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ARTICLES

Parathyroid Hormone-Related Peptide Expression in the Epiphyseal Growth Plate of the Juvenile Chicken: Evidence for the Origin of the Parathyroid Hormone-Related Peptide Found in the Epiphyseal Growth Plate

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Abstract Parathyroid hormone-related peptide (PTHrP) has been shown to be essential for normal endochondral bone formation. Along with Indian hedgehog (Ihh), it forms a paracrine regulatory loop that governs the pace of chondrocyte differentiation. However, the source of PTHrP for this regulatory loop is not clear. While one hypothesis has suggested the periarticular perichondrium as the source of PTHrP for growth plate regulation, other data utilizing immunohistochemistry and in situ hybridization would indicate that growth plate chondrocytes themselves are the source of this peptide. The data described in this report supports the view that postnatal growth plate chondrocytes have the ability to synthesize this important regulatory peptide. Immunohistochemistry of tissue sections showed that PTHrP protein was evident throughout the chick epiphysis. PTHrP was seen in chondrocytes in the periarticular perichondrium, the perichondrium adjacent to the growth plate, the prehypertrophic zone of the growth plate, and the hypertrophic zone of the growth plate. However, cells in the proliferative zone, as well as some chondrocytes in the deeper layers of articular cartilage were predominantly negative for PTHrP. PTHrP was detected by Western blotting as a band of 16,400 Da in extracts from hypertrophic chondrocytes, but not from proliferative cells. RT-PCR detected PTHrP mRNA in both proliferative and hypertrophic growth plate chondrocytes, as well as in articular chondrocytes. PTH/PTHrP receptor mRNA was detected by Northern blotting in growth plate, but not articular chondrocytes. Thus, we conclude that most of the PTHrP present in the epiphyseal growth plate of the juvenile chick originates in the growth plate itself. Furthermore, the presence of large amounts of PTHrP protein in the hypertrophic zone supports the concept that PTHrP has other functions in addition to regulating chondrocyte differentiation. J. Cell. Biochem. 80:504–511, 2001.

Key words: PTHrP; Ihh; chondrocyte differentiation; endochondral bone formation; apoptosis; immunohistochemistry; Western blotting; immunocytochemistry; RT-PCR; Northern blotting

Parathyroid hormone-related peptide (PTHrP) has emerged as a key player in the sequence of events essential for normal growth plate development and endochondral bone growth. Research with transgenic mice has demonstrated that PTHrP functions to regulate the pace of endochondral bone formation. Growth plates from PTHrP and PTH/PTHrP receptor null mice display a distinct phenotype characterized by inadequate proliferation, premature differentiation, and accelerated endochondral bone formation [Amizuka et al., 1994; Karaplis et al., 1994; Lanske et al., 1996; Lee et al., 1996]. In contrast, growth plates from mice in which PTHrP is overexpressed are marked by delayed chondrocyte differentiation, persistence of prehypertrophic chondrocytes, and total absence of ossification [Amling et al., 1997; Weir et al., 1996]. Growth plates of transgenic mice carrying an activating mutation in the PTH/PTHrP receptor display similar features: decelerated maturation of chondrocytes, delayed vascular invasion, and absence of

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mineralization [Schipani et al., 1997]. Thus, it is well documented that a lack of PTHrP or PTH/PTHrP receptor signaling leads to accelerated endochondral bone formation, while excessive PTHrP or PTH/PTHrP receptor signaling results in delayed endochondral bone formation.

It has been demonstrated that PTHrP and Indian hedgehog (Ihh) participate in a paracrine regulatory loop that governs the pace of chondrocyte differentiation and endochondral bone formation [Lanske et al., 1996; Vortkamp et al., 1996]. This signaling pathway is thought to involve the epiphyseal growth plate, the perichondrium, and the periarticular perichondrium. In the model proposed by Vortkamp et al. [1996], Ihh is expressed by chondrocytes in the epiphyseal growth plate as they enter into hypertrophy. Secreted Ihh binds to its receptor Patched in the nearby perichondrium. This signal directly or indirectly stimulates PTHrP expression in the periarticular perichondrium. PTHrP presumably diffuses into the growth plate and binds to PTH/PTHrP receptors located primarily in the prehypertrophic zone. Binding of PTHrP to its receptor decreases the rate at which cells enter into hypertrophy, thus forming a negative feedback loop.

