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Discovery of Sonic Hedgehog Expression in Postnatal Growth Plate Chondrocytes: Differential Regulation of Sonic and Indian Hedgehog by Retinoic Acid

Licia N.Y. Wu, Min Lu, Brian R. Genge, George Y. Guo, Daotai Nie, and Roy E. Wuthier*

Department of Chemistry and Biochemistry, University of South Carolina, 329 Graduate Research Science Center, Columbia, South Carolina 29208

Abstract Sonic hedgehog (Shh) is a key signal protein in early embryological patterning of limb bud development. Its analog, Indian hedgehog (Ihh), primarily expressed during early cartilage development in prehypertrophic chondrocytes, regulates proliferation and suppresses terminal differentiation of postnatal growth plate (GP) chondrocytes. We report here for the first time that both Shh and Ihh mRNA are expressed in the GP of rapidly growing 6-week-old broiler-strain chickens. They are also expressed in other tissues such as articular chondrocytes, kidney, and bone. In situ hybridization and RT-PCR analyses reveal *Shh* in all zones of the GP, with peak expression in late hypertrophy. Using primary cultures of GP chondrocytes in serum-containing medium, we followed the patterns of Shh and Ihh mRNA expression as the cultures matured and mineralized. We find a cyclical expression of both hedgehog genes during the early period of culture development between day 10 and 14; when one is elevated, the other tended to be suppressed, suggesting that the two hedgehogs may play complementary roles during GP development. Retinoic acid (RA), a powerful modulator of gene expression in cell differentiation, stimulates GP chondrocytes toward terminal differentiation, enhancing mineral formation. We find that RA strongly suppresses *Ihh*, but enhances expression of *Shh* in this system. While Ihh suppresses maturation of GP chondrocytes to hypertrophy, we hypothesize that Shh acts to push these cells toward hypertrophy. J. Cell. Biochem. 87: 173–187, 2002.

Key words: Sonic hedgehog; Indian hedgehog; retinoic acid; postnatal growth plate chondrocytes; regulation of mRNA expression; calcification; bone formation

The hedgehog proteins constitute a conserved family of macromolecules that provide crucial signals in embryonic patterning in many organisms [Echelard et al., 1993; Krauss et al., 1993; Hammerschmidt et al., 1997; Tabin and McMahon, 1997]. The segment polarity gene, *hedgehog*, was originally identified in *Drosophila* [Nusslein-Volhard and Wieschaus, 1980] and this led to the isolation of vertebrate homologs. In higher vertebrates there are at least three hedgehog genes: *sonic hedgehog* (*Shh*), *Indian hedgehog* (*Ihh*), and *desert hedgehog* (*Dhh*) [For review, see Hammerschmidt et al., 1997]. One important role of Shh protein, among many, is the regulation of anterior–posterior patterning in avian and mammalian limb bud development [Riddle et al., 1993]. In vertebrate limb buds, a subset of posterior mesenchymal cells, termed the zone of polarizing activity (ZPA) regulates anterior–posterior digit identity [For review, see Pearse and Tabin, 1998]. At this early stage of limb development Shh is apparently the only member of the hedgehog family expressed. At later stages of embryonic development during formation of the skeletal elements Ihh, a homologue of Shh, appears in the middle of the condensing cartilage elements. Its expression in mice starts in prechondrocytes in embryonic development, but after birth it is restricted in the growth plate (GP) to the prehypertrophic zone [Bitgood and McMahon, 1995]. Ihh has been shown to regulate the pace of chondrocyte differentiation

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*Correspondence to: Roy E. Wuthier, PhD, Department of Chemistry and Biochemistry, 329 Graduate Research Science Center, University of South Carolina, Columbia, South Carolina 29208. E-mail: wuthier@mail.chem.sc.edu

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[Lanske et al., 1996; Vortkamp et al., 1996; Wallis, 1996]. Ectopic expression of Ihh delays hypertrophy and subsequent replacement of cartilage with bone [Vortkamp et al., 1996].

In fact, a wide variety of clinical disorders are manifested by disturbances in skeletal growth and development. They can vary in severity from asymptomatic lesions to lethal, and in localization from a specific anatomic region (e.g., increased number of digits, broad thumbs, craniofacial malformation) to extensive involvement of the whole musculoskeletal system. Mouse knockouts that disrupt *Shh* gene expression show extremely foreshortened limbs, complete absence of vertebrae, and loss of distal structures [Chiang et al., 1996]. This suggests that Shh plays important roles not only in anterior–posterior patterning, but also in skeletogenesis of developing limbs. Ihh also plays an extensive role in skeletal development. *Ihh* null mutants exhibit greatly shortened limbs, reduced chondrocyte proliferation, inappropriate timing of maturation, and a failure of osteoblastic development in endochondral bone [St-Jacques et al., 1999]. These discoveries point out that much additional research is needed for an adequate understanding of the molecular and cellular mechanisms underlying the critical hedgehog signaling pathway [McMahon, 2000].

Detailed analyses of the hedgehog receptors, *patched* (*Ptc*) and *Gli*, in postnatal tissues indicate that they are expressed both in less differentiated chondrocytes and in late hypertrophic chondrocytes, proximal and distal to *Ihh*-expressing cells in the primary ossification center [Vortkamp et al., 1998]. Their expressions are necessary for target tissues to respond to the hedgehog proteins; and are expressed at all known sites where hedgehog signaling occurs [Tabin and McMahon, 1997]. In addition to signal peptide cleavage in the secretory pathway, the hedgehog proteins have the intrinsic ability to cleave themselves into two smaller units, a ~19 kDa N-peptide, and ~25 kDa C-peptide, by an efficient auto catalytic reaction that also involves attachment of cholesterol to the C-terminus of the N-peptide [Porter et al., 1996a,b]. While Ihh has been assumed to be the only hedgehog expressed in the postnatal GP, we present new evidence that Shh is also expressed there, as well as in cultured GP cells.

Previous studies have demonstrated that systemic retinoids play an essential role in signaling GP chondrocyte maturation and endochondral calcification during avian limb skeletogenesis [Pease, 1962; Roberts and Sporn, 1984; Eichele, 1989]. Both hyper- and hypovitaminosis A lead to disturbances in the patterns of chondrocyte maturation in the GP and in the rates and degree of mineralization of hypertrophic cartilage in long bones of growing animals [Wolbach and Hegsted, 1953; Howell et al., 1967; Vasan, 1975; Woodard et al., 1997]. How the retinoids and retinoid-dependent mechanisms are coordinated in normal skeletal development is unclear. Signaling through retinoic acid (RA) receptors presumably plays a role in chondrocyte maturation. A number of in vitro investigations using various culture systems have previously shown that RA modulates completion of GP chondrocyte maturation [Shapiro and Poon, 1976; Pacifici et al., 1991; Ballock et al., 1994; Iwamoto et al., 1994] and stimulates mineralization of hypertrophic chondrocytes [Iwamoto et al., 1993, 1994; Wu et al., 1997]. Here we explored the mechanism by which RA regulates maturation of cells in our primary GP culture system. We find that RA stimulates *Shh*, while inhibiting *Ihh* mRNA expression in the chondrocyte cultures. This observation suggests that RA may act through these proteins in stimulating endochondral ossification.

