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Expression of parathyroid hormone-related peptide (PTHrP) and its receptor (PTH1R) during the histogenesis of cartilage and bone in the chicken mandibular process

Qiong Zhao,[1] Philip R. Brauer,[1] Lei Xiao,[3] Michael H. McGuire[2] and John A. Yee[1]

[1]*Department of Biomedical Sciences,* [2]*Department of Surgery, Division of Orthopaedic Surgery and*
[3]*Department of Pharmacology, Creighton University, School of Medicine, 2500 California Plaza, Omaha, NE 68178, USA*

Abstract

The purpose of this study was to examine the expression and actions of parathyroid hormone-related protein (PTHrP) when skeletal histogenesis occurs in the chicken mandible. Prior to the appearance of skeletal tissues, PTHrP and PTH1R were co-expressed by cells in the ectoderm, skeletal muscle, peripheral nerve and mesenchyme. Hyaline cartilage was first observed at HH stage 27 when many but not all chondroblasts expressed PTHrP and PTH1R. By stage 34, PTHrP and PTH1R were not detected in chondrocytes but were expressed in the perichondrium. Alkaline phosphatase (AP)-positive preosteoblasts and woven bone appeared at stages 31 and 34, respectively. Preosteoblasts, osteoblasts and osteocytes co-expressed PTHrP and PTH1R. Treatment with chicken PTHrP (1–36) increased cAMP in mesenchyme from stage 26 embryos. Continuous exposure to chicken PTHrP (1–36) for 14 days increased cartilage nodule number and decreased AP while intermittent exposure did not affect cartilage nodule number and increased AP in cultures of stage 26 mesenchymal cells. Adding a neutralizing anti-PTHrP antibody to the cultures reduced cartilage nodule number and did not affect AP. These findings show that PTHrP and PTH1R are co-expressed by extraskeletal and skeletal cells before and during skeletal tissue histogenesis, and that PTHrP may influence skeletal tissue histogenesis by affecting the differentiation of mandibular mesenchymal cells into chondroblasts and osteoblasts.
Key words intramembranous ossification; mandible development; Meckel's cartilage; membrane bone; perichondrium.

Introduction

During development, skeletal histogenesis occurs by intramembranous and endochondral ossification. The former produces the flat bones in the craniofacial skeleton, while the bones in the vertebral column, limbs and some craniofacial bones develop by the latter. Both processes begin as a condensation of mesenchymal cells, but then diverge along separate morphogenetic pathways. The mesenchymal cells differentiate into osteoblasts that deposit woven bone at sites where membrane bones form, whereas differentiation to chondroblasts and hyaline cartilage formation occurs in areas of endochondral ossification. While the morphological details associated with both types of bone formation are well established, the molecules that regulate the differentiation of mesenchymal cells and growth of the skeletal tissues are only now being discovered.

Locally produced molecules influence one or more steps in the patterning, histogenesis and growth of bone tissue during development. Recent studies on gene-null mice and normal chick embryos have established that skeletal development and growth require parathyroid hormone-related protein (PTHrP) and the PTH/PTHrP receptor (PTH1R) (Philbrick et al. 1996). Although PTHrP (Karaplis et al. 1994) and PTH1R (Lanske et al. 1996, 1998, 1999) gene-null mice die before or shortly after birth, the fetuses/neonates exhibit similar appendicular and craniofacial skeletal abnormalities that include shortened limb bones, a shortened mandible and a domed skull (Karaplis et al. 1994). The dwarfed limbed phenotype occurs as a result of abnormal

Correspondence
John A. Yee, PhD, Department of Biomedical Sciences, School of Medicine, Creighton University, 2500 California Plaza, Omaha, NE 68178, USA. Tel.: (402) 280 2916; fax: (402) 280 5556; e-mail: jayee@creighton.edu

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interstitial growth of the epiphyseal growth cartilage. In the absence of PTHrP or its receptor, chondrocytes undergo premature hypertrophy (Amizuka et al. 1994; Karaplis et al. 1994; Lanske et al. 1996; Chung et al. 1998). This reduces the number of resting and proliferating chondrocytes and significantly decreases longitudinal bone growth (Amizuka et al. 1994; Karaplis et al. 1994). Chondrocyte differentiation is also inhibited and longitudinal bone growth retarded in transgenic mice that overexpress PTHrP (Weir et al. 1996). These results demonstrate that PTHrP, acting through the PTH1R, regulates longitudinal bone growth by controlling chondrocyte differentiation in the epiphyseal growth cartilage (Amizuka et al. 1994; Karaplis et al. 1994; Lanske et al. 1996; Vortkamp et al. 1996; Chung et al. 1998).

The reduced growth of limb bones in PTHrP or PTH1R knockout mice is corrected when either PTHrP (Philbrick et al. 1998) or PTH1R (Schipani et al. 1997) expression by chondrocytes is rescued using PTHrP or PTH1R transgenes under control of the procollagen α1 (II) promoter. However, restoring PTHrP expression by chondrocytes in gene-null mice failed to correct the shortening of the maxilla and mandible (Philbrick et al. 1998). This suggests that abnormal development of the mandible is not due to reduced growth of hyaline cartilage in the mandibular process. To explain this finding it was suggested that shortening of the mandible in PTHrP gene null mice is due to reduced membrane bone growth. However, this is inconsistent with a report that interstitial growth of Meckel's cartilage is the primary mechanism for outgrowth of the mandible (Diewert, 1980). Thus, the exact role of PTHrP on mandible development remains unclear. To clarify this point we have examined the temporal and spatial distribution of cells that express PTHrP and PTH1R before and during the histogenesis of hyaline cartilage and membrane bone tissues in the embryonic chicken mandible, and determined the effects of PTHrP on endpoints of chondrogenesis and osteoblast differentiation by mandibular mesenchymal cells *in vitro*.

Materials and methods

Morphological experiments and methods

Tissue preparation

Pathogen-free fertilized white leghorn chicken eggs were purchased from Hy-Vac Laboratories (Adel, IA, USA). The eggs were incubated at 38 °C. After 53 h, 3, 4, 5, 5.5, 7 and 8 days (stages 16, 18, 23, 26, 27, 31, and 34, respectively) embryos were collected, and bisected transversely caudal to the developing heart. The rostral part of each embryo was fixed in 10% formalin in PBS (pH 7.4) for 1 h at room temperature (RT) and embedded in paraffin. Serial 7-μm-thick sagittal sections through the mandibular process were mounted on glass slides and used for immunostaining, histochemical localization of alkaline phosphatase (AP), alcian blue staining to localize sulphated glycosaminoglycans (cartilage matrix), biotinylated hPTHrP (1–34) binding, and *in situ* hybridization histochemistry.

PTHrP and PTH1R immunohistochemistry

A polyclonal antibody provided by Dr Jane M. Moseley (St. Vincent's Institute for Medical Research, Melbourne, Australia) that was raised in rabbits against synthetic human PTHrP (1–34) was used for PTHrP immunostaining. The antibody recognizes synthetic PTHrP (1–34) as well as recombinant PTHrP (1–84), PTHrP (1–108) and PTHrP (1–141) (Danks et al. 1990). The antiserum used for PTH1R immunostaining was raised in rabbits against a synthetic 20 amino acid peptide sequence in the extracellular N-terminal region of the human PTH1R. Dr Cary W. Cooper (University of Texas Medical Branch at Galveston, Galveston, TX, USA) provided this antiserum. The secondary antibody was goat antirabbit IgG conjugated to horseradish peroxidase (HRP) (Sigma, St. Louis, MO, USA).