The report by Reynolds et al. [1998] supports the concept that the periarticular perichondrium is the source of PTHrP in the growth plates of postnatal chicks. This conclusion was based upon the observation that PTHrP mRNA expression was detected in articular, but not growth plate tissue or isolated cells. We questioned whether PTHrP from the periarticular perichondrium could serve as the major source of PTHrP for growth plate signaling in the postnatal (4 week) chick for two reasons: (1) distances required for peptide diffusion would be large; and (2) there is no evidence for a gradient of PTHrP across the growth plate from the perichondrium.

The purpose of this report is to describe the distribution of PTHrP protein and PTHrP mRNA in the juvenile chick epiphysis, and in so doing to provide evidence that the primary source of PTHrP for growth plate signaling is the growth plate itself. We used immunostaining to localize PTHrP protein in tissue sections of the epiphysis and Western blotting to characterize the protein. Reverse transcription and the polymerase chain reaction were used to detect mRNA for PTHrP in articular, proliferative, and hypertrophic chondrocytes. Northern blotting was used to detect mRNA for the PTH/PTHrP receptor in articular and growth plate chondrocytes.

METHODS

Tissue Preparation and Immunohistochemistry

Male broiler Peterson × Arbor Acre chicks raised on commercial broiler feed at the Pennsylvania State University Poultry Education and Research Center were used to supply tissue. Birds were sacrificed at 4 weeks of age by cervical dislocation and their proximal tibiotarsi were removed using a protocol approved by IACUC (No. 94R120D097).

Epiphyses were excised, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), rinsed in PBS, processed by standard methods, and embedded in paraffin. Serial 5 μm sections were deparaffinized and rehydrated prior to immunostaining. Sections were immersed in 0.3% hydrogen peroxide for 30 min, rinsed in PBS, and pretreated with hyaluronidase (1 mg/ml) for 30 min at 37°C. After rinsing in PBS, sections were blocked in 10% normal goat serum in PBS for 30 min. The primary antibody was a rabbit polyclonal antibody raised against residues 34–53 of human PTHrP (Calbiochem, San Diego, CA, No. PC09). Sections were incubated in primary antibody (4 μg/ml) in PBS for 1.5 h, rinsed in PBS, incubated in biotinylated goat anti-rabbit IgG (Sigma, St. Louis, MO, No. B-7389) in PBS (1:100) for 30 min, and rinsed in PBS. Sections were then incubated in ABC reagent (Vectastain kit, Vector Laboratories, Inc., Burlingame, CA) for 20 min, rinsed in PBS, and rinsed in 1% Triton-X 100 in PBS. Sections were incubated with diaminobenzidine tetrahydrochloride for 8 min. Following a rinse in distilled water, sections were counterstained with Gill's hematoxylin for 30 sec, rinsed, and mounted with 90% glycerol in PBS for microscopic examination. All rinses in PBS consisted of 35-min rinses. For negative controls, rabbit IgG (4 μg/ml) (Sigma, St. Louis, MO, No. I-5006) was substituted for the primary antibody. Specificity of antibody binding to tissue sections was demonstrated by preincubation with a 60-fold concentration of PTHrP peptide (Calbiochem, San Diego, CA, No. PP14) that contained residues 34–53 of human PTHrP.