To assess the localization of expression of the hedgehog mRNA in the GP, and to compare their response to the administration of RA, we also examined expression of other GP mRNAs to provide a context for our current findings. We present RT-PCR analyses of genes whose expression in the GP is known to be affected in chondrocyte differentiation: *vascular endothelial growth factor* (*VEGF*), *osteogenic protein-1* (*OP-1 = BMP-7*), and *Bcl-2*. VEGF, an angiogenic factor thought to promote ingrowth of blood vessels at the terminus of the GP, is expressed at highest levels in the hypertrophic zone [Flamme et al., 1995; Gerber et al., 1999]. OP-1, a factor crucial for bone formation, is also expressed at high levels in the hypertrophic zone [Houston et al., 1994; Vukicevic et al., 1994; Helder et al., 1995]. In contrast, Bcl-2, a small membrane-associated protein that appears to be protective against programmed cell death [Vaux et al., 1988; Korsmeyer, 1992; Amling et al., 1997], is expressed at highest levels in the proliferative and prehypertrophic zone.

To establish the effects of RA, we also present RT-PCR analyses of three collagen genes whose expressions are sensitive to RA and are maturation specific: *type II*, *type X*, and *type I*. Type II, a cartilage-specific collagen, is expressed in the proliferative and prehypertrophic zones; type X is hypertrophic zone specific. In the context of the GP, type I collagen is limited to osteoblastic bone deposited on calcified cartilage and to the perichondrial fascia. In avians, there is evidence that some hypertrophic cells survive and differentiate into osteoblasts [Wu et al., 1997].

MATERIALS AND METHODS

Cell Culture

Chondrocytes were isolated from epiphyseal GP cartilage of the proximal tibiae of 6–8 week-old hybrid broiler-strain chickens and plated in 35-mm dishes as described [Wu et al., 1989, 1992; Ishikawa et al., 1997]. Cultures were started in 2 ml of DMEM with 10% FBS in a 5% CO_2: 95% air atmosphere. Media were changed every 3–4 days, with a fresh supplement of ascorbic acid (50 μg/ml), until the end of the experiments. On day 7, cells were switched to DATP5 mineralization medium containing 1.9 mM total Pi and 1.8 mM total Ca^{2+} [Ishikawa et al., 1997]. For treatment of cells with RA, on day 7 and at each media change thereafter, all-trans-RA was delivered to the cultures in an ethanol stock solution diluted 1000-fold to minimize the ethanol level in the medium. Control dishes received the same volume of ethanol (1.0 μl per ml of medium).

Total RNA Extraction

Total RNA was extracted from cultured GP chondrocytes and various tissue samples using the acid guanidinium thiocyanate-phenol-chloroform method [Chomczynski and Sacchi, 1987] with modifications recently described by Nie et al. [1998]. RNA precipitates were dissolved in 50–100 μl of 0.1 mM EDTA in diethylpyrocarbonate (DEPC)-treated water for RT-PCR. RNA was preserved immediately after isolation as an ethanol precipitate.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA (5 μg or less) was reversed-transcribed using random hexamers and MMLV reverse transcriptase, as described by the manufacturer (Epicenter Technologies). After 60 min at 37°C, to the RT reaction tube was added 1/10 volume of 3 M sodium acetate buffer (pH 5.2) and 2.5 volumes of ethanol. The cDNA precipitates were washed twice with 70% ethanol and suspended in double-distilled water. Aliquots equivalent to 0.25–0.50 μg total RNA of each cDNA sample were used to set up the PCR reaction, as done previously [Nie et al., 1998]. For all samples in which expression levels were directly compared, equal amounts of total RNA were amplified and RT-PCR amplifications were run simultaneously under exactly the same conditions. G3PDH was used as an internal standard for monitoring mRNA loading. Primer sequences used to identify Shh and Ihh hedgehog genes and chondrocyte differentiation-associated gene expression are listed in Table I. A 10 μl aliquot of each sample was analyzed using 1.5% agarose TAE (40 mM Tris-acetate, 2 mM EDTA) gels and visualized by ethidium bromide staining. The sizes of the various PCR products are shown on the right in Table I. To verify the authenticity of the PCR products, DNA sequence analyses were performed after cloning into TA vector (Invitrogen).

In Situ Hybridization

In situ hybridization was carried out on the dewaxed tissue sections as described by Hicks et al. [1997] and O'Keefe et al. [1997a,b]. Further details are given in the figure legends.

RESULTS

Expression of *Shh, VEGF, OP-1,* and *Bcl-2* in Chicken GP Chondrocyte Cultures

Previous studies had shown that our primary GP cultures recapitulate most in vivo processes. The chondrocytes reach confluence in 2 weeks and become hypertrophic. They then form numerous cell-surface blebs, exfoliate matrix vesicles, and subsequently mineralize the extracellular matrix. Analyzing such GP chondrocyte cultures with RT-PCR, the expression of *Shh* is compared with several maturation-related genes (Fig. 1). We report first the discovery that the *Shh* gene is expressed at significant levels in postnatal GP chondrocyte cultures. The mRNA of other maturation-related genes (*VEGF*, *OP-1*, and *Bcl-2*) are detected by RT-PCR in our GP cultures as well. We show here the nucleotide sequence of two of these, *Shh* (Table III), and *VEGF* (Table IV) to document that these PCR products were

TABLE I. Primer Sequences for RT-PCR

Name	S/A[a]	Sequence	Product size (bp)
Shh	S	5′-TGCTAGGGATCGGTGGATAG	197
	A	5′-ACAAGTCAGCCCAGAGGAGA	
Ihh	S	5′-ACAACCCCCGACATCATCTTC	302
	A	5′-GCGCCTTGGACTCGTAGTAG	
G3PDH	S	5′-ACCACTGTCCATGCCATCACAGCC	433
	A	5′-TCCAAACTCATTGTCATACCAGGAA	
VEGF	S	5′-CTCTGAGCAAGGCTCACAGTG	301/229
	A	5′-CAGGCCATCCTGTGTGCCTCT	
OP-1	S	5′-TGGTGGAGCATGACAGAGAG	351
	A	5′-TTTGCCCATCTATGCTTTCC	
Bcl-2	S	5′-TTCGATTTCTCCTGGATCTCTCTGA	328
	A	5′-GTGATGACAGATACAACTTGCAGAG	
Type I collagen	S	5′-CCTTCTCTCAGACCCAAGGATTATG	672
	A	5′-ACAGACTGGGCCAATGTGCAAACCG	
Type II collagen	S	5′-CGACATCAAACTCTGCCATCCCGAG	554
	A	5′-GATCGGCTCCGCCAATGTCCTAAGG	
Type X collagen	S	5′-GTTAAGGGAGTTCCAGGTATTAATG	675
	A	5′-GTTAGATTTGAGCAAATAGGAAACC	

[a]S, sense; A, antisense.

authentic. Note in Figure 1 that the 229 bp variant in *VEGF* expression is present in higher levels than the 301 bp form. The nucleotide sequences of the other PCR products (*Ihh*, *G3PDH*, *OP-1*, and *Bcl-2*) were identical to those published in the gene bank and are not shown.

