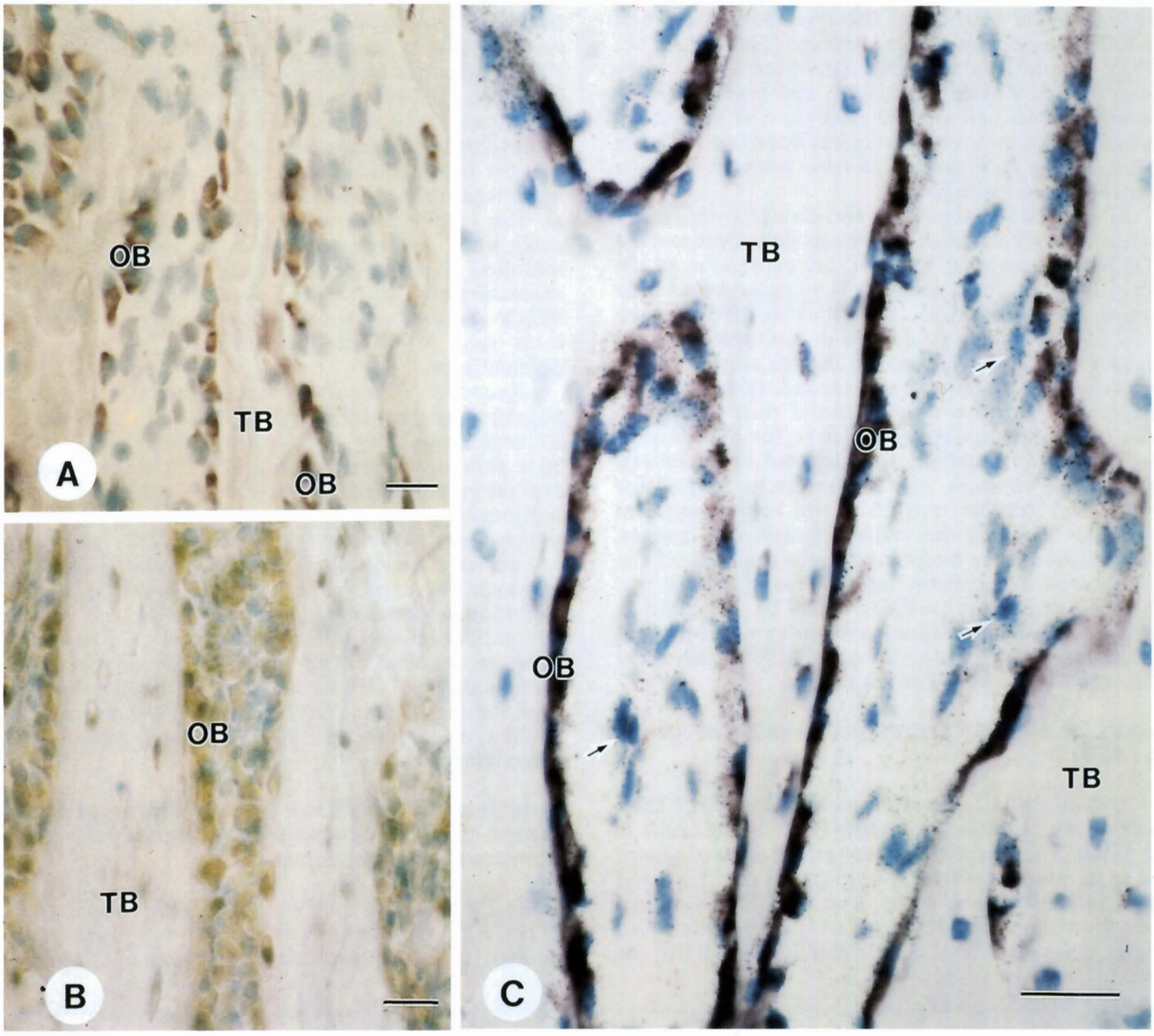


Fig. 5. A, B. The localization of the gene and protein of PTHrP in bone tissue. In situ hybridization (A) and immunocytochemistry (B) for PTHrP in bone tissue (quoted from Amizuka et al., 1996a). The PTHrP gene is expressed in osteoblasts (OB) as shown by the brown color (A). Weak immunoreactivity for PTHrP (brown) is seen in osteoblasts located on trabecular bones (TB) (B). Bar: 20μm. **C.** Double-labeled in situ hybridization for PTHrP and the PTH/PTHrP receptor in bone tissue. PTHrP and the PTH/PTHrP receptor are represented by the brown and by silver grains, respectively. Osteoblasts show co-localization of genes encoding the ligand and the receptor, therefore suggesting a paracrine/autocrine mode of action of the ligand. The receptor gene is also broadly expressed in preosteoblastic cells. Note silver grains, but not red brown color in the preosteoblastic cells (arrows). Bar: 20μm