Chondrocyte Isolation

Articular and epiphyseal growth plate chondrocytes were isolated using a modification of the procedure first described by Rosselot et al. [1992]. Tissue was taken from the proximal tibiotarsi of 4-week-old chicks (Fig. 1). For the isolation of articular chondrocytes, the periarticular perichondrium was removed and discarded. Small slices of articular cartilage were then harvested and diced. Tissue from the proliferative zone was obtained by lightly scraping the exposed growth plate following complete removal of the articular cartilage. The lower proliferative and prehypertrophic zones were then discarded before obtaining slices of tissue from the hypertrophic zone. Harvested tissue was placed in F-12 Ham nutrient media (F-12) containing 0.083% trypsin and 0.017% hyaluronidase. Tissue was incubated in separate spinner flasks with slow agitation for 15 min at 37°C. After two rinses in F-12, proliferative, hypertrophic, and total growth plate tissues were incubated in F-12 containing 0.10% collagenase for 3 h in spinner flasks with slow agitation at 37°C. Articular cartilage tissue required 4 h of incubation in 0.15% collagenase. At the end of the incubation period, dispersed chondrocytes of each type were filtered through 149-μm nylon mesh, and centrifuged for 10 min at 1000 rpm. The cell pellets obtained were resuspended and rinsed in F-12, then centrifuged two more times. The final pellets were resuspended in modified Webber's medium described by Rosselot et al. [1992]. Articular, proliferative and hypertrophic chondrocytes were frozen at −80°C for subsequent protein extraction or for RNA purification.

Western Blot Analysis

Protein extracts were prepared from proliferative and hypertrophic chondrocytes as described by Luan et al. [1996]. One hundred microgram samples of total protein from proliferative and hypertrophic extracts were subjected to Western blot analysis. Initially, no PTHrP could be detected in total protein extracts either from proliferative chondrocytes or hypertrophic chondrocytes by immunoblotting. Therefore, PTHrP was partially purified using gel filtration chromatography. Large volumes (750–1000 μl) of proliferative and hypertrophic protein extracts were applied to a column bed of sephadex G-50 beads. Fractions eluted after the void volume of the column (fractions No. 4–11) were dialyzed, lyophilized, and reconstituted so as to be concentrated 10–15-fold. Semi-purified, concentrated extracts from proliferative and hypertrophic chondrocytes were electrophoresed on a 12% sodium dodecyl sulfate polyacrylamide gel and electroblotted onto a nitrocellulose membrane. The Western blotting procedure was carried out using the ECL Western blotting system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's instructions. In order to detect PTHrP, membranes were incubated in rabbit antiserum to chicken PTHrP 1-34 (Peninsula Laboratories, Inc., Belmont, CA, No. IHC 6161).

RT-PCR

Total RNA was extracted from articular, proliferative, and hypertrophic chondrocytes with TRIzol reagent (Gibco BRL, Grant Island, NY) according to the manufacturer's instructions. Four-day chick embryo RNA was used as a positive control. Two micrograms of total RNA were reverse transcribed in You-Prime First Strand Ready-To-Go tubes (Amersham Pharmacia Biotech, Piscataway, NJ) with hexanucleotide random primers (Roche, Indianapolis, IN). The 5′ PCR primer was 5′-ATC-AGC-CGC-AGA-CTC-AAA-CG-3′ and corresponded to bp 106–126 of the published cDNA sequence for PTHrP in the 10-day chick embryo (Thiede and Rutledge, 1990), while the 3′ PCR primer was 5′-AGC-TCG-CCT-CTT-CTT-TTT-CTT-CT-3′ and corresponded to the complement of bp 428–447 of the published sequence. These primers should yield a 341 bp PCR product. Conditions for the PCR reaction were 5 min of initial

Fig. 1. Localization of PTHrP protein in tissue sections of the chick epiphysis. Diagram at the top of the figure shows the location of photographs taken. Immunostaining was seen in chondrocytes of the periarticular perichondrium (**A**), the intermediate layer of articular cartilage (**B**), the prehypertrophic zone (**D1**), hypertrophic zone (**E1**), and the perichondrium adjacent to the growth plate (**F**). Chondrocytes in the early proliferative zone (**C**) were negative for PTHrP, while chondrocytes in the late proliferative zone were positive for PTHrP. Negative controls for the prehypertrophic zone and hypertrophic zone are shown in (**D2**) and (**E2**), respectively.