Sagittal sections through the mandibular process were incubated in a blocking solution (2% bovine serum albumin (BSA) and 0.1% Tween-20 in PBS) and then exposed to the primary antibody (1 : 200) overnight at 4 °C in a humidified box. Sections were then washed, placed in 0.3% hydrogen peroxide to block endogenous peroxidase activity (Meacock et al. 1992), and then incubated in the secondary antibody (1 : 500) for 1 h at RT. Peroxidase activity was detected using 3-amino-9-ethylcarbazole (AEC, Zymed Laboratories, Inc., San Francisco, CA, USA). The sections were counterstained for 5 s in 10× diluted Mayer's haematoxylin (Vector Laboratories, Inc., Burlingame, CA, USA). Coverslips were applied using aqueous mounting medium (Crystal/Mount, Biomeda, Foster City, CA, USA) and sealed with clear nail polish. Immunohistochemical controls included: (1) omission of primary antibody, (2) replacing the primary antibody with non-immune rabbit serum, (3) omission of the secondary antibody and (4)

pre-absorption of the PTHrP antiserum with 2 μM [Tyr^{36}] chicken PTHrP (1–36) amide (chicken PTHrP (1–36), Peninsula Laboratories, Belmont, CA, USA) before immunostaining.

Biotinylated PTHrP binding

PTHrP target cells in the mandibular process were located by their ability to bind biotinylated human PTHrP (1–34) (Peninsula Laboratories, Inc., Belmont, CA, USA). Histological sections were incubated in a solution containing biotinylated human PTHrP (1–34) (10 ng μL^{-1} in blocking buffer) overnight at 4 °C. The sections were then washed, placed in 0.3% hydrogen peroxide, washed, and then incubated in streptavidin-biotin-HRP (ABC-HRP, Vector Laboratories). Peroxidase activity was detected using AEC. Sections were then counterstained with haematoxylin and coverslipped with aqueous mounting medium (see above). Controls to assess the specificity of biotinylated human PTHrP (1–34) binding included: (1) omission of the biotinylated human PTHrP (1–34) from the binding solution, and (2) addition of 500-fold excess of unlabelled chicken PTHrP (1–36) (5 μg μL^{-1}) to the incubation solution containing biotinylated human PTHrP (1–34).

Double staining for AP and extracellular matrix sulphated glycosaminoglycans

Histochemical staining for AP was used to identify cells in the osteoblast lineage, and staining with alcian blue at pH 1.0 was used to detect sulphated acidic proteoglycans in hyaline cartilage extracellular matrix. AP was detected by incubating sections in Tris buffer (pH 8.5) containing 0.01% (w/v) naphthol AS-MX phosphate (Sigma) and 0.07% (w/v) fast red violet LB (Sigma) in the dark. After washing with Tris buffer, the sections were then incubated in 0.1 N HCl (pH 1.0) containing 0.1% alcian blue (Sigma), washed in 0.1 N HCl followed by several changes of Tris buffer (pH 8.5), and coverslipped as above.

In situ hybridization

Preparation of PTHrP and PTH1R cDNAs

A 495-bp fragment of chicken PTHrP cDNA corresponding to the region +130 to +625 (Thiede & Rutledge, 1990) was isolated from total RNA of stage 18 embryos using RT-PCR. The sequence of the upstream (sense) primer was 5′-TAT CAG AGC ACC AGC TAC TG-3′; and the sequence of the downstream (antisense) primer was 5′-AAT GAG GCC TTG ACC TCA CA-3′. The chicken PTHrP cDNA products were then subcloned into the vector pCR2.1 (InVitrogen, San Diego, CA, USA) according to the manufacturer's protocol and the sequence confirmed using a DNA Sequencer (ABI Model 373, Applied Biosystems, Foster City, CA, USA). A 500-bp PCR cDNA fragment that corresponded to the 5-prime end of the human PTH1R cDNA was obtained from Dr Matthew Gillespie (St. Vincent's Institute of Medical Research, Melbourne, Australia). This cDNA fragment was inserted in the EcoRI site of the Bluescript II KS (+) plasmid. Plasmids with cDNA fragments in opposite directions were selected to produce sense and antisense riboprobes. The riboprobes were synthesized and labelled with digoxigenin (DIG) using an RNA labelling kit from Roche Molecular Biochemicals (Indianapolis, IN, USA). The sense and antisense probes for chicken PTHrP were generated by T7 RNA polymerase transcription after plasmids were linearized by BamHI. The sense and antisense probes for the human PTH1R were generated by T7 or T3 RNA polymerase transcription after linearization of the plasmid.

In situ *hybridization*

Embryos were fixed in 10% buffered formalin for 2 h at 4 °C, and embedded in paraffin under RNase-free conditions. Sagittal sections containing the mandibular process were incubated in 0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0), rinsed in 2× standard saline citrate (SSC) for 5 min and then equilibrated in prehybridization buffer (50% formamide in 0.3 M NaCl, 0.06 M Tris-HCl, and 0.004 mM EDTA) at 37 °C. The hybridization solution contained DIG-labelled riboprobe (1 μg mL^{-1}), 5× SSC, 50% formamide, 1× Denhardt's solution, 10% dextran sulphate, 100 μg mL^{-1} salmon sperm DNA, and 100 μg mL^{-1} yeast tRNA. Hybridization was performed in a humidified chamber for 18 h at 57 °C. The sections were then washed with 50% formamide in 2× SSC, two changes of 1× SSC, and three changes of 0.5× SSC. After blocking with Tris-buffered saline (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) containing 2% horse serum, the hybridized probe was detected by immunostaining with an AP-conjugated anti-DIG antibody (1 : 500). AP was detected using NBT-BCIP and X-phosphate (Roche Molecular Biochemicals). Sections without counterstaining were coverslipped as described above. Controls for *in situ* hybridization

included: (1) omission of labelled antisense riboprobe, (2) pretreatment of sections with RNase A (200 μg mL^{-1} in 2× SSC for 90 min at 37 °C) before hybridization and (3) hybridization with the labelled sense riboprobe.

Western blotting

Western blotting was used to verify the specificity of the antibodies used for immunostaining and to establish the apparent molecular weights of the immunodetectable proteins in developing chicken embryos. The primary and secondary antibodies were the ones used for immunohistochemistry. PTHrP protein was evaluated in stage 18, 23, 26, 27, 31, and 34 embryos and PTH1R protein was evaluated in stage 26 embryos. Embryos were homogenized in ice-cold PBS containing 1% Triton X-100 (Sigma Chemical Co., St. Louis, MO, USA). The crude homogenate was then centrifuged at 20 000 ***g*** for 10 min at 4 °C, and the protein concentration in the supernatant measured (BCA protein assay kit, Sigma Chemical Co.). Samples containing 150–200 μg protein were diluted in 2× sodium dodecyl sulphate (SDS) sample buffer containing 5% β-mercaptoethanol and electrophoresed on 4–15% linear SDS-polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were then transferred to nitrocellulose membrane (0.22 μm, Micron Separations Inc., West Borough, MA, USA). The membrane was pre-incubated in blocking solution (5% casein, 1% BSA, 0.1% Tween-20 in Tris buffered saline) overnight, and then incubated with the primary antibodies diluted 1: 2000 in blocking solution for 2 h at RT. After incubation with the antibodies, the membranes were washed with Tris-buffered saline, quenched in 0.3% hydrogen peroxide, and then incubated with the goat antirabbit IgG-HRP (1 : 10 000) for 1 h at RT. Peroxidase activity was detected with an enhanced chemiluminescence kit (ECL, Pierce, Rockford, IL, USA).