cell nuclear antigen (Amizuka et al., 1996b). It seems likely, therefore, that the inhibition of chondrocyte proliferation observed in the PTHrP deficient mice was primarily due to a reduction in the interaction of PTHrP with the PTH/PTHrP receptor on proliferating chondrocytes. In the murine growth plate, localization of PTHrP mRNA and protein was restricted to resting chondrocytes, to chondrocytes at the junction between proliferative and hypertrophic zones (Amizuka et al., 1994, Weir et al., 1996), and differentiated osteoblasts (Amizuka et al., 1996a, Suda et al., 1996, Lomri et al., 1997). Northern analysis has also revealed expression of the PTHrP gene in epiphyseal cartilage (Tsukazaki et al., 1995). In addition, cultured osteoblastic cell lines demonstrated PTHrP expression and binding of the protein to their cell surface (Enomoto et al., 1993). Taken together, these observations indicate that PTHrP is synthesized in cartilage and in bone where it acts in a paracrine/autocrine fashion.

In contrast to osteoblasts and chondrocytes, many studies have reported that osteoclasts do not possess the PTH/PTHrP receptor, however, the expression of the PTH/PTHrP receptor gene in osteoclasts has recently been discussed (Athanasou and Sabokbar, 1999; Langub et al., 1999). Langub et al. have demonstrated that the receptor gene could be detected in both normal human and patients suffering from secondary hyperparathyroidism. Yet, it was observed the most osteoclasts from patients were immunopositive for the receptor protein, while no staining was detected in osteoclasts from normal subjects. They, therefore, predicted that PTH/PTHrP receptor mRNA would be detectable in osteoclasts in both normal and hyperpatathyroid patients, but translation to the receptor protein is achieved only in patients with hyperparathyroidism. It is of interest that PTH/PTHrP receptor mRNA would be subjected to translation in the osteoclasts under pathological circumstances, suggesting the possibility of the direct effect of PTH and PTHrP to osteoclasts.