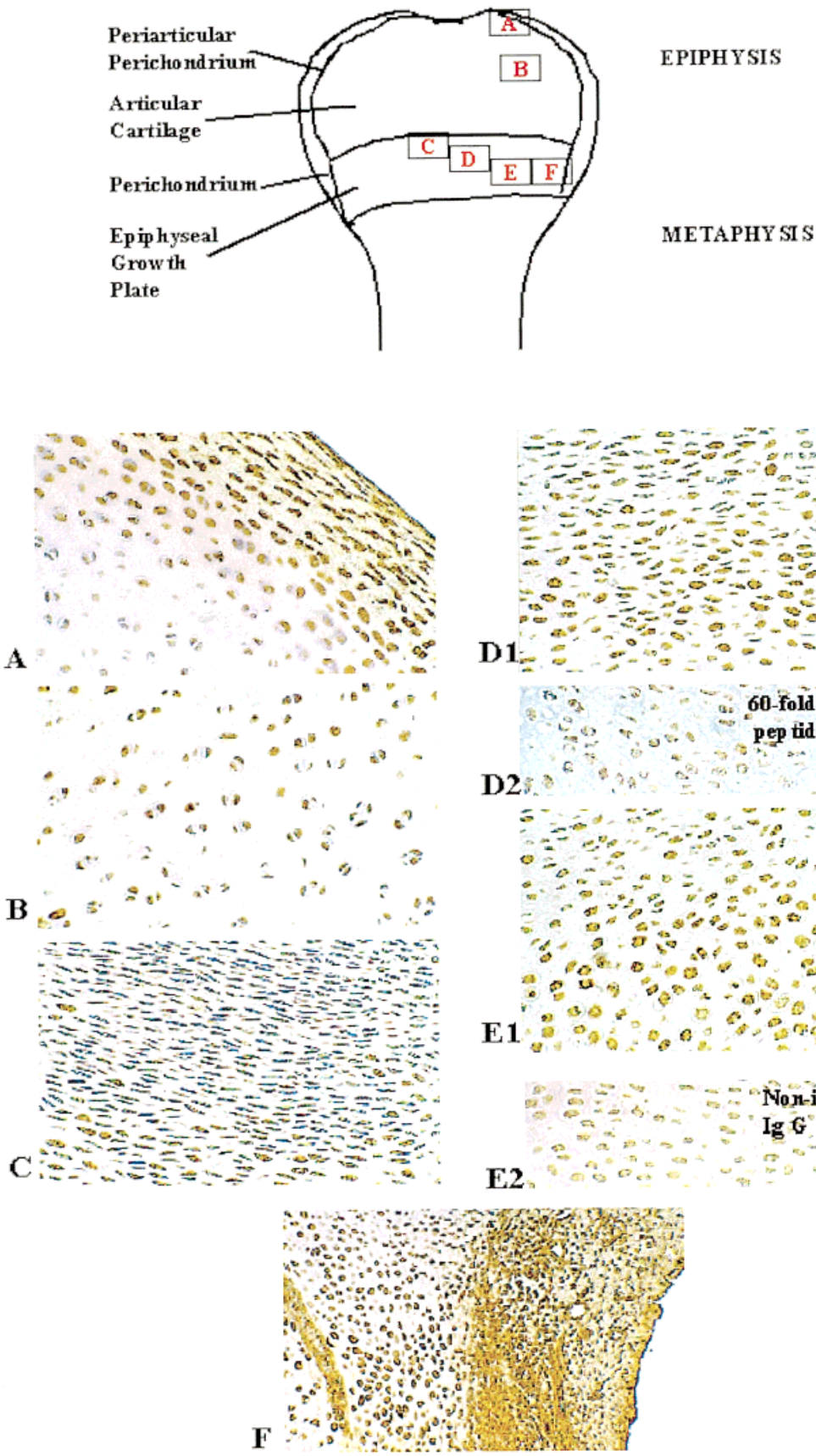
Periarticular Perichondrium
Articular Cartilage
Perichondrium
Epiphyseal Growth Plate
A
B
C
D
E
F
EPIPHYSIS
METAPHYSIS
A
B
C
D1
60-fold PTHrP peptide
D2
E1
Non-immune Ig G
E2
F

Fig. 1.

denaturation at 95°C followed by 40 cycles of denaturing at 95°C for 1 min, annealing at 58°C for 1.5 min, and extension at 72°C for 1.5 min.