Detection of Sonic Hedgehog (Shh) mRNA in Postnatal Chicken Tissues

Shh is one of the earliest signals governing the development of the normal limb bud during embryogenesis. It first appears at Stage 17 in chick embryo development, reaches a peak at Stage 23, and then seems to disappear. In contrast, *Ihh* is expressed in the developing cartilage elements at Stage 26 and following, and continues to be expressed in postnatal GP of chickens and mice [Iwasaki et al., 1997; Tabin and McMahon, 1997]. However, the possibility of *Shh* expression in later development and in adult tissues has received little attention. We present here clear evidence that Shh mRNA is constitutively expressed in the postnatal avian

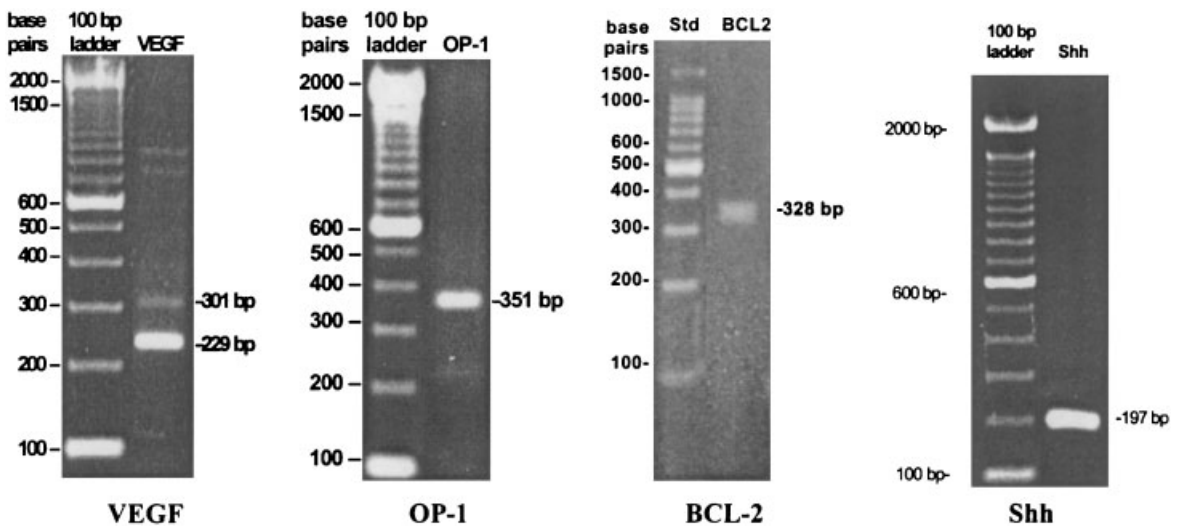

Fig. 1. Expression of VEGF, OP-1, BCL-2, and Shh mRNA in chicken GP chondrocyte cultures. RT-PCR shows that vascular endothelial growth factor (VEGF), osteogenic protein-1 (OP-1), BCL-2, and Sonic hedgehog (Shh) mRNA are expressed in cultured chondrocytes. Total RNA was isolated and the RT-PCR reaction was performed using *VEGF, OP-1, BCL-2,* and *Shh* primers as described in the text. The authenticity of *VEGF, OP-1, BCL-2* and *Shh* was each verified by DNA sequence analysis of each PCR product after cloning into TA vector (Invitrogen). *VEGF* has several isoforms generated by alternative splicing, a 229 bp fragment corresponding to VEGF 164 (soluble glycoprotein) and a 301 bp fragment corresponding to VEGF 188 bound to proteoglycan of the cell surface [Flamme et al., 1995].

TABLE II. Differential Expression of Sonic Hedgehog mRNA in Sequential Zones of Chicken Growth Plate Cartilage

Tissue slice	Zone	Sonic hedgehog[a]
1	Resting	1.122 ± 0.141[b]
2	Proliferating	0.576 ± 0.123
3	Prehypertrophic	0.933 ± 0.062[b]
4	Hypertrophic	0.921 ± 0.181[n.s.]
5	Late hypertrophic/calcified	1.372 ± 0.069[c,d]
6	Primary spongiosa	1.116 ± 0.028[e]

[a]Difference between indicated zone and proliferating zone. [b]$P \sim 0.05$; [e]$P \sim 0.02$; [c]$P \sim 0.01$; n.s., not significant.
Difference between prehypertrophic and late hypertrophic/calcified zone. [d]$P \sim 0.01$.
Serial horizontal tissue slices from the metatarsal GP of 6-week-old broiler-strain chickens were collected in sterile tubes. The sequential slices represent the following zones: 1, resting; 2, proliferating; 3, prehypertrophic; 4, hypertrophic; 5, late hypertrophic/calcified; and 6, primary spongiosa. Total RNA was isolated and RT-PCR was performed using *Shh* primers as described earlier. Glyceraldehyde 3-phosphate dehydrogenase (G3PDH), a constitutively expressed enzyme in GP cartilage was used as an internal standard for monitoring mRNA loading. The RT-PCR products were analyzed using 1.5% agarose TAE gels and visualized by ethidium bromide staining. For quantitation, the gels were scanned and digitized. To normalize data, the intensity of the Shh bands were expressed as a ratio of the G3PDH band for each lane and the average ratios for all zones calculated. The level of mRNA expression in each zone was calculated as a ratio to the mean of the ratios of all zones. The ratios of Shh mRNA expression in each zone for the three separate experiments were averaged and the SEM calculated.

GP (Fig. 2; top panel). Our RT-PCR data show that *Shh* is expressed in fresh, cleanly dissected avian GP tissue (lane 2), and in primary cultures of avian GP chondrocytes (lane 3). Shh mRNA is also present in bone (lane 4), articular chondrocytes (lane 5), and kidney (lane 6). For comparison, the expression of *Ihh* in the various tissues was also investigated by RT-PCR (Fig. 2; bottom panel). *Ihh* was strongly expressed in GP tissue (lane 1) and in primary cultures of GP chondrocytes (lane 5), but was also expressed in bone (lane 2), articular cartilage (lane 3), and kidney (lane 4). DNA analysis showed that the *Ihh* PCR product had the correct sequence.

TABLE III. Nucleotide Sequence of the Shh PCR Product

ACAAGTCAGCCCAGAGGAGACTAAAAGAGCGCAGATG
AAGCCCACCAAGAGAATTCTTGTCAACAGCAGCATTT
CGTCCATTGAATCCAATTACTTCACAGCTCTCTGTGC
CTATCCCTGGCTGTCTCTCTAGAGCTCTCTCTCCTCC
TATGTCCTTGTCTGCTTTCTGATCGTATACTATCCAC
CGATCCCTAGCA

After RT-PCR amplification, a TA cloning kit (Invitrogen, San Diego, CA) was used to clone the 197-bp Shh PCR product for confirmation of its identity. Plasmids were isolated from 2-ml colony overnight cultures using an UltraClean mini-plasmid prep kit (MO BIO, Solana, CA). DNA sequencing was performed. One hundred percent identity is obtained comparing to chicken Shh mRNA of GenBank accession L28099.

To further establish the specific area(s) of the GP where *Shh* and *Ihh* are expressed, the metatarsal GP of 6-week-old chickens was dissected into six different zones (resting, proliferating, prehypertrophic, hypertrophic, late hypertrophic, and primary spongiosa); total RNA was extracted and then RT-PCR was performed using equal amounts of total RNA. The metatarsal GP was chosen for dissection because of its relatively flat surface, making it easier to dissect into serial layers for detection of gene expression by zonal RT-PCR analyses. We demonstrate the validity of the zonal dissection of the GP by showing that levels of type X collagen mRNA, a specific hypertrophic zone marker, are elevated in that fraction of the GP (Fig. 3). Type X collagen mRNA was absent in bone, serving as a negative control. Figure 4 shows that *Shh* is abundantly expressed in layer 5 (late hypertrophic zone), distal to where *Ihh* is maximally expressed in layer 3 (the prehypertrophic zone) where others had previously noted maximal expression of *Ihh* [Murakami and Noda, 2000; van der Eerden et al., 2000]. We found Shh mRNA expressed throughout the GP, being most strongly expressed in layer 1 (resting zone) and in layer 5 (late hypertrophic/calcified zone). The intensity of the densitometric scans of the ethidium bromide-stained RT-PCR bands of Shh for the three separate zonal dissection experiments were calculated as ratios of the *glyceraldehyde-3-phosphate dehydrogenase* (G3PDH) band, and normalized to the mean of all zones (Table II). Here it is apparent that Shh expression was lowest in the proliferating zone and highest in the prehypertrophic and late hypertrophic/calcified resting, zones. In contrast, Ihh expression was lowest in the resting zone, and highest in the prehypertrophic and early hypertrophic zones. Both hedgehogs were expressed significantly in the primary spongiosa where osteoblasts deposit bone on spicules of calcified cartilage.