Mesenchymal cell isolation and cell culture

Mandibular processes from stage 26 chick embryos were digested in PBS (pH 7.4) containing 0.25% trypsin and 0.43% porcine pancreatin at 4 °C. After 30 min the tissues were transferred into Medium 199 containing 50% fetal bovine serum (FBS, Life and Science Technology, Gaithersburg, MA, USA), and the mesenchyme was separated from the epithelium by microdissection. The mesenchymal tissue was washed several times in serum-free Medium 199 and its cells placed into suspension by vigorous pipetting in Medium 199 supplemented with 10% FBS, 10 000 U mL^{-1} penicillin and 10 mg mL^{-1} streptomycin (P/S). Cells were pelleted by centrifugation, washed twice with culture medium, resuspended, and plated into 24-well culture plates at an initial density of 40 000 cells per well. After 18 h the cultures were changed to fresh medium containing chicken PTHrP 1–36 (cPTHrP 1–36) (see below). The culture medium was changed every 48 h. The cells were maintained in Medium 199 supplemented with 10% FBS and P/S for 7 days, and then changed to BGJb medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS, 50 μg mL^{-1} ascorbic acid and P/S.

Effect of cPTHrP (1–36) on cAMP in ectoderm-free chicken mandibular mesenchyme

After removing the ectoderm pieces of stage 26 mesenchyme were pre-incubated in serum-free medium containing 0.5 mM isobutylmethylxanthine (IBMX, Sigma Chemical Co.) for 30 min and then exposed to 0 (control), 50, 500 and 1000 nM cPTHrP (1–36) in IBMX containing medium for 20 min. Cyclic AMP in tricarboxylic acid extracts of the mesenchyme was measured by enzyme immunoassay (EIA) using a kit purchased from Amersham Life Science, Inc. (Arlington Heights, IL, USA) according to the manufacturer's instructions.

Effect of cPTHrP (1–36) on cartilage nodule number and ap activity in mandibular mesenchymal cell cultures

Cultures of chick mandibular mesenchymal cells were treated with 0 (control), 0.001, 0.01, 0.1, 1 or 10 nM cPTHrP (1–36) for 14 days. Exposure to the peptide was either continuous or intermittent. For intermittent treatment, cPTHrP (1–36) was added to the medium, and after 6 h the medium was removed, the cells washed with peptide-free medium, and the cultures maintained in PTHrP-free medium for the remainder of the 48-h period. This procedure was repeated for each of the seven 48-h treatment periods over the 14 days. For continuous treatment, cells were exposed to cPTHrP (1–36) for the entire time. At the end of the treatment period, some cultures were fixed with 80% methanol for 30 min at RT, washed with 1.0 mM

Tris-buffer (pH 8.5), stained with alcian blue, and the number of cartilage nodules in each well counted. The cells in parallel cultures were lysed in deionized water containing 0.1% (v/v) Triton X-100. Cell lysates were centrifuged at 12 000 ***g*** for 10 min at 4 °C. The supernatants and pellets were separated and protein in the supernatant was measured as described above. AP activity in the supernatant was measured with the Sigma 104 alkaline phosphatase assay kit (Sigma Chemical Co.) as described previously (Yee, 1985). The intermittent exposure experiment was repeated three times and the continuous exposure experiment was repeated four times.

Effect of anti-PTHrP antibody on cartilage nodule number and AP activity in cultures of chick mandibular mesenchymal cells

Cultures of stage 26 mandibular mesenchymal cells were exposed to various dilutions (1 : 16 000 to 1 : 250) of rabbit antihuman PTHrP (1–34) antiserum. After 13 days the cultures were analysed for cartilage nodules and AP activity as described above.

Statistical methods

The data from replicate experiments to determine the effect of cPTHrP (1–36) on cartilage nodule number and AP activity were normalized as percentage of control and pooled for statistical analysis. Differences between treated and control groups were determined by one-way ANOVA and the Student–Newmann–Keuls multiple range test.

Results

Western blotting

An immunoblot of proteins extracted from stage 18, 23, 27, 31 and 34 chick embryos is shown in Fig. 1. The rabbit antihuman PTHrP antibody cross-reacted with synthetic aminoterminal cPTHrP (1–36) (lane 1) that has a molecular weight of 10 kDa. An immunodetectable band at 10 kDa was also detected in the chick embryo protein extracts at every stage studied (lanes 2–6). The anti-PTHrP antibody also cross-reacted with a protein of 37 kDa. The antihuman PTH1R antiserum reacted most strongly and consistently with a 60-kDa protein on Western blots of proteins extracted from stage 26 chicken embryos (Fig. 2). This antibody also detected two additional proteins that migrated at 45 and 85 kDa on some immunoblots (data not shown).

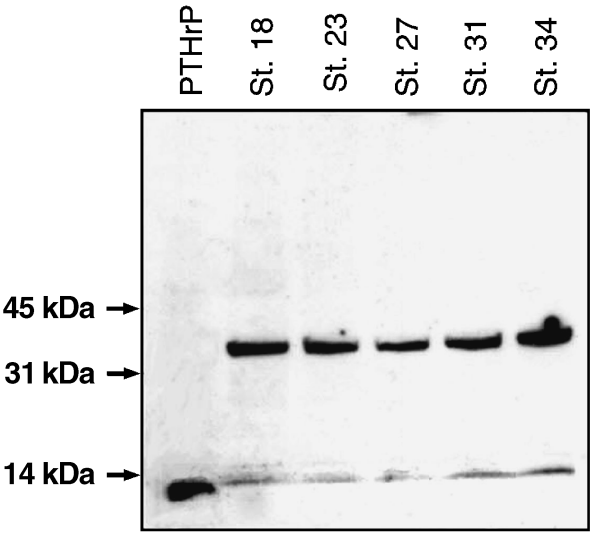


Fig. 1 Immunoblot of PTHrP in chicken embryos. Western blot of chicken proteins immunostained with rabbit anti-hPTHrP antiserum. The lane labelled PTHrP was loaded with 150 ng cPTHrP (1–36). Two proteins, which migrated at 37 kDa and 10 kDa, were detected in samples from stages 18 (St. 18), 23 (St. 23), 27 (St. 27), 31 (St. 31) and 34 (St. 34) chicken embryos. The arrows indicate the positions of the 45-, 31- and 14-kDa protein markers.