Northern Blotting

Total RNA was extracted from articular, proliferative, and hypertrophic chondrocytes using TRIzol reagent according to the manufacturer's instructions. Ten microgram samples of total RNA were electrophoresed through a 1% agarose formaldehyde gel and visualized with ethidium bromide staining. RNA was blotted onto a nylon membrane using a Turboblotter (Schleicher and Schuell, Keene, NH) and UV crosslinked. ^{32}P-labeled probes were hybridized to blots at a concentration of 1×10^6 cpm/ml (5×10^5 for 18S rRNA) using standard procedures. Blots were exposed to film and/or a phosphorimager. Blots were sequentially hybridized to PTH/PTHrP receptor and 18S rRNA cDNA probes. The probe used for the PTH/PTHrP receptor was a 0.975 kb *Bam*HI-*Eco*RI fragment of plasmid for chicken PTH/PTHrP receptor [Vortkamp et al., 1996] and was provided by Clifford J. Tabin of Harvard Medical School.

RESULTS

Localization of PTHrP Protein in Tissue Sections of the Chick Epiphysis

Immunohistochemistry showed that PTHrP protein is present in tissue sections of the 4-week-old chick epiphysis (Fig. 1). Intense immunostaining was seen in the chondrocytes of the periarticular perichondrium (superficial layer of articular cartilage) and the perichondrium adjacent to the epiphyseal growth plate. Equally intense staining for PTHrP was seen in the transitional chondrocytes of the prehypertrophic zone and in mature chondrocytes of the hypertrophic zone of the growth plate. Some chondrocytes in the intermediate and deep layers of articular cartilage were positive, while others were negative for PTHrP. Chondrocytes in the early proliferative zone were negative for PTHrP, while chondrocytes in the late proliferative zone were positive for PTHrP. Immunostaining for PTHrP was localized to cells, with the exception of the staining seen in the matrix of the perichondrium lateral to the growth plate. In negative control experiments, diminished staining was seen after preincubation of the PTHrP antibody with a 60-fold excess of PTHrP peptide or after substitution of non-immune IgG for the primary antibody.

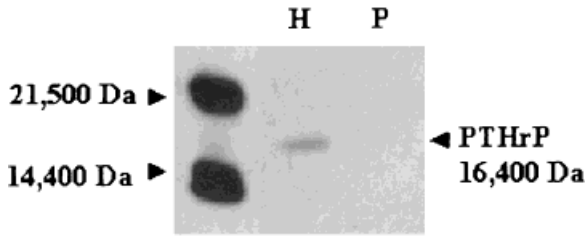

Fig. 2. PTHrP protein in chondrocyte protein extracts. Western blotting shows that PTHrP was present in hypertrophic (H), but not proliferative (P) chondrocytes. The size of the band detected in the semi-purified, concentrated hypertrophic protein extract was 16,400 Da.

PTHrP Protein in Chondrocyte Protein Extracts

After partial purification of chondrocyte protein extracts, we were able to detect PTHrP protein in hypertrophic chondrocytes, but not in proliferative chondrocytes (Fig. 2). This finding corresponds to our results from immunohistochemistry, which showed intense immunostaining in hypertrophic chondrocytes, but not in proliferative chondrocytes. The size of the band detected was 16,400 Da.

PTHrP Message in Articular and Growth Plate Chondrocytes

We were previously unable to detect mRNA for PTHrP by Northern blotting, however reverse transcription and the polymerase chain reaction allowed detection of PTHrP mRNA in RNA purified from articular and growth plate chondrocytes (Fig. 3). A product corresponding to the predicted 341 bp product was seen in articular, proliferative, and hypertrophic chondrocytes, as well as the control 4 day embryo

Fig. 3. PTHrP mRNA in articular and growth plate chondrocytes. PTHrP mRNA was detected by RT-PCR in 4-day chick embryo (E), articular (A), proliferative (P), and hypertrophic chondrocytes (H). Lane (B) contained all PCR reagents, but no substrate.