Regulation of PTHrP expression by indian hedgehog and TGFß

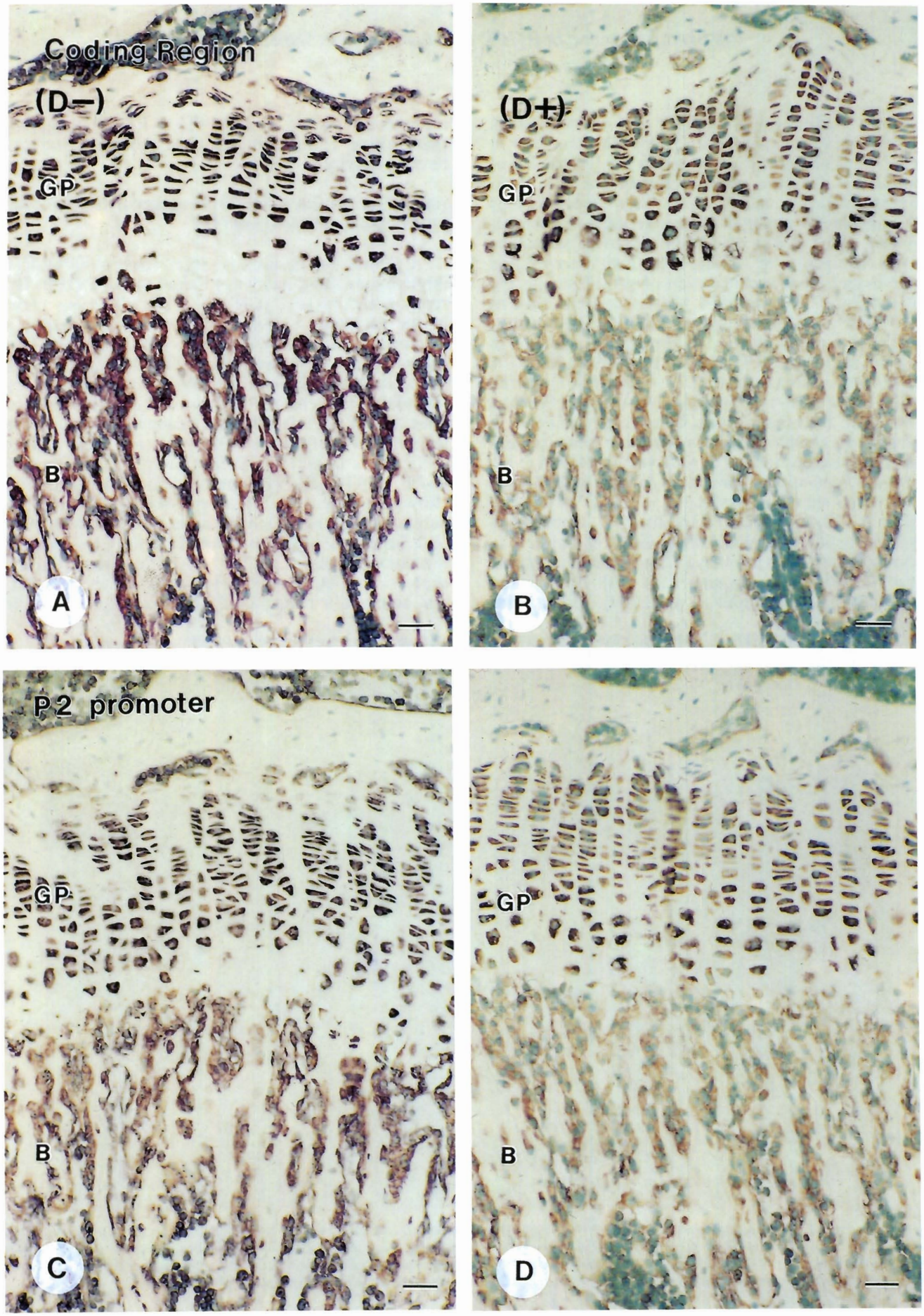
A mouse with targeted deletion of the PTH/PTHrP receptor has also been generated in Boston (Lanske et al., 1996). The receptor deficient mouse showed almost the same abnormalities in epiphyseal cartilage and endochondral bone as those seen in PTHrP deficient mice. However, the PTH/PTHrP receptor-null mice died in mid-gestation. This was attributed to the fact that the activity of maternal PTHrP was blocked in these mice. In contrast to the PTHrP deficient animal, a mouse in which PTHrP overexpression was driven by collagen II promoter, developed at Yale University, exhibited enhanced chondrocyte proliferation, delayed chondrocyte maturation, and inhibition of endochondral bone formation (Weir et al., 1996). This transgenic model, therefore, demonstrated the abnormalies in endochondral bone formation which were reverse of those seen in the PTHrP and PTH/PTHrP receptor deficient mice. To further examine the pathways linked to PTHrP signalling during bone development, investigators in Boston examined the regulation of PTHrP by indian hedgehog (IHH), one of the family of hedgehog morphogens known to be involved in bone formation. Those studies demonstrated IHH expression in developing cartilage was restricted to a sub-set of cells at the junction between the proliferative and hypertrophic zones (Vortkamp et al., 1996). Vortkamp et al. also employed a model of IHH-misexpression in chick limb cartilage, to demonstrate that the protein inhibited chondrocyte differentiation and stimulated expression of PTHrP mRNA. It was hypothesized that PTHrP was downstream of IHH in a negative feedback loop that modulates the rate of chondrocyte differentiation.

Recently, Serra et al. have demonstrated that transformimg growth factor ß (TGFß) and PTHrP act in a common signalling cascade to regulate endochondral bone formation (Serra et al., 1999). Using an organ explant model of embryonic murine metatarsal bone rudiments, they demonstrated that TGFß inhibited chondrocyte proliferation, hypertrophic differentiation, and matrix mineralization. Treatment with TGFß1 also promoted the expression of PTHrP, resulting in the inhibition of hypertrophic diffferentiation and matrix mineralization but did not affect cell proliferation. However, terminal differentiation of chondrocytes was not inhibited by TGFß when the in matatarsal rudiments were removed from PTHrP-null mice, suggesting that TGFß acts upstream of PTHrP to inhibit chondrocyte differentiation.