RA Treatment Elevates the Level of Shh mRNA and Changes the Level of Collagen mRNA

GP chondrocyte cultures were treated with 50 nM RA in the DATP5 medium (5% FBS) from Day 7 to 14 then RNA was isolated. Using RT-PCR, we compared the steady state levels of Shh and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA in the presence and

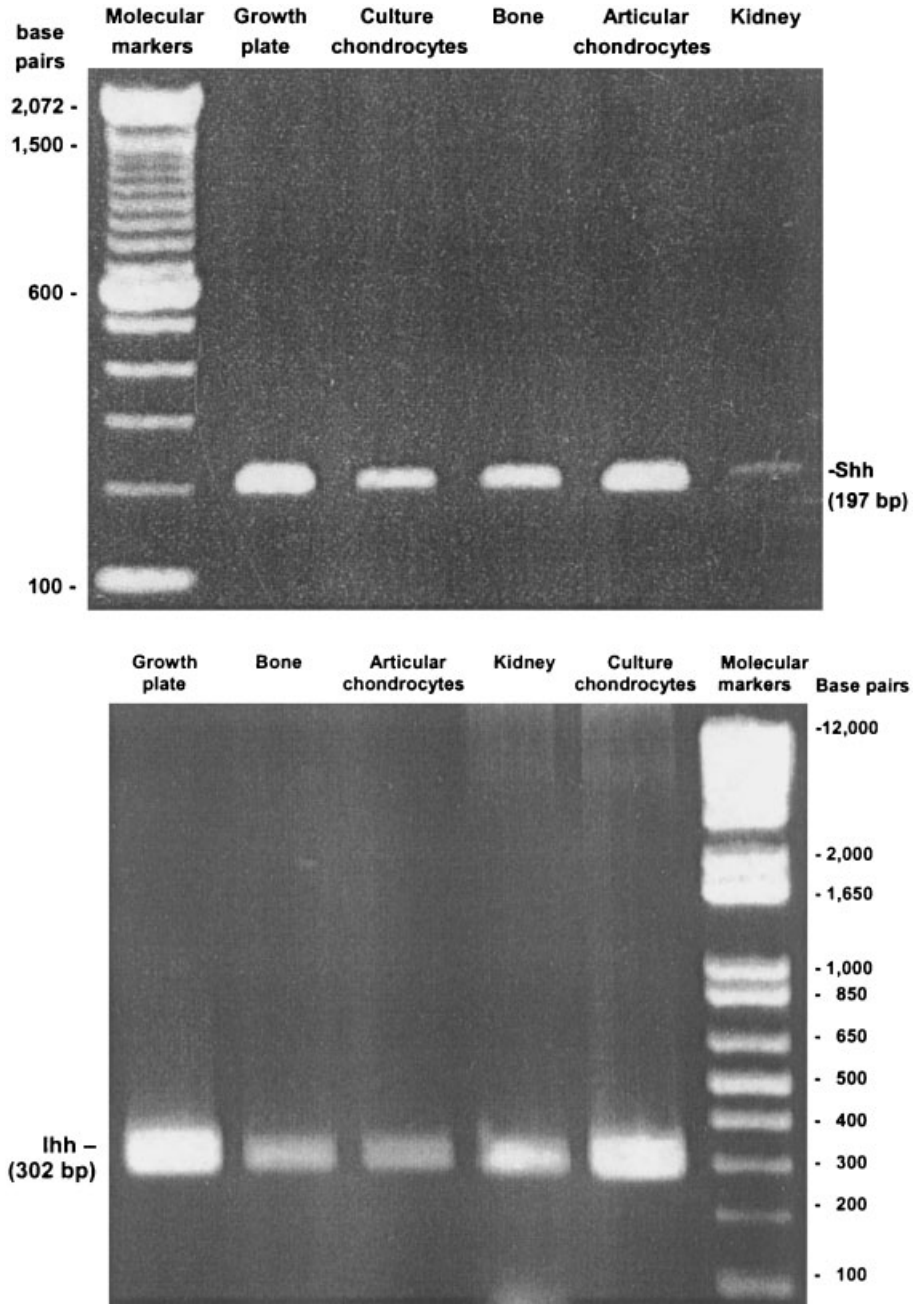

Fig. 2. Expression of Shh and Ihh mRNA in postnatal chicken tissues. RT-PCR analysis shows that Sonic hedgehog (Shh, upper figure), and Indian hedgehog (Ihh, lower figure) mRNA are present in GP chondrocytes. The total RNA was isolated from the various tissues and cultured chondrocytes. Identical amounts of RNA were used for the RT-PCR reaction, which was performed using *Ihh* and *Shh* primers as described in the text. The authenticity of *Ihh* and *Shh* in these bands was verified by DNA sequence analysis of each PCR product after cloning into TA vector (Invitrogen).

absence of RA (Fig. 5). Our data show that there was a marked increase in the level of Shh mRNA expression in the presence of 50 nM RA, while the mRNA level of the internal standard *G3PDH* remained essentially unchanged (Fig. 5).

The effects of RA on the expression of genes associated with GP chondrocyte differentiation (i.e., *collagens II*, *X*, and *I*) were also investigated (Fig. 6). The influences of graded dosages of RA on the expression of collagen mRNA were assessed by RT-PCR. There was no detectable

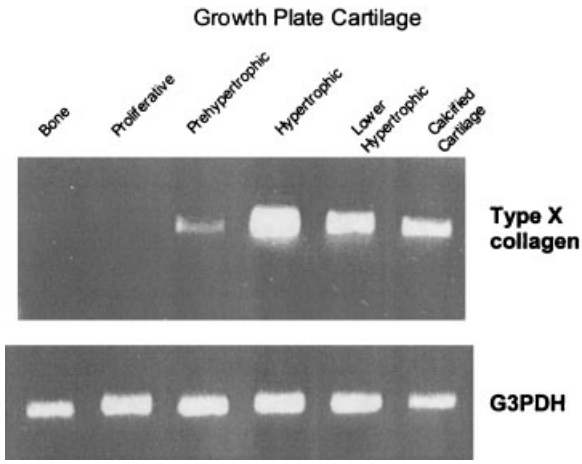

Fig. 3. Type X mRNA distribution in avian growth plate tissue. RT-PCR showing that type X collagen mRNA is enriched in the hypertrophic zone of avian growth plate tissue. Total RNA was isolated from serial horizontal slices dissected from metatarsal growth plate cartilage. RT-PCR was performed using *type X* primers (**top panel**). Glyceraldehyde 3-phosphate dehydrogenase was used as an internal control (**bottom panel**). This localization of *type X* message levels in the native GP cartilage using zonal RT-PCR analysis indicates that our methods for localization and estimation of *Shh* message levels using similar levels of total RNA for PCR should be valid.

collagen I mRNA in the control (no added RA) GP chondrocytes (lane 1); increasing dosages of RA (10–250 nM) stimulated its expression (lanes 2–4) with near maximal effects being seen at 50 nM. In contrast, the control chondrocytes expressed maximal levels of collagen II mRNA (lane 5). Marked decrease was observed in the presence of only 10 nM RA, and increasing levels of RA to 250 nM completely suppressed *collagen II* expression (lanes 6–8). Control GP chondrocytes expressed significant levels of collagen X mRNA (lane 9); low concentrations of RA (10 and 50 nM) caused enhanced collagen X mRNA expression (lanes 10–11), but higher concentration (250 nM) suppressed its expression (lane 12). Thus, depending on the level of exposure, RA was capable of altering the maturation status of cultured chondrocytes, affecting the expression of both *Shh* and the *collagen* genes.