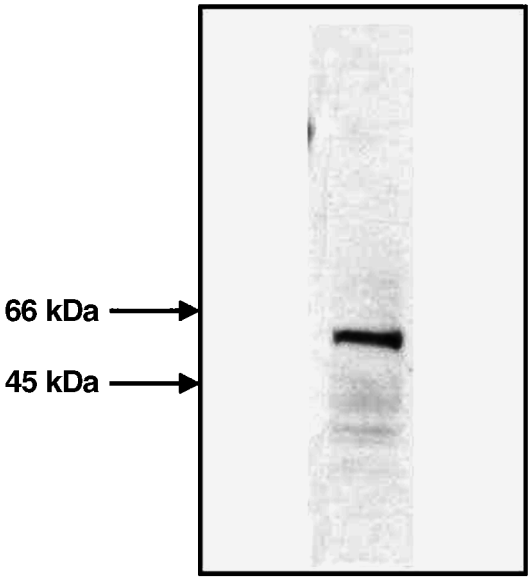


Fig. 2 Immunoblot PTH1R in stage 26 chicken embryos. Western blot of chicken proteins immunostained with rabbit anti-hPTH1R antiserum. The antiserum detected a protein that migrated at 60 kDa. The arrows indicate the positions of 66- and 45-kDa protein markers.

Immunohistochemistry and *in situ* hybridization histochemistry

Stage 23

Since the morphology and staining patterns observed in the chick mandibular process at stages 16, 18 and 23 were similar, only stage 23 is described. Parasagittal sections of the stage 23 chick mandibular process stained with alcian blue (pH 1.0) or haematoxylin are shown in Fig. 3A and B, respectively. At this stage the mandibular process consisted of a core of mesenchyme covered by a simple cuboidal epithelium. In the mesenchyme were skeletal muscle cells, peripheral nerves, and a morphologically homogeneous population of mesenchymal cells. All of the epithelial cells in the ectoderm were immunostained by the PTHrP (Fig. 3C) and the PTH1R (Fig. 3D) antisera. Immunostaining for both proteins was also observed in some, but not all, mesenchymal cells. Immunopositive mesenchymal cells were primarily located in the ventral part of the mandibular process. Skeletal muscle cells and axons and/or glial cells of the peripheral nerves were also positive for PTHrP and PTHR1. The cellular distribution of PTHrP and PTH1R mRNA transcripts was identical to that observed for the proteins, except mRNAs were not observed in axons of the peripheral nerves (Fig. 3E,F). No immunostaining was visible at this or any other stage when the primary antibodies were omitted from the staining procedure (Fig. 3B), or when the antihuman PTHrP antiserum was pre-absorbed with 5 μM chick PTHrP (1–36) before exposure to the tissue sections (not shown). Neither PTHrP nor PTH1R mRNAs were detected in sections hybridized with digoxigenin-labelled sense riboprobes (Fig. 3G,H).

Stage 27

Histochemical staining for hyaline cartilage tissue was observed in the mesenchyme of the mandibular process at stage 27. The blastema of Meckel's cartilage was a distinct region of extracellular matrix that lightly stained with alcian blue (Fig. 4A). Immunostaining for PTHrP (Fig. 4C,D) and its receptor (Fig. 4G,H) was observed on many chondroblasts surrounded by this extracellular matrix. PTHrP (Fig. 4K,L) and PTH1R (Fig. 4O,P) mRNA transcripts exhibited a similar pattern of cellular localization.

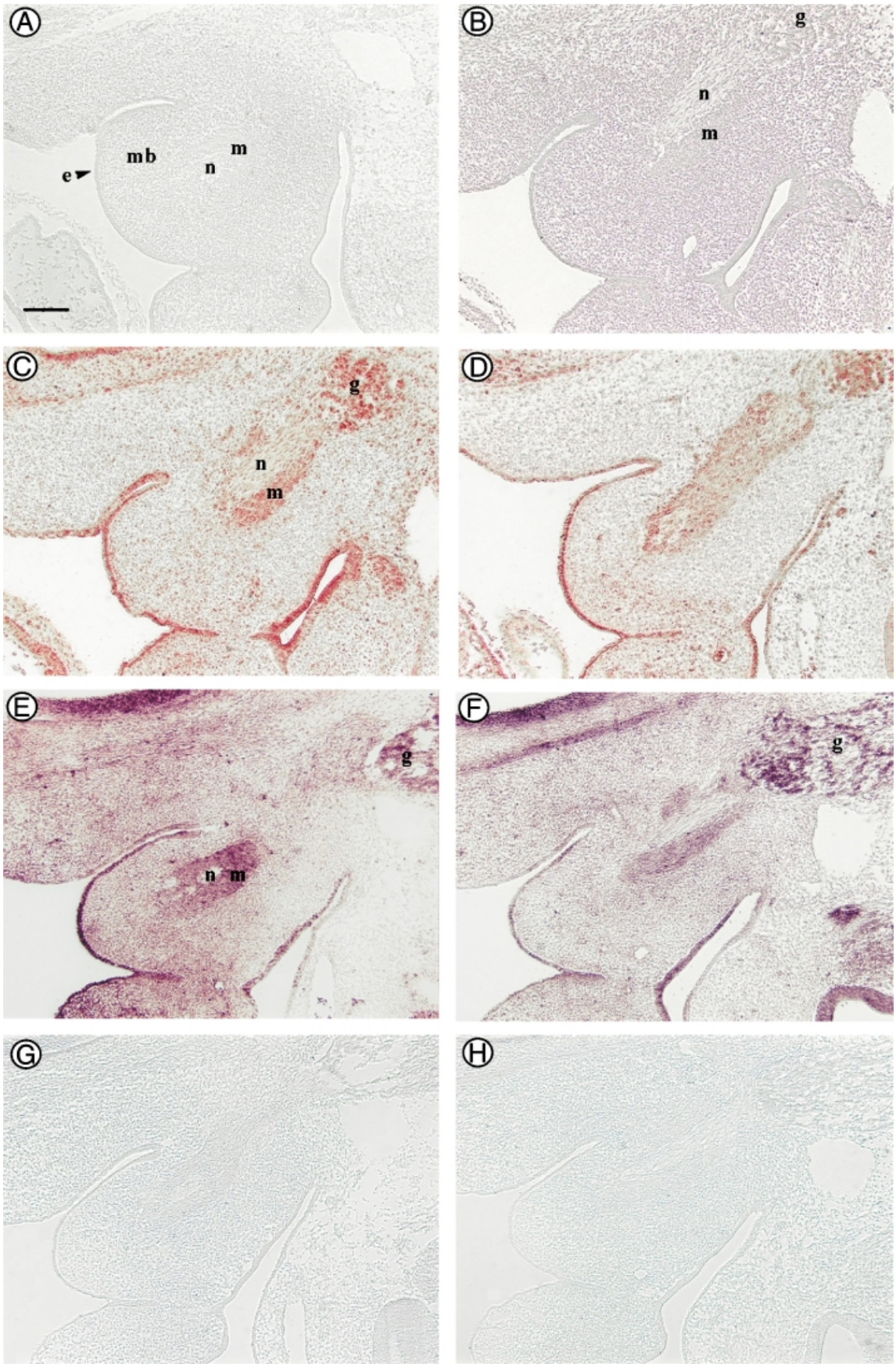
Stage 31

The mandibular process of a stage 31 embryo that was double-stained for AP and with alcian blue is shown in Fig. 4(B). Mature hyaline cartilage tissue and histochemical evidence of osteogenic cell differentiation characterized this stage. The extracellular matrix of Meckel's cartilage was intensely stained with alcian blue and the boundary between this tissue and the mesenchyme was marked by a morphologically distinct perichondrium. PTHrP and PTH1R proteins (Fig. 4E,F and 4I,J) and mRNAs (Fig. 4M,N and 4Q,R) were observed in the perichondrium at the ventral end of Meckel's cartilage. Some chondrocytes were immunopositive for PTHrP (Fig. 4F) and PTH1R (Fig. 4J) proteins, but few exhibited mRNAs for these genes (Fig. 4N,R). An aggregate of AP-positive cells located adjacent to Meckel's cartilage revealed osteoblast differentiation and served as the first evidence of membrane bone formation (Fig. 4B). The cells in these aggregates were positively stained for PTHrP and PTH1R proteins (Fig. 4E,F and 4I,J) and mRNA transcripts (Fig. 4M,N and 4Q,R).