Mutations in the PTH/PTHrP receptor gene in Jansen-type metaphyseal chondrodysplasia and blomstrand chondrodysplasia

In 1995, a point mutation in the gene encoding the PTH/PTHrP receptor was discovered in a patient with Jansen-type metaphyseal chondrodysplasia, which is a

Fig. 6. A, B. *In situ* hybridization to detect gene expression of the PTH/PTHrP receptor coding region transcripts in the control and $1\alpha,25(OH)_2D_3$-treated mouse tibiae (quoted from Amizuka et al., 1999). **A:** In the control tibiae (D-), hybridization signals (dark brown) corresponding to expression of the PTH/PTHrP receptor coding region were detected in metaphyseal osteoblasts and in chondrocytes of the growth plate (GP). **B.** The $1\alpha,25(OH)_2D_3$-treated tibia (D+) showed only a weak hybridization signal (faint brown color) in osteoblasts. Chondrocytes maintained an intense signal indicating strong PTHR coding region expression in the growth plate (GP). **C, D.** *In situ* hybridization detecting gene expression of P2 promoter-specific transcripts in control and $1\alpha,25(OH)_2D_3$-treated mouse tibiae. **C:** Control tibiae showed strong gene expression of P2 specific transcripts (brown color) in metaphyseal osteoblasts and in chondrocytes of the growth plate. **D:** $1\alpha,25(OH)_2D_3$-treated tibiae showed reduced expression of P2-specific transcripts in osteoblasts. Strong gene expression was still observed in the growth plate chondrocytes. B: bone. Bar: 50 μm.


Coding Region
(D−)
GP
B
A
(D+)
GP
B
B
P2 promoter
GP
B
C
GP
B
D

heritable form of dwarfism associated with hypercalcemia (Schipani et al., 1995). The mutation in the first intracellular domain results in the substitution of a conserved histidine residue, at position 223, to an arginine. Another mutation, which substitute a threonine with a proline at position 410 in the sixth membrane-spanning domain, has also been identified in Jansen patients (Schipani et al., 1996). These mutations result in ligand-independent accumulation of cAMP suggesting that residues 223 and 410 play a critical role in PTH/PTHrP receptor signal transduction. In contrast, Jobert et al. recently reported an inactivating mutation in the gene encoding the PTH/PTHrP receptor. The G/A substitution at nucleotide 1176, inherited from the mother, cause an 11 amino acid deletion in the first part of the fifth transmembrane domain and results in Blomstrand's chondrodysplasia, which is characterized by premature endochondral bone formation (Jobert et al., 1998). The mutant receptor can not bind PTH or PTHrP, and failed to stimulate production of cAMP or inositol phosphate. More recently, Karaplis et al. identified a single nucleotide exchange, which results in a proline to leucine substitution at position 132 in the amino terminal extracellular domain, in an infant with Blomstrand chondrodysplasia (Karaplis et al., 1999). This mutant receptor also failed to bind PTH or PTHrP, suggesting that proline 132 is critial for the receptor's intrinsic binding activity.

Regulation of the PTH/PTHrP receptor transcription by 1α,25-dihydroxyvitamin D3

Calcium homeostasis is largely controlled by PTH through signalling pathways linked to the PTH/PTHrP receptor and by $1\alpha,25(OH)_2D_3$. PTH, which is secreted by the parathyroid gland in response to a reduction in extracellular fluid calcium, mobilizes calcium from bone and stimulates its reabsorption in the kidney (Muff et al., 1992; Backsai and Friedman, 1995). $1\alpha,25(OH)_2D_3$ also stimulates bone resorption and calcium release from bone, and in addition enhances calcium absorption in the intestine (Wasserman and Fullmer et al., 1983; Deluca, 1985; Hock et al., 1986). The interplay between PTH and $1\alpha,25(OH)_2D_3$ is complex. $1\alpha,25(OH)_2D_3$ has been shown to downregulate expression of PTH in the parathyroid glands (Delmez et al., 1989; Naveh-Many and Silver, 1990) whereas PTH acts to promote formation of active $1\alpha,25(OH)_2D_3$ by stimulating the expression of the 1α-hydroxylase enzyme in the kidney that converts the inactive 25-hydroxy precusor to the