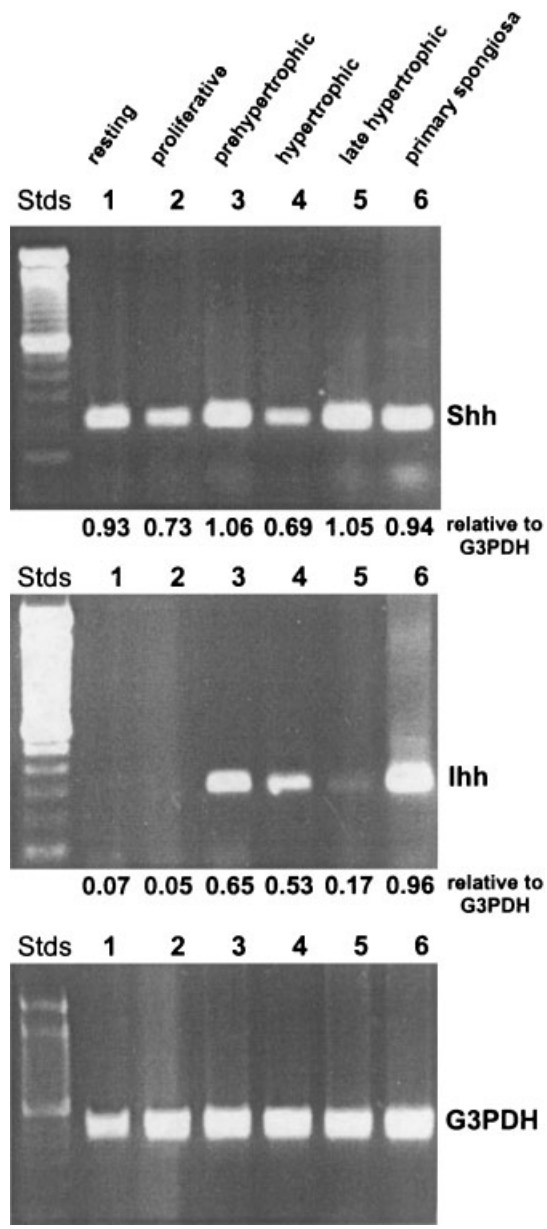

Fig. 4. Analysis of the expression of Shh, Ihh, and G3PDH mRNA in successive zones of the avian growth plate. Serial horizontal tissue slices from the metatarsal growth plate of 6-week-old broiler-strain chickens encompassing resting, proliferating, prehypertrophic, hypertrophic, late hypertrophic, and primary spongiosa were collected into six sterile tubes. Total RNA was isolated and RT-PCR was performed using *Shh* and *Ihh* primers as previously described. Each sample used for PCR contained cDNA derived from 0.25 μg total RNA in each reverse transcription reaction. Glyceraldehyde 3-phosphate dehydrogenase, a constitutively expressed enzyme in GP was used as an internal standard for monitoring mRNA loading. Shown here are representative results from one of three separate experiments. Note that *Ihh* is expressed in maximal levels in the prehypertrophic zone of the GP (where others had previously noted maximal expression) and is also expressed at even higher levels in primary spongiosa. In contrast, *Shh* is expressed in a cyclical pattern. Peak expression of *Shh* occurs in the resting zone, prehypertrophic zone, and hypertrophic zone; substantial expression also occurs in the primary spongiosa. The relative band intensities were assessed by scanning densitometry; ratios relative to G3PDH are shown below each lane. PCR product sizes—*Shh* (197 bp), *Ihh* (302 bp), *G3PDH* (433 bp).

TABLE IV. Nucleotide Sequence of the VEGF PCR Product

CTCTGAGCAAGGCTCACAGTGAAAGCTGGGTGGTTTG
TACCGGCCTTTCTTGCGCTTTCTCTTTTGACCCTTCC
CCTTTCCTCGCTTTGATTTTTTTTCTTGTTTATTTTT
GACATCTTTCTTTGGTCTGCAGTCACATTTACTGTGC
TGTAAGAAGCTCATGTGCGCTATGTGCTGACTCTGAT
GGGGTTTAATTCTTGCGATCTCCATCGTGACGTTGTA
CACATCCACAGGGACACATTCTAGGCCCTCATCGCCG
CAGCAACCCGCACATCTCATCAGAGGCACACAGGATG
GCCTG

After RT-PCR amplification, a TA cloning kit (Invitrogen) was used to clone the 301-bp VEGF PCR product for confirmation of its identity. Plasmids were isolated from 2-ml colony overnight cultures using an UltraClean mini-plasmid prep kit (MO BIO). DNA sequencing was performed. One hundred percent identity is obtained comparing to chicken VEGF mRNA of GenBank accession no. S79680.

Inhibitory Effect of RA on Indian Hedgehog (Ihh) Expression

We then examined the effect of RA on Ihh and Shh mRNA expression in a more systematic manner, using continuous administration of 50 nM RA in serum-containing media from day 7 (preconfluent cultures) to day 26 when the cultures had mineralized (Fig. 7). Total RNA was isolated from the cell layer on specified days and equal amounts of RNA were loaded for *Ihh, Shh*, and *G3PDH* amplification using simultaneous RT-PCR. We found that levels of Ihh mRNA in control cultures (no RA) varied in a cyclical pattern: on day 10, 18, and 26, peak levels were observed; on day 14, expression was undetectable, and on day 22 mRNA levels were below that seen on day 18 and 26. While the levels of Shh mRNA in the control cultures were steadier than those of *Ihh*, there was minimal early expression (day 10) of *Shh* when high levels of Ihh mRNA were present (Fig. 7). On day 14, when there were undetectable levels of *Ihh*, a transient maximum in *Shh* expression was evident. On day 18, when levels of *Shh* declined, *Ihh* levels rose. Gradually increasing levels of Shh mRNA were seen thereafter as the cultures matured and mineralized (day 22 and 26).

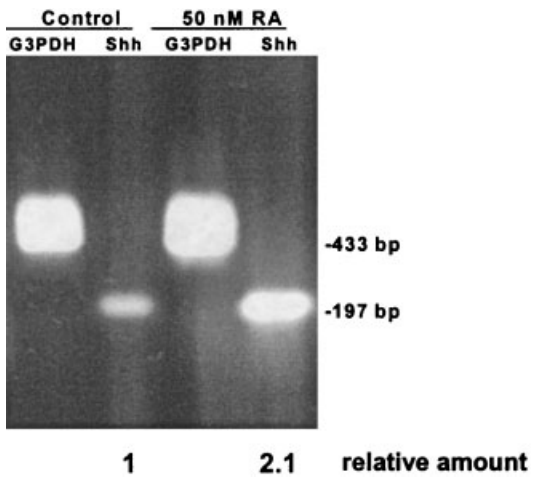

Fig. 5. Enhancement of Shh mRNA expression by RA. RT-PCR analysis shows that Shh mRNA is constitutively expressed in primary cultures of postnatal GP chondrocytes and is upregulated by RA. Cultures were treated with 50 nM RA in the DATP5 serum-containing medium from day 7 to 14, and then harvested on day 20. Steady-state levels of Shh mRNA (197 bp) and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) mRNA (433 bp), in the presence and absence of RA, were compared using RT-PCR analysis. The numbers below the Shh lanes are the ratios of the intensity of the mRNA band of the control versus the RA-treated cells. Note the 2.1-fold increase in the level of Shh mRNA expression in the presence of low levels of RA (50 nM). G3PDH mRNA levels used as an internal standard for monitoring mRNA show that loading was essentially constant.