Stage 34

Membrane bone surrounding Meckel's cartilage represented the major morphological change in the mandibular process at stage 34 (Fig. 5). Osteoblasts and osteocytes associated with the bone tissue exhibited positive staining for PTHrP and PTH1R proteins (Fig. 5A,B) and mRNAs (Fig. 5C,D). Immunostaining for PTHrP (Fig. 5E) and PTH1R (Fig. 5F) was clearly visible in perichondrial cells, and PTHrP and PTH1R mRNA transcripts were also detected in these cells (not shown). The expression of PTHrP and PTH1R mRNAs (Fig. 5H,I)

Fig. 3 Localization of PTHrP and PTH1R proteins and mRNA transcripts in sagittal sections through the mandibular process of stage 23 chicken embryos. The sections were: (A) double-stained with alcian blue and for AP activity; (B) negative control (omission of primary antibody) for immunostaining and counterstained with haematoxylin; (C) immunostained for PTHrP and counterstained with haematoxylin; (D) immunostained for PTH1R and counterstained with haematoxylin; (E) hybridized with PTHrP antisense riboprobe; (F) hybridized with PTH1R antisense riboprobe; (G) hybridized with PTHrP sense riboprobe; (H) hybridized with PTH1R sense riboprobe. Mandible (mb); epithelium (e); skeletal muscle (m); mandibular nerve (n); trigeminal ganglion (g). Scale bar = 100 μm.



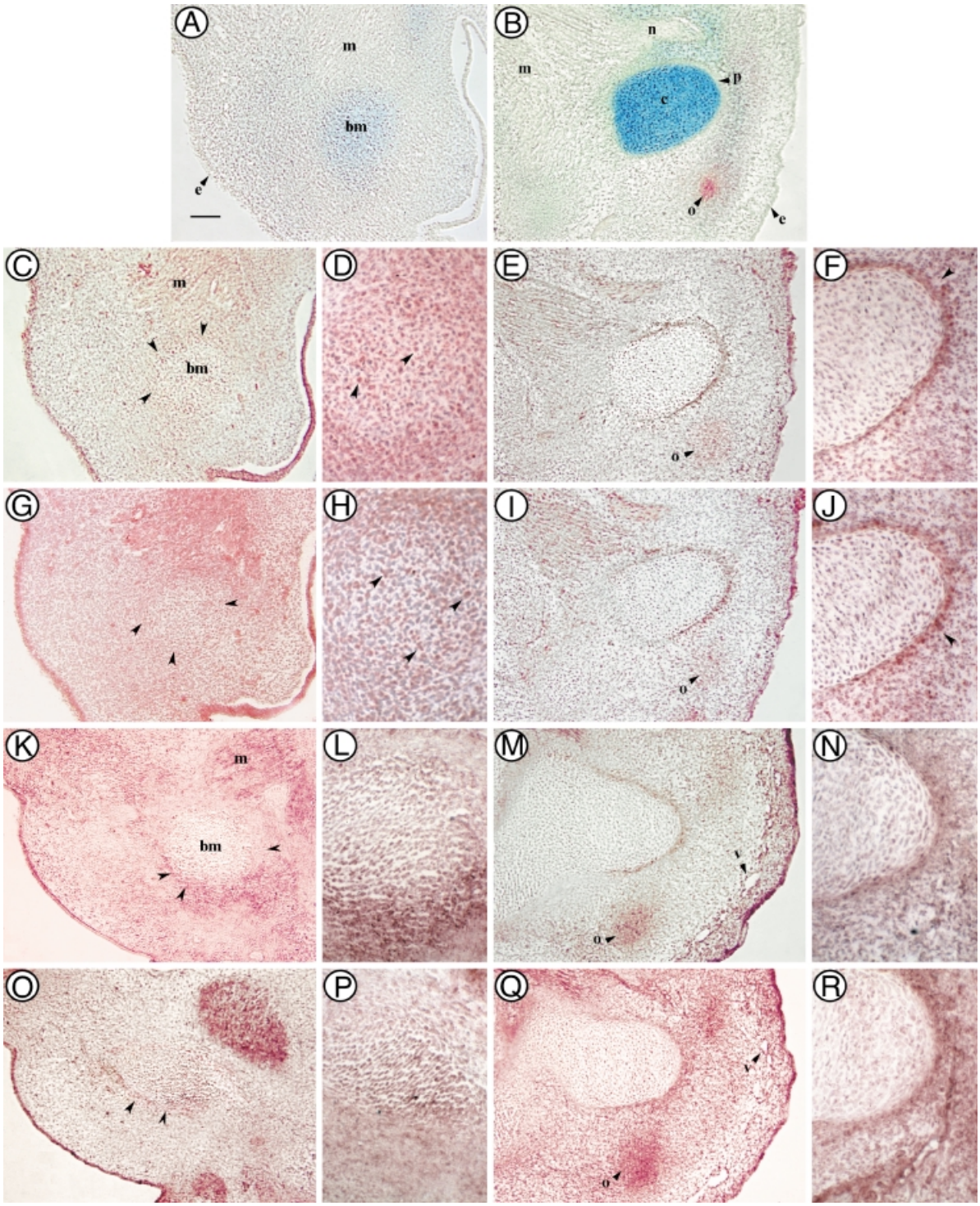


Fig. 4 Localization of PTHrP and PTH1R proteins and mRNA transcripts in sagittal sections through the mandibular process of stage 27 and 31 chicken embryos. Sections from stage 27 chicks are shown in A, C, D, G, H, K, L, O and P; sections from stage 31 chicks are shown in B, E, F, I, J, M, N, Q and R. The sections were: (A,B) double-stained with alcian blue and for AP activity; (C–F) immunostained for PTHrP and counterstained with haematoxylin; (G–J) immunostained for PTH1R and counterstained with haematoxylin; (K–N) hybridized with PTHrP antisense riboprobe; (O–R) hybridized with PTH1R antisense riboprobe. Epithelium (e); skeletal muscle (m); blastema of Meckel's cartilage (bm); arrowheads indicate cells that immunostained for PTHrP or PTH1R or contained PTHrP or PTH1R mRNAs detected by hybridization to antisense riboprobes; Meckel's cartilage (c); perichondrium (p); aggregate of osteogenic cells (o); mandibular nerve (n); and blood vessel (v). Scale bar = 100 μm.

and proteins (not shown) was especially prominent in the perichondrium and adjacent mesenchyme at the ventral tip of Meckel's cartilage. No evidence of PTHrP or PTH1R expression was observed in chondrocytes at either the protein or mRNA level (Fig. 5A–D,E,F). The distribution of PTHrP target cells in the stage 34 chick mandibular process was further confirmed by binding of biotinylated human PTHrP (1–34) by osteoblasts, osteocytes, perichondrial cells and mesenchymal cells (Fig. 5G).