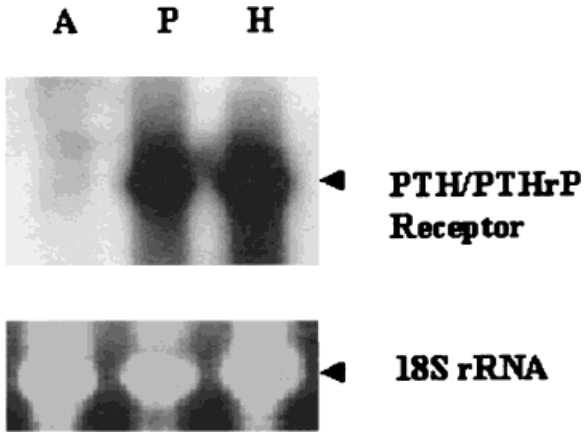

Fig. 4. PTH/PTHrP Receptor mRNA in articular and growth plate chondrocytes. PTH/PTHrP receptor mRNA was detected by Northern blotting in proliferative (P) and hypertrophic (H) chondrocytes, but not in articular (A) chondrocytes.

RNA. Digestion of the PCR product with Pst I yielded fragments similar to the 123 and 218 bp fragments predicted by the cloned sequence, and samples of RNA which had not been reverse transcribed yielded no product (data not shown).

PTH/PTHrP Receptor Message in Articular and Growth Plate Chondrocytes

PTH/PTHrP receptor mRNA was detected by Northern blotting in RNA extracted from growth plate chondrocytes (Fig. 4). Receptor message was repeatedly seen in proliferative and hypertrophic chondrocytes, but never seen in articular chondrocytes.

DISCUSSION

We report here that PTHrP protein can be detected in sections of juvenile (4-week-old) chick growth plate tissue and in semi-purified protein extracts from chick hypertrophic chondrocytes. Moreover, PTHrP mRNA was also detected in chick growth plate chondrocytes. These data suggest that the PTHrP found within the growth plate is endogenous to the growth plate. These observations allow for an alternative, but simpler explanation of the origin of growth plate PTHrP than previously proposed for embryonic tissues [Vortkamp et al., 1996]. In agreement with the observations by Van Der Eerden et al. [2000] on the postnatal rat growth plate, we believe that in postnatal stages, a PTHrP paracrine feedback loop confined to the growth plate seems more feasible and takes into account the data we have presented here.

Our results show that PTHrP is clearly present in the prehypertrophic and hypertrophic zones of the growth plate, in the perichondrium lateral to the growth plate and in the perichondrium surrounding the articular cartilage. PTHrP is absent from cells early in the proliferative zone of the growth plate but becomes more evident as the cells progress to prehypertrophy. Reports from the literature describe the localization of PTHrP in the growth plate and surrounding tissues. In embryonic mice, Lee et al. [1996] found PTHrP protein to be most concentrated in the prehypertrophic zone, less evident in the hypertrophic zone, and absent from perichondrial and proliferative chondrocytes. Kartsogiannis et al. [1997] localized PTHrP protein in embryonic and newborn (Day 7) mice and observed PTHrP in the proliferative, prehypertrophic, and hypertrophic zones. Tsukazaki et al. [1995] found PTHrP in the lower two-thirds of the 3-week-old rat growth plate and also in chondrocytes of rat articular cartilage, and reported that expression of PTHrP in articular cartilage fluctuated during development. PTHrP has been observed in the prehypertrophic and hypertrophic zones of the postnatal rat growth plate but was absent from the perichondrium [Van Der Eerden et al., 2000].

Our Western blots detected a band for PTHrP in hypertrophic chondrocyte protein extracts, which averaged 16,400 Da. The chicken PTHrP gene encodes a protein of 139 amino acids [Thiede and Rutledge, 1990] with a predicted molecular weight of 15,500 Da. Since we used an antibody directed against chicken PTHrP 1-34, our band contains the N-terminus and may represent the full-length chicken PTHrP peptide. Additionally, since an N-terminal species of PTHrP can be glycosylated by some cell types [Stewart et al., 1991; Wu et al., 1991], some degree of glycosylation may be present in the chicken PTHrP peptide.