Treatment of the cells with 50 nM of RA caused a marked suppression of Ihh mRNA at each time point, except on day 14 when control levels were undetectable (Fig. 7). There was little change in the level of G3PDH mRNA that served as a positive internal control for both integrity and amount of total RNA loaded. The effects of RA on Shh mRNA expression were opposite of those on *Ihh*. RA clearly stimulated *Shh* expression, especially on day 10, 18, and 22 when control *Shh* levels tended to be low. RA was without effect on *Shh* on day 14 and 26 (Fig. 7). The pattern that emerged was: 1) In control chicken GP chondrocyte cultures, Shh and Ihh mRNA were co-expressed, except on day 14 when Ihh mRNA is absent. 2) In cultures treated with RA, Shh mRNA dominated; there was little Ihh mRNA expressed.

Localization of Shh by In Situ Hybridization

To establish its localization in the postnatal GP we performed in situ hybridization according to the procedure described by Hicks et al. [1996] and O'Keefe et al. [1997a]. Type X collagen was used as a hypertrophic zone marker: the upper zones of the GP did not express type X, whereas in the lower hypertrophic zone its expression was abundant. Thus, it is evident that peak expression of Shh mRNA is localized to the late hypertrophic zone of the postnatal GP (Fig. 8), which is in agreement with data from the zonal dissections (Figure 4 and Table II). The large cells in the calcified cartilage region well below the tips of the invading capillary buds were heavily stained with Shh mRNA. Above the tips of the invading capillaries in the

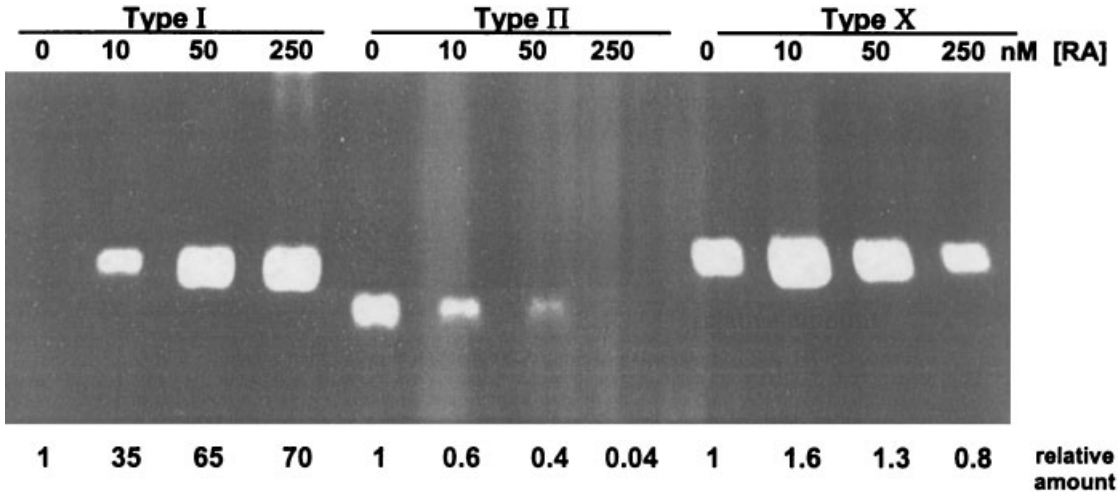

Fig. 6. Regulation of collagen mRNA expression by RA in primary cultures of avian GP chondrocytes. Cultures were treated with 0, 10, 50, and 250 nM RA from day 7 to 14. On day 14, total RNA was isolated from the cell layer and RT-PCR reactions were performed using primers specific to *type I, type II*, and *type X collagen*. As shown above, RA had potent effects on the level of expression of these collagens genes. For example, while there was no detectable expression of mRNA for *type I collagen* in the control sample, 10 nM RA treatment dramatically *increased* the expression of its message; higher doses of RA potentiated this effect. In contrast, RA exerted an opposite effect on the expression of type II collagen mRNA showing a dose-dependent *inhibition*. Expression of Type X collagen mRNA was maximally increased by 10 nM RA, but inhibited at higher levels of RA. The numbers below each lane are the ratios of the intensity of the mRNA band of the control versus the RA-treated cells. The internal standard, G3PDH mRNA remained at constant levels in the presence of 10, 50, and 250 nM RA (data not shown). Our previous studies have also demonstrated that G3PDH levels in chondrocytes are not affected by treatment with these concentrations of RA (see Nie et al., 1998 *J Cell Biochem* 68, 90–99). PCR product sizes—*type I* 672 bp; *type II* 554 bp; *type X* 675 bp.

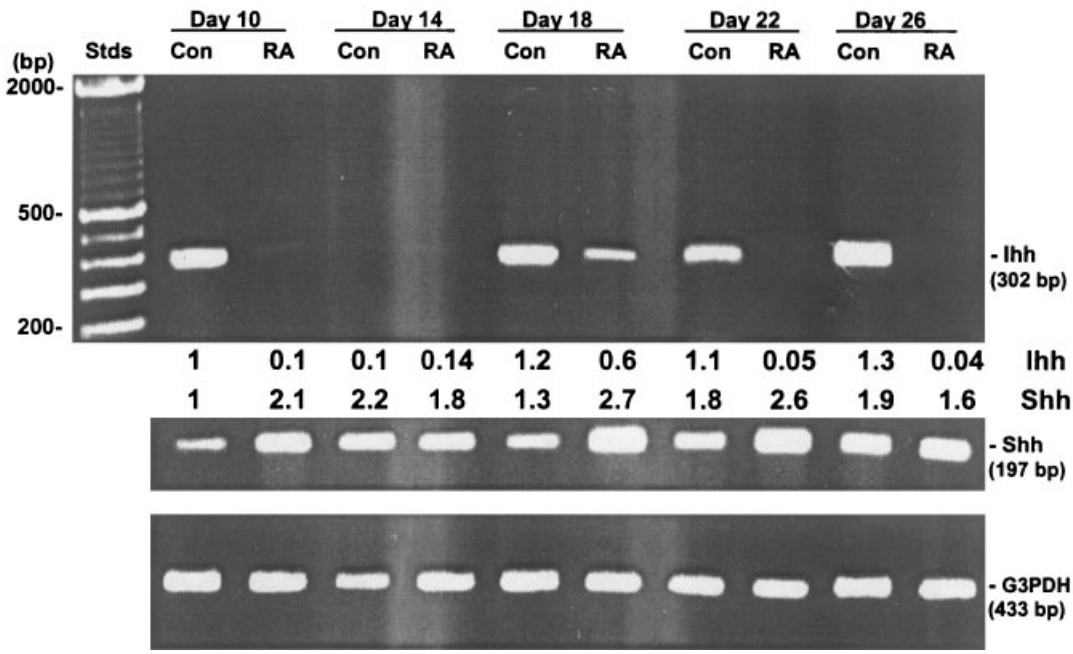

Fig. 7. Time dependent upregulation of Shh and downregulation of Ihh mRNA expression by RA. Chondrocyte cultures were treated with 50 nM RA in DATP5 medium from day 7 (preconfluent) to day 26. Total RNA was isolated on day 10, 14, 18, 22, and 26. The steady-state levels of Ihh (302 bp), Shh (197 bp), and G3PDH (433 bp) mRNA, in the presence and absence of RA were compared using RT-PCR. Note the cyclical expression of the *Ihh* gene in the control cultures, and the dramatic *decrease* in level of expression of Ihh mRNA in the presence of this low level of RA, while the internal control, *G3PDH*, remained unchanged. In contrast, expression of the *Snn* gene in the control cultures was initially very low, but steadily increased with time. RA significantly *increased* Shh mRNA levels, especially on day 10, 18, and 22. The intensity of the *Ihh* and *Shh* bands were expressed as a ratio normalized to the day 10 control of *Ihh* and *Shh*, respectively.