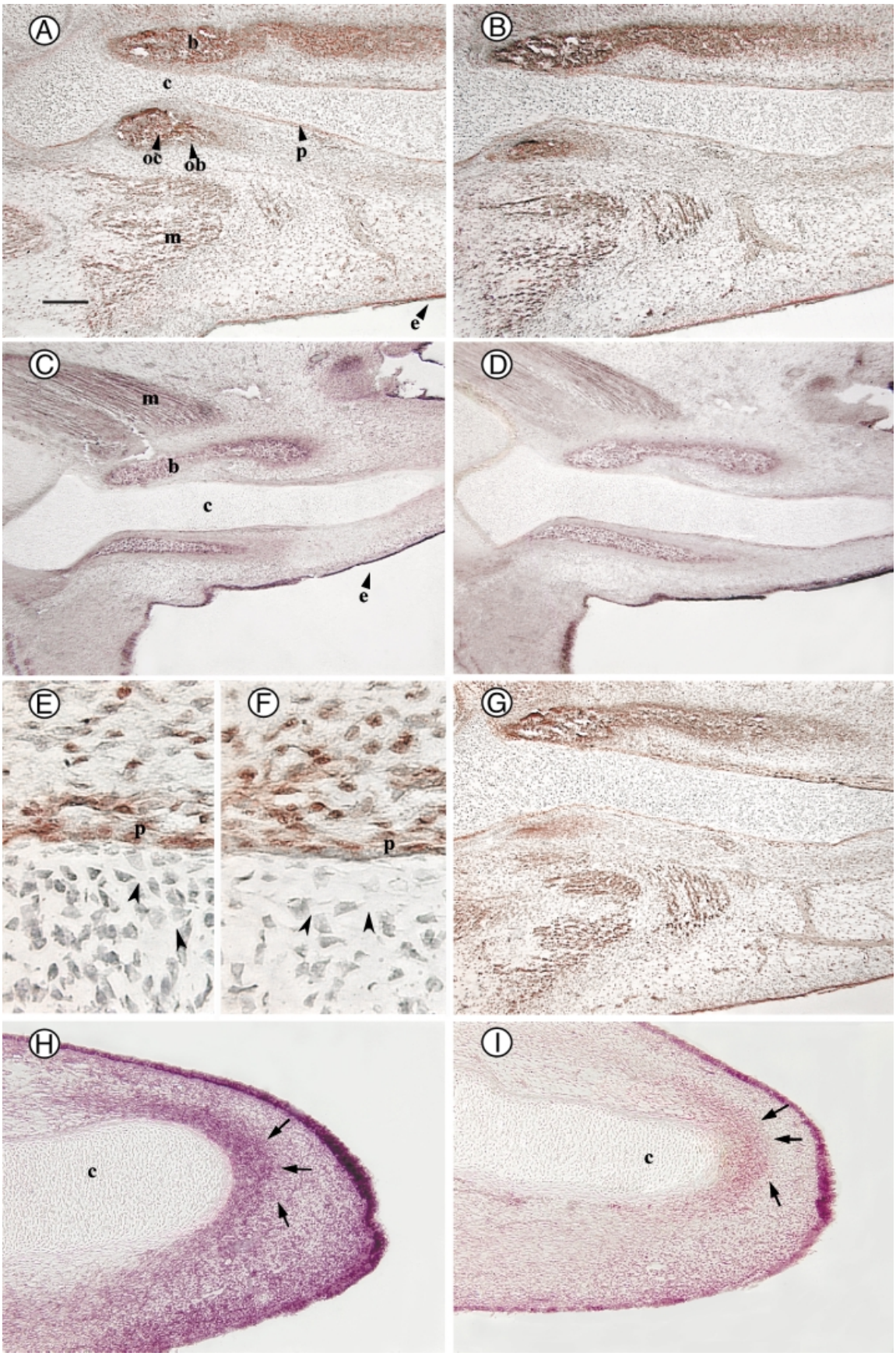
Effects of cPTHrP (1–36) and anti-human PTHrP (1–34) antiserum on chick mandibular mesenchymal cells *in vitro*

Both ectoderm-free mandibular mesenchyme and primary cultures of mandiblular mesenchymal cells were used to study the functional response of the PTH1R to cPTHrP (1–36). Cells and tissue from stage 26 embryos were selected because immunohistochemistry demonstrated that PTHrP and PTH1R proteins were associated with mesenchymal cells in the mandibular process at this stage of development. In order to assess PTH1R expressed by mesenchymal cells the experiments were done on ectoderm-free mesenchyme and primary cultures of mesenchymal cells. Since the PTH1R is coupled to adenylate cyclase, the effect of cPTHrP (1–36) on cAMP accumulation was determined. Treatment with 50, 500 or 1000 nM cPTH (1–36) for 20 min caused an increase in cAMP of, respectively, $145 \pm 5\%$ ($P \leq 0.01$), $165 \pm 2\%$ ($P \leq 0.001$) and $205 \pm 7\%$ ($P \leq 0.001$) over the control. Next we examined whether continuous or intermittent exposure to cPTHrP (1–36) affected chondrogenic or osteogenic differentiation of mandibular mesenchymal cells *in vitro*. Continuous exposure of cells isolated from stage 26 mandibular mesenchyme to cPTHrP (1–36) for 14 days caused a dose-related increase in the number of cartilage nodules at concentrations ranging from 0.1 to 10 nM. Intermittent exposure to the same concentrations increased cartilage nodule number, but the magnitude was less and not dose-related (Fig. 6). Intermittent cPTHrP (1–36) had a biphasic dose-related effect on AP activity (Fig. 7); concentrations between 0.001 and 0.1 nM increased AP activity, whereas higher concentrations had no effect. Continuous exposure to 1 and 10 nM cPTHrP (1–36) decreased AP activity. Compared to untreated controls, the number of cartilage nodules that developed in cultures of stage 26 chick mesenchymal cells was decreased by 50% in the presence of a 1 : 250 dilution of anti-PTHrP antibody (Fig. 8). The antibody had no effect on AP activity.

Discussion

The present study demonstrates that multiple cell types in the mandibular process of the developing chick express PTHrP and PTH1R. Non-skeletal tissues that expressed PTHrP and its receptor throughout the time period studied included undifferentiated mesenchymal cells, epithelial cells in the ectoderm, skeletal muscle cells and peripheral nerve tissue. The histogenesis of cartilage and bone in the mandibular process was accompanied by the appearance of tissue-specific cells that expressed PTHrP and its receptor. Between stages 27 and 34 the cells associated with chondrogenesis that exhibited immunodetectable proteins and hybridizable mRNAs shifted from chondroblasts in the blastema of Meckel's cartilage (stage 27) to cells in the perichondrium (stage 34). Alkaline phosphatase-positive osteogenic cells that form aggregates that marked the initiation of membrane bone formation at stage 31 co-expressed PTHrP and its receptor. Although difficult to determine unequivocally for chondrogenic cells, PTHrP and PTH1R were co-expressed by 100% of the osteogenic cells in the cell aggregates (stage 31) and all of the osteoblasts and osteocytes associated with woven bone (stage 34). The functional capacity of the PTH1R was established by several observations. First, treatment with aminoterminal cPTHrP (1–36) stimulated cyclic AMP accumulation in ectoderm-free pieces of mandibular mesenchyme. Second, cPTHrP (1–36) increased cartilage nodule number and AP activity in cultures of stage 26 chick mandibular mesenchymal cells. Finally, cartilage nodule formation was reduced when an anti-PTHrP antibody that neutralizes the action of PTHrP (Danks et al. 1990) was added to the mesenchymal cell cultures. These results are consistent with the conclusion that PTHrP is a local regulator of skeletogenesis in the chick mandible. This is consistent with observations that development of the mandible in mice is abnormal in the absence of functional PTHrP and PTH1R (Karaplis et al. 1994; Lanske et al. 1996; Vortkamp et al. 1996; Lanske et al. 1998, 1999; Suda et al. 2001).