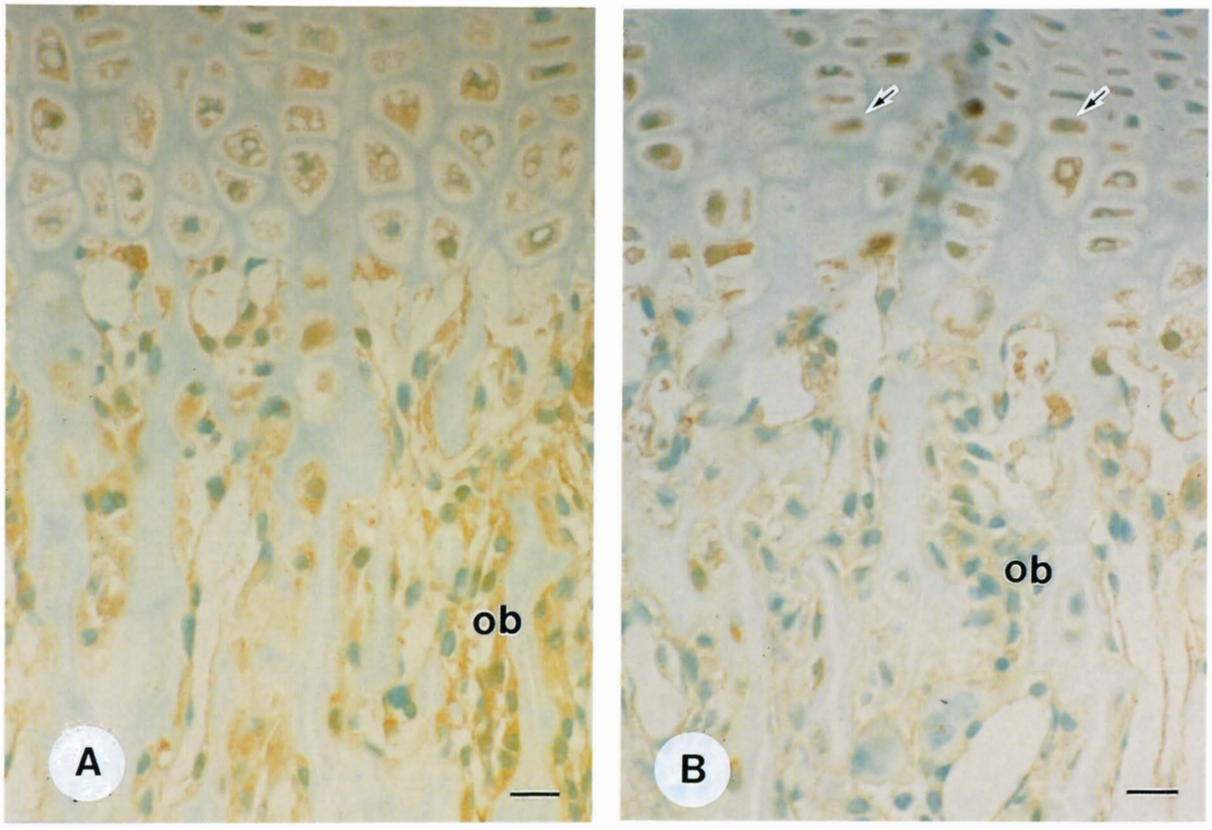


Fig. 7. A, B. Immunohistochemical analysis of PTH/PTHrP receptor protein in the control (A) and $1\alpha,25(OH)_2D_3$-treated (B) bone (quoted from Amizuka et al., 1999). **A.** PTH/PTHrP receptor-immunoreactivity (brown color) is observed in osteoblasts (ob) and chondrocytes in the growth plate (GP) of control animals. **B.** PTH/PTHrP receptor immunoreactivity in chondrocytes (arrowheads) from $1\alpha,25(OH)_2D_3$-treated animals was not altered significantly, whereas immunoreactivity in osteoblasts (ob) was noticably decreased after $1\alpha,25(OH)_2D_3$-treatment. Bar: 20 μm.

active 1α, 25-dihydroxy form of D3 (Goltzman and Hendy, 1995).