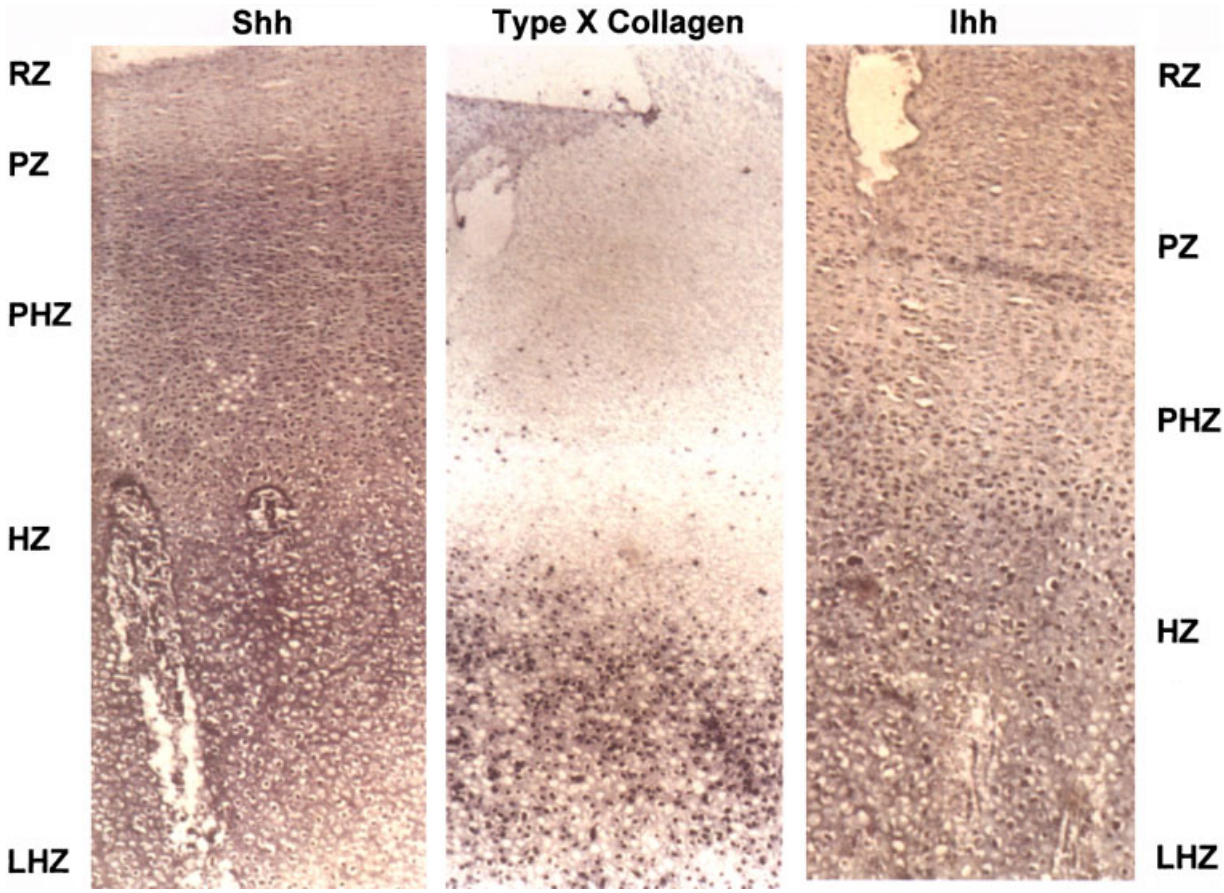

Fig. 8. In situ hybridization of *Ihh* and *Shh* expression in tibial growth plate cartilage of 6-week-old broiler-strain chickens. In situ hybridization was performed according to procedures described in Materials and Methods. Briefly, dewaxed tissue sections were rehydrated and permeabilized with proteinase K. After acetylation and prehybridization steps to minimize nonspecific binding, the slides were hybridized the dUTP-digoxigenin-labeled antisense cDNA probes that bind specifically to Shh, Type X collagen, or Ihh mRNA within the cell. Detection of the hybrid involves use of an antidigoxigenin antibody conjugated with alkaline phosphatase. Bound antibody is then detected using an alkaline phosphatase substrate that gives a blue stain. Note the strongest staining for Ihh mRNA is in the prehypertrophic zone above the tip of the vascular front. Shh mRNA is predominately expressed in the lower hypertrophic cartilage below the tip of the capillary and it is also in the other areas with varying intensity. Type X collagen mRNA was selectively expressed in the hypertrophic zone of the growth plate (middle panel). [Magnification = 125–160×] RZ, resting zone; PZ, proliferating zone; PHZ, prehypertrophic zone; HZ, hypertrophic zone; LHZ, late hypertrophic zone. Black and white image is shown.

prehypertrophic zone, levels of Shh mRNA were lower; in contrast, Ihh mRNA was maximally expressed in that region. At higher magnification, it is evident that the large round hypertrophic cells of the calcified cartilage region were stained intensely for Shh mRNA (Fig. 9).

DISCUSSION

We report here the discovery that *Shh*, in addition to *Ihh*, is expressed at significant levels in postnatal GP cartilage. Using a variety of methods and conditions we detected Shh mRNA throughout the GP with variable levels of intensity; with Ihh, lowest levels were expressed in the resting zone, and highest levels were present in prehypertrophic zone. In primary cultures of avian GP chondrocytes, during the early stages when *Ihh* is expressed abundantly, *Shh* is expressed at low levels. Later, on day 14 as the cultures mature, levels of Shh mRNA increased, whereas *Ihh* disappeared. Using RNA isolated from zonal dissection of GP tissue coupled with RT-PCR, we find that in regions where *Ihh* is poorly expressed (e.g., resting zone) *Shh* is strongly expressed. Further, using in situ hybridization methods, we find that *Ihh* is maximally expressed in the prehypertrophic area of the GP above the point of vascular penetration, whereas *Shh* is most strongly

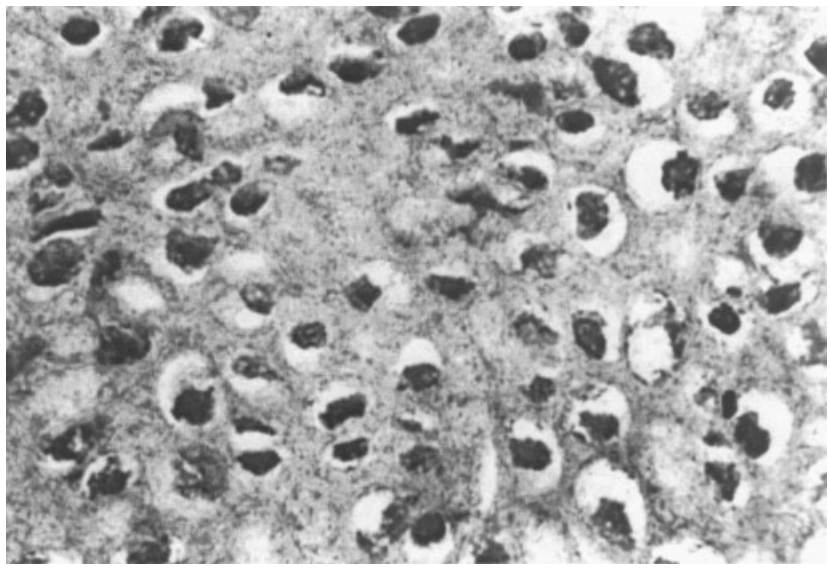

Fig. 9. Higher magnification of in situ hybridization of *Shh* expression in the hypertrophic zone of tibial growth plate cartilage. In situ hybridization and detection were performed according to the procedure detailed in Figure 8. Slides were hybridized with an antisense dUTP-digoxigenin-labeled cDNA probe that binds complementarily to Shh mRNA in the cell. Note the strong staining for Shh mRNA in the hypertrophic chondrocytes. Magnification = 475×.

expressed in the late hypertrophic/calcified cartilage zone below the terminus of the vascular buds where type X collagen is known to be specifically expressed.