We were not able to detect PTHrP mRNA using Northern blotting. However, RT-PCR allowed us to detect message for PTHrP not only in RNA derived from articular chondrocytes, but also from proliferative and hypertrophic chondrocytes in the juvenile chick. The fact that PTHrP message in the epiphyseal growth plate is difficult to detect may be related

to the status of PTHrP as an early response gene [Allinson and Drucker, 1992; Holt et al., 1994]. Such genes have constitutively low levels of expression and unstable mRNA leading to rapid mRNA turnover. This situation may be advantageous in the fine-tuned regulation of a process like endochondral bone growth. Reynolds et al. [1998] used RNaase protection to detect an abundance of PTHrP mRNA in articular chondrocytes of the juvenile chick, but were not able to detect it in chick growth plate chondrocytes. More recent research by these investigators [Pateder et al., 2000] has confirmed the presence of PTHrP mRNA in the epiphysis (articular cartilage) by RNaase protection. Lower levels of mRNA were detected in growth plate chondrocytes. The amount of PTHrP expression was highest in proliferative chondrocytes and declined with increasing maturation of other cell populations. Because of the much higher level of expression in the epiphyseal (articular) chondrocytes, these authors still consider this tissue to be the major source of PTHrP for growth plate tissue. However, in growth plate tissue, there is an inverse relationship between the amount of PTHrP mRNA detected by Pateder et al. [2000] and PTHrP protein as observed by our immunostaining. These conflicting observations reinforce the concept that it may be misleading to base conclusions exclusively on the quantitation of mRNA levels. This problem is highlighted by the report of Rosselot et al. [1995] who found correlations between tissue insulin-like growth factor-I mRNA and peptide levels to be poor.

In situ hybridization has detected PTHrP mRNA in the perichondrium and hypertrophic zone of embryonic rat tibiae [Lee et al., 1995], in the perichondrium of embryonic mice [Lee et al., 1996], and in the proliferative, prehypertrophic, and hypertrophic zones of embryonic and newborn mice [Kartsogiannis et al., 1997]. These data do not exclude the possibility that the growth plate itself is the source of PTHrP needed for signaling there.

Using Northern blotting, we have demonstrated that PTH/PTHrP receptor message can readily be detected in RNA extracts from growth plate chondrocytes. The distribution of PTH/PTHrP receptor message in the growth plate has been described previously by others. In situ hybridization has indicated that PTH/PTHrP receptor message is present in maturing (prehypertrophic) growth plate chondrocytes of embryonic rats and mice [Lee et al., 1995, 1996]. While PTH/PTHrP receptor expression is restricted to the prehypertrophic growth plate chondrocytes of the 3-week posthatch chick [Ben-Bassat et al., 1999; Vortkamp et al., 1996], there is weak expression in the proliferative zone as well as the strong expression seen in the prehypertrophic and hypertrophic zones of the postnatal rat [Van Der Eerden et al., 2000].

While we observe PTHrP in the perichondrium we think that it is unlikely to diffuse far enough into the postnatal growth plate to control chondrocyte differentiation. We can only speculate as to the function of the PTHrP localized within the perichondrium. One possibility would be control of the differentiation of cells localized within these tissues.

Our localization of PTHrP and its receptor in the prehypertrophic and hypertrophic zone are consistent with the well-established role of this signaling pathway in controlling the pace of chondrocyte differentiation [Kronenberg et al., 1998]. Our observations support a model proposed by Van Der Eerden et al. [2000], where PTHrP and its receptor form part of a regulatory loop that is confined to the prehypertrophic and hypertrophic zone of the postnatal growth plate. In addition to controlling differentiation, a recent study by Lanske et al. [1999] suggests that PTHrP may function in other roles. These roles may involve vascular invasion of cartilage or regulation of chondrocyte apoptosis. Our observation that there is intense immunostaining for PTHrP in hypertrophic chondrocytes would be consistent with these functions.

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