The expression of PTHrP and PTH1R mRNAs or proteins in the developing mandible has also been observed in rats (Lee et al. 1993) and mice (Yamazaki



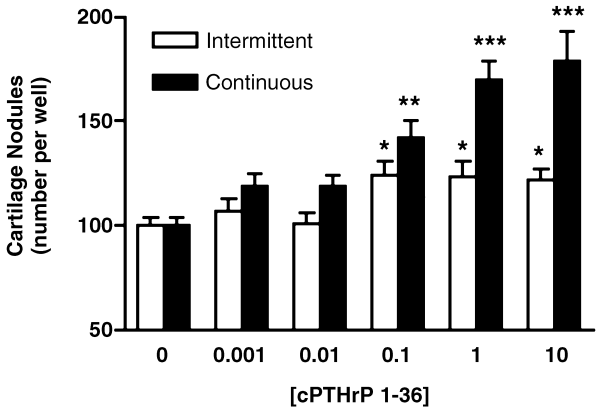


Fig. 6 Effect of continuous and intermittent treatment with cPTHrP 1–36 on cartilage nodule number. Cultures of stage 26 mandibular mesenchymal cells were treated continuously or intermittently (see Materials and methods) with cPTHrP (1–36) for 14 days and the number of alcian blue stained cartilage nodules in each culture counted. The mean ± SEM are shown for pooled data from four separate experiments with 3–4 cultures at each cPTHrP (1–36) concentration in each experiment; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$.

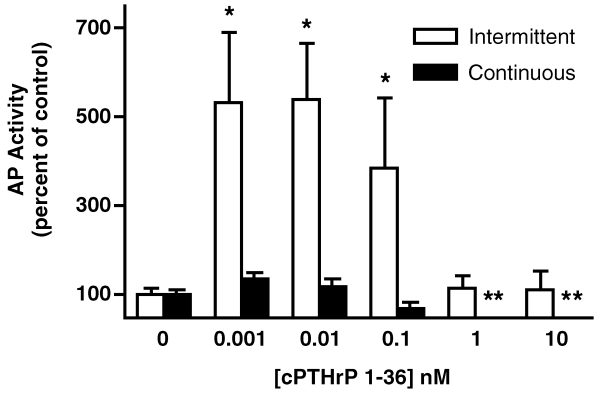


Fig. 7 Effect of continuous and intermittent treatment with cPTHrP 1–36 on AP activity. Cultures of stage 26 mandibular mesenchymal cells were treated continuously or intermittently (see Materials and methods) with cPTHrP (1–36) for 14 days and the AP activity in each culture was measured in lysates of the cells. The mean ± SEM are shown for pooled data from four separate experiments with 3–4 cultures at each cPTHrP (1–36) concentration in each experiment; $*P \leq 0.05$; $**P \leq 0.01$.

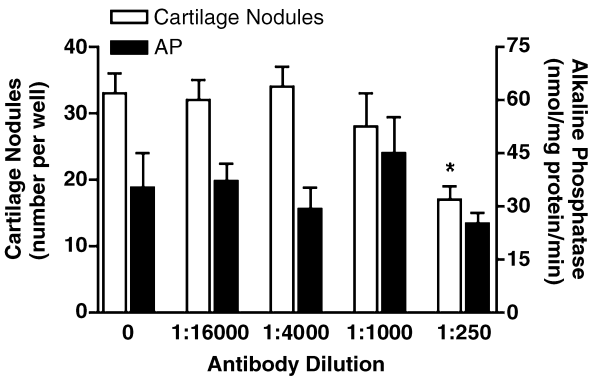


Fig. 8 Effect of anti-hPTHrP antiserum on cartilage nodule number and AP activity. Various concentrations of anti-hPTHrP antiserum were added to cultures of stage 26 mandibular mesenchymal cells (see Materials and methods) for 13 days and the number of cartilage nodules and AP activity in each culture was measured. The mean ± SEM is shown for four cultures at each antibody dilution; $*P \leq 0.05$.

et al. 1997). Comparing observations in mammals with our results indicates that the pattern of expression is most similar in mice and chicks where cells appear to co-express PTHrP and PTH1R. The two genes are expressed by phenotypically distinct but anatomically related cell types in rats (Lee et al. 1993). During development of the mandible in chicks the expression of PTHrP and PTH1R shifted from chondroblasts at stage 27 to perichondrial cells at stage 34. A similar transient pattern of PTHrP and PTH1R protein immunostaining was observed in cartilage cells between embryonic days 13 and 18 in mice (Yamazaki et al. 1997). These observations imply that the maturation of chondroblasts to chondrocytes in Meckel's cartilage is associated with a loss in PTHrP production and responsiveness. This differs from hyaline cartilage of long bone epiphyseal growth plates where prehypertrophic chondrocytes express PTH1R during development and growth of the limbs (Lanske et al. 1996; Vortkamp et al. 1996). It should be noted that while the early spatiotemporal

Fig. 5 Localization of PTHrP and PTH1R proteins and mRNA transcripts in sagittal sections through the mandibular process of stage 34 chicken embryos. The sections were: (A) immunostained for PTHrP and counterstained with haematoxylin; (B) immunostained for PTH1R and counterstained with haematoxylin; (C) hybridized with PTHrP antisense riboprobe; (D) hybridized with PTH1R antisense riboprobe; (E) immunostained for PTHrP and counterstained with haematoxylin; (F) immunostained for PTH1R and counterstained with haematoxylin; (G) incubated with biotinylated human PTHrP (1–34) and counterstained with haematoxylin; (H) hybridized with PTHrP antisense riboprobe; (I) hybridized with PTH1R antisense riboprobe. Epithelium (e); skeletal muscle (m); Meckel's cartilage (c); perichondrium (p); woven bone (b); osteoblasts (ob); osteocytes (oc); chondrocytes (arrowheads); perichondrium at ventral end of Meckel's cartilage (arrows). Scale bar = 250 μm (A–D,G); = 100 μm (H,I); = 25 μm (E,F).

expression of PTHrP and PTH1R by chondrogenic cells in Meckel's cartilage is similar in mice and chicks, this tissue has distinctly different fates in the two species. In mice the anterior region of Meckel's cartilage undergoes endochondral ossification and contributes to the mandible, the posterior region undergoes endochondral ossification to form the malleus and incus of the middle ear, and the central region degenerates and gives rise to the sphenomandibular ligament (Bhaskar et al. 1953). By contrast, Meckel's cartilage is retained in the chick mandible and is surrounded by the dentary, suprangular, angular and opercular bones that form by intramembranous ossification (Noden, 1991).