We have examined the effect of $1\alpha,25(OH)_2D_3$ on PTH/PTHrP receptor gene expression in bone and cartilage. Expression of the PTH/PTHrP receptor is controlled by at least two promoters, P1 and P2 (McCuaing et al., 1994, 1995). The downstream promoter, P2 is ubiquitously expressed, whereas expression of the upstream promoter, P1 is largely restricted to kidney (Amizuka et al., 1997). *In situ* hybridization with an antisense probe recognizing P1-specific transcript, revealed only weak signals in both osteoblasts and chondrocytes. Conversely, the downstream promoter P2 is highly expressed both in growth plate chondrocytes and in osteoblasts lining the bony trabeculae (Amizuka et al., 1999). The distribution of PTH/PTHrP receptor coding sequences detected in mouse tibiae was essentially identical to that of P2-specific sequences. $1\alpha,25(OH)_2D_3$-treatment *in vivo* markedly decreased the expression of the PTH/PTHrP receptor coding region in both preosteoblasts and osteoblasts (Figs. 6A,B, 7). In contrast, PTH/PTHrP receptor expression in chondrocytes remained high. A similar pattern of differential regulation in osteoblasts and chondrocytes was observed when tibiae from $1\alpha,25(OH)_2D_3$-treated animals were analyzed with a probe complementary to P2-specific transcripts (Amizuka et al., 1999) (Fig. 6C,D). The inhibitory effects on $1\alpha,25(OH)_2D_3$ were also observed *in vitro* in the osteoblastic cell line, MC3T3-E1, but not in CFK 2 chondrocytic cells. Gene transfer experiments suggested that this inhibition was due to cell-specific repression of P2 function by $1\alpha,25(OH)_2D_3$ in osteoblasts but not in chondrocytes (data not shown). The regulation of PTH/PTHrP receptor gene expression by $1\alpha,25(OH)_2D_3$ in osteoblasts but not in chondrocytes indicates that the