While Shh and Ihh proteins may have similar signaling capabilities once bound to Patched (Ptc) their common receptor [Carpenter et al., 1998], it is likely that their individual roles during embryonic development and thereafter depend on when and where they are expressed. *Shh* emerges early in the posterior mesenchyme of the limb bud (Stages 17–23) [Riddle et al., 1993; Pearse and Tabin, 1998]; *Ihh* has been shown to express later in limb development during the formation of the skeletal elements (Stage 26 and following) [Bitgood and McMahon, 1995]. Although the continued expression of *Ihh* is well established, our discovery that *Shh* is also expressed in significant amounts in the postnatal GP is a new finding. Our localization of *Ihh* is exactly where many other investigators have previously mapped it [Lanske et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999; van der Eerden et al., 2000], but no one has previously reported the presence of *Shh* in postnatal GP tissues.

Recent studies have identified Ihh as an important coordinating molecule in controlling longitudinal bone growth. Parathyroid related protein (PTHrP) appears to mediate the action of Ihh in GP development [Lanske et al., 1996]. In the embryonic period, Ihh secreted by cells in the prehypertrophic zone stimulates PTHrP expression in periarticular perichondrial cells [Vortkamp et al., 1996]; these are thought to be components of a feedback loop that regulates the relative proportions of proliferating and hypertrophic chondrocytes. However in the postnatal period, recent data indicate that GP chondrocytes themselves are the source of the PTHrP peptide, providing a paracrine feedback loop confined to the GP [Medill et al., 2001]. But there is evidence that Ihh can exert its effects through both PTHrP-dependent and PTHrP-independent processes [Lanske et al., 1996]. Further, in *Ihh*-null mice, a more severe form of dwarfism is seen than in those lacking PTHrP [St-Jacques et al., 1999; Guo et al., 2001]. Ihh is also necessary for osteogenesis in the formation of the bony collar around the cartilage anlagen [Chung et al., 2001]. Thus, Ihh appears to play an extensive role in coordinating diverse aspects of skeletal morphogenesis during development.

Our new data revealing the expression of both *Shh* and *Ihh* in the postnatal GP suggest that Shh also has a role beyond its known involvement in the very early patterning of embryonic limb development. Vortkamp et al. [1998] found that the expressions of the hedgehog receptors,

Patched (*Ptc*) and its downstream responsive element, *Gli*, occur in discrete bands both above and below the prehypertrophic zone where *Ihh* is maximally expressed in postnatal tibia by in situ hybridization. The expression of Ptc has been confirmed by RT-PCR to be in maturationally distinct, Percoll-fractionated chondrocytes isolated from the GP of postnatal chickens [Farquharson et al., 2001]. While considering that Ihh might signal in both directions, Vortkamp et al. [1998] commented that there might be another hedgehog gene expressed in the GP. Our finding that *Shh* expression is very high in the late hypertrophic zone where vascular penetration and mineralization of cartilage occur suggests that it may be the other hedgehog gene.

We postulate that Shh may be directly involved in chondrocyte hypertrophy and mineralization, not only because of its high level of expression in this region, but also because its responsive genes, *Ptc* and *Gli*, are both expressed close by [Vortkamp et al., 1998]. Secreted *Shh*, attached to cholesterol at the C-terminus of the cleaved N-terminal fragment, binds to *Ptc* receptors on target cells [McMahon, 2000]. High levels of Ihh mRNA expression in the prehypertrophic zone of GP presumably regulate the rate of cellular maturation, balanced with the effect of *Shh* also expressed in this zone. Later, when *Shh* becomes more dominantly expressed, it apparently pushes the cells forward toward hypertrophic differentiation. This concept is consistent with previous in vitro work showing that retrovirally-mediated over-expression of *Shh* in chondrogenic cultures promotes development of characteristics typical of hypertrophic chondrocytes [Stott and Chuong, 1997]. Overexpression of *Shh* in these limb bud micromass cultures induces heightened expression of alkaline phosphatase, type X collagen, and formation of multilayer cellular nodules. In contrast, there is a report that Shh N-terminal peptide inhibits matrix mineralization and decreased the size of the hypertrophic area when applied to embryonic metatarsal bone rudiment organ cultures [Alvarez et al., 2002]. The broader expression of Shh mRNA in the postnatal GP also merits comment. The high level of *Shh* expression in the resting zone may in fact be the trigger that initiates cellular proliferation, a concept consistent with its critical role in limb bud development. Further, its general presence in the adolescent postnatal GP suggests that it is involved in rapid longitudinal growth of bone during this period.

Turning to the effects of RA, long ago it was documented that proper levels of retinoids are essential for normal bone formation. In growing animals ingestion of both excessive [Wolbach and Hegsted, 1953; Pease, 1962; Vasan, 1975] and deficient levels of vitamin A [Havivi, 1967; Howell et al., 1967] lead to disturbances in GP chondrocyte maturation and mineralization. In vitro, RA has been shown to enhance chondrocyte maturation and to stimulate mineralization of hypertrophic chondrocytes [Iwamoto et al., 1993; Woodard et al., 1997; Wu et al., 1997]. How this is orchestrated remains unclear, despite the fact that RA is known to alter the level of expression of several collagen proteins [Iwamoto et al., 1994; Wu et al., 1997; Koyama et al., 1999; Sakano et al., 1999]. Our in vitro finding that RA stimulates Shh expression, but inhibits Ihh expression in GP chondrocyte cultures provides a new clue. The opposing effects of RA on the expression of the two hedgehog genes are important for several reasons. First, how the expressions of *Ihh*, and now *Shh*, are regulated in GP development in vivo is not well established. We now identify RA as a potent upstream regulator of their expression. Second, the ability of RA to attenuate Ihh gene expression could be a means of controlling the rate of GP differentiation. The fact that RA is tightly bound to serum proteins [Smith et al., 1973; Chen and Heller, 1977; Hodam and Creek, 1996] would limit its access to cells deep within the avascular, tightly woven, proteoglycan-rich extracellular matrix of the postnatal GP. The validity of this concept has been established with the discovery that normal GP cartilage exhibits classic signs of essential fatty acid deficiency even though serum levels are normal [Adkisson et al., 1991]. Like RA, fatty acids are carried in blood bound to albumin and their delivery to chondrocytes in the bulk of the GP is clearly compromised. Thus, vascular penetration into the distal GP would increase the availability of RA, enhancing production of Shh, enabling prehypertrophic chondrocytes to advance to the hypertrophic state.

In a recent study, RA was shown to transiently *increase* levels of *Ihh* expression in micromass cultures of rabbit GP chondrocytes [Yoshida et al., 2001]. These effects required direct exposure to high dosages of RA (100 nM) in serum-free medium; at an even higher and

clearly super-physiological level (1000 nM) the stimulatory effects of RA on *Ihh* expression were even more pronounced. On the other hand, lower more physiologic levels of RA (10 nM) caused a reduction in Ihh mRNA levels, in agreement with our current findings. Hodam and Creek [1996] and Creek et al. [1989] have shown that when RA is administered to cells in serum-free medium, about one-third is rapidly taken up (10 min) and then is quickly metabolized to inactive polar compounds that are released back into the medium. In contrast, when RA is given as a bovine serum albumin (BSA) complex, uptake was much slower; the maximum levels attained were only about one-tenth that seen after treatment with free RA, and RA was much less susceptible to degradation. (Our cells were treated with 50 nM RA in serum-containing medium