Experiments on knockout mice and normal chick embryos established that PTHrP regulates endochondral bone growth by a direct action on prehypertrophic chondrocytes (Lanske et al. 1996; Vortkamp et al. 1996). Thus, it is not surprising that reduced long bone growth in PTHrP gene null mice is corrected when PTHrP expression is established in chondrocytes by a transgene driven with the procollagen-II promoter (Philbrick et al. 1998). However, an interesting outcome is that shortening of the mandible is not corrected in these animals. To explain this it was proposed that outgrowth of the mandible depends on PTHrP-driven membrane bone growth. While absence of PTHrP on membrane bone growth in the mandible has not been measured in knockout mice, the histology of membrane bone in the mandible is altered in these animals (Suda et al. 2001). Moreover, two findings in our study support that PTHrP can influence membrane bone formation in the developing chick mandible. First, osteoblasts at sites of membrane bone formation in the chick mandible express PTH1R. This confirms previous reports that PTH1R is expressed by osteoblasts at sites of membrane bone formation in humans (Moseley et al. 1991; Lomeri et al. 1997), rats (Lee et al. 1995), mice (Yamazaki et al. 1997) and rabbits (Kartsogiannis et al. 1997). Second, intermittent exposure to cPTHrP 1–36 increased AP activity, a measure of osteoblast differentiation, in cultured stage 26 chick mandibular mesenchymal cells. At this stage of development, the targets for PTHrP action in the mandible were epithelial cells, skeletal muscle cells and undifferentiated mesenchymal cells. Of these, only mesenchymal cells are osteoblast precursors. Therefore, we conclude that intermittent exposure to PTHrP stimulates their differentiation into osteoblasts. This is consistent with the observation that intermittent treatment with aminoterminal PTHrP (and PTH) is anabolic in skeleton *in vivo* (Hock et al. 1989; Weir et al. 1992; Stewart et al. 2000) and *in vitro* (Canalis et al. 1990; Yee, 1985; Jongen et al. 1993; Ishizuya et al. 1997) and supports the suggestion that PTHrP modulates proliferation and differentiation of osteoblasts from mesenchymal precursors during intramembranous ossification (Kartsogiannis et al. 1997). While our observations are not sufficient to prove that outgrowth of the chick mandible depends on PTHrP-stimulated growth of membrane bones, they are consistent with such a notion. It should be noted that AP activity assessed by colorimetric assay of cell lysates might include AP expressed by osteoblasts as well as hypertrophic chondrocytes in cartilage nodules. However, the finding that AP histochemistry/alcian blue staining of parallel cultures failed to demonstrate AP-positive chondrocytes (data not shown) supports the conclusion that the assay reflects enzyme activity expressed by osteoblasts.

Whether or not membrane bone formation is a major factor for normal outgrowth of the mandible is questionable. A study in rats showed that growth of the mandible in the sagittal plane is primarily due to growth of Meckel's cartilage (Diewert, 1980). Since hyaline cartilage can increase in size by interstitial and appositional growth, PTHrP could affect one or both of these processes. We found that few chondrocytes, the primary cells responsible for interstitial growth, express PTH1R in Meckel's cartilage at stages 31 and 34. Therefore, unless the PTH1R gene is expressed at a later time not examined in this study, PTHrP does not influence interstitial growth of Meckel's cartilage. This is consistent with the finding that reduced growth of the mandible in the PTHrP gene null mouse is not corrected when PTHrP expression by chondrocytes is rescued by the procollagen II-driven transgene (Philbrick et al. 1998), and with the observation that development and growth of hyaline cartilage in the respiratory tract is not affected in PTHrP-gene null mice (Karaplis et al. 1994).

By contrast, two findings from our study suggest that PTHrP might influence appositional growth of Meckel's cartilage. First, perichondrial cells, particularly at the ventral aspect of Meckel's cartilage, expressed PTHrP and PTH1R. Second, treatment with PTHrP increased the number of cartilage nodules in cultures of stage 26 mandibular mesenchymal cells. Although PTHrP reduces differentiation of prehypertrophic chondrocytes in the epiphyseal growth cartilage, aminoterminal PTHrP and/or PTH stimulate mitosis and differentiation

of chondrogenic cells *in vitro* (Koike et al. 1990; Ishikawa et al. 1997). If cells in the cambial layer of the perichondrium and chondrogenic precursors in the mandibular mesenchyme of stage 26 chick embryos are similar in their functional potential, the increase in nodule number suggests that PTHrP stimulates chondroblast differentiation and cartilage formation equivalent to what occurs during appositional growth. Since perichondrial cells do not express type II collagen, they would not express PTHrP in the transgenic PTHrP-knockout mice. This would explain why mandible growth is not corrected when PTHrP expression by chondrocytes is re-established in PTHrP-knockout mice (Philbrick et al. 1998).

Several procedures were used to validate our results using rabbit polyclonal antibodies raised against human peptides to locate these antigens in chicks (Burry, 2000). First, replacing either of the primary antibodies with non-immune rabbit serum eliminated all immunostaining. This result verified that the reaction product generated by the peroxidase enzyme activity was dependent on binding of the primary antibody to antigens in the histolological sections of chick tissue. Second, antibody specificity was further assessed by immunoblotting and, in the case of the anti-PTHrP antibody, pre-absorption of the antibody with chicken PTHrP (1–36). The antibodies used for immunohistochemistry cross-reacted with molecules of appropriate size for PTHrP (Bui et al. 1993; Sandhu et al. 1993; Funk & Wei, 1998) and its receptor (Bisello et al. 1996; Karpf et al. 1987) on Western blots of proteins extracted from whole chick embryos. Moreover, the rabbit antihuman PTHrP antibody exhibited clear cross-reactivity toward 10 kDa synthetic aminoterminal chicken PTHrP (1–36), suggesting that the immunoreactive peptide of similar size detected in proteins extracted from stages 18–34 chicken embryos was most likely aminoterminal PTHrP. The finding that pre-absorption with chicken PTHrP (1–36) eliminated all detectable immunostaining observed with the anti-PTHrP antibody further supports this conclusion. The anti-PTHrP antibody also cross-reacted with a peptide of approximately 37 kDa. Larger than expected PTHrP molecules, including a 39-kDa peptide in fetal human heart, have been detected by Western blotting and may represent polymerization of PTHrP (1–139) or PTHrP (1–141) molecules (MW 16–20 kDa) (Bui et al. 1993). Finally, the similar distribution of cells that immunostained with the anti-PTH1R antibody and bound biotinylated human PTHrP (1–34) confirmed that the anti-PTH1R receptor antibody accurately located PTHrP target cells in the chick mandibular process. These results establish that the immunohistochemical methods used in this study accurately located PTHrP and the PTH/PTHrP receptor proteins in histological sections of the developing chick mandible.

The present study combined *in situ* hybridization, immunohistochemistry and functional studies to characterize the temporal and spatial distribution of PTHrP-producing cells and PTHrP target cells during skeletal tissue histogenesis in the developing chicken mandible. Chondrogenic cells associated with the blastema of Meckel's cartilage were found to express both PTHrP and its receptor. However, at later stages of development, only cells in discrete areas of the perichondrium and in subperichondrial regions of Meckel's cartilage produced and/or were targets for PTHrP. Preosteoblasts, osteoblasts and osteocytes associated with membrane bone development in the mandible also produced, and were target cells for, PTHrP. Furthermore, treatment with cPTHrP (1–36) stimulated differentiation of both chondrogenic and osteogenic cells in cultures of mandibular mesenchymal cells. We conclude that PTHrP influences the histogenesis and/or growth of skeletal tissues in the chicken mandible via an autocrine pathway mediated by the PTH1R.

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