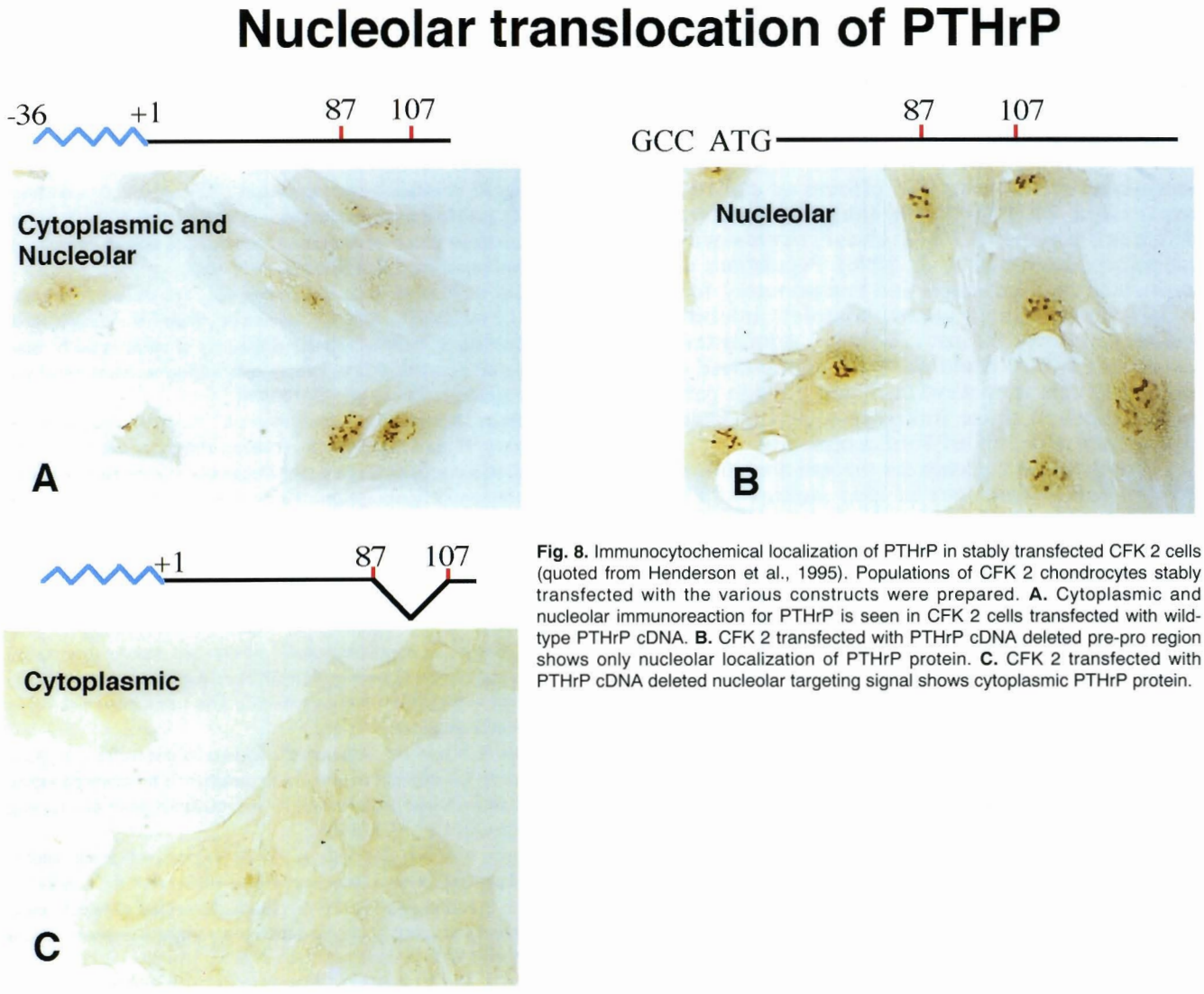


Fig. 8. Immunocytochemical localization of PTHrP in stably transfected CFK 2 cells (quoted from Henderson et al., 1995). Populations of CFK 2 chondrocytes stably transfected with the various constructs were prepared. **A.** Cytoplasmic and nucleolar immunoreaction for PTHrP is seen in CFK 2 cells transfected with wild-type PTHrP cDNA. **B.** CFK 2 transfected with PTHrP cDNA deleted pre-pro region shows only nucleolar localization of PTHrP protein. **C.** CFK 2 transfected with PTHrP cDNA deleted nucleolar targeting signal shows cytoplasmic PTHrP protein.

interplay between the two signalling pathways is specific to their overlapping roles in controlling calcium mobilization in bone, and not to their complementary effects on proliferation and maturation of chondrocytes.

Nuclear targeting of PTHrP

It is currently believed that the majority of the biological activity of PTHrP is mediated through the interaction of its amino terminus with the common PTH/PTHrP cell surface receptor. However, recent work has established an alternative, intracellular mechanism of action for PTHrP. Thus, amino acids 87-107 of PTHrP represent a bipartite nuclear targetin signal that is homologus to nucleolar targeting signal (NTS) found in certain retroviral regulatory proteins such as Rex, Rev and Tat. Henderson et al. have demonstrated that the PTHrP NTS directed passage of both PTHrP and heterologous, cytoplasmic proteins to the nuclear compartment of the transfected cells (Henderson et al., 1995) (Fig. 8). PTHrP has been localize to the nucleus and nucleoli of a sub-set of cells *in vitro* and *in vivo*. Immunoelectron microscopy localized PTHrP to the coarse fibrillar component of the nucleolus, which is where newly transcribed ribosomal RNA is found. In addition, the presence of nucleolar PTHrP was shown to protect serum-deprived CFK 2 chondrocytic cells from apoptotic cell death. The effects of constitutive expression of PTHrP on the proliferation and differentiation of CFK 2 chondrocytes was also examined (Henderson et al., 1996). Populations of cells expressing both secretory and non-secretory forms of PTHrP demonstrated almost complete inhibition of mRNA expression for matrix proteins. Administration of exogenous PTHrP could not fully mimic these effects suggesting that they were mediated through pathways independent of those linked to signal transduction downstream of the PTH/PTHrP receptor.

Corroborative evidence for the anti-apoptotic role of PTHrP comes from our *in vivo* studies of PTHrP-deficient mice (Amizuka et al., 1996b). In the lower region of the hypertrophic zone of the epiphyseal cartilage of the homozygous mice, many apoptotic chondrocytes were identified *in situ* by endo-labeling of fragmented DNA with TUNEL staining. In contrast, age-matched wild type littermates demonstrated few such apoptotic chondrocytes suggesting that apoptosis was accelerated in the growth plates of PTHrP-deficient mice.

With respect to the potential function of PTHrP in the nucleolus, Aarts et al. recently demonstrated that PTHrP associated with endogenous and homopolymeric RNA through its NTS (Aarts et al., 1999). Site-directed mutagenesis revealed that the association was dependent on preservation of a GXKKXXK motif embedded in the PTHrP NTS, which is conserved amongst double stranded RNA binding proteins. Taken together, with the location of PTHrP in the coarse fibrillar component of the nucleolus, it is hypothesized that its RNA-binding activity predicts a role for PTHrP in the regulation in the nucleoli of a sub-set of randomly cycling cells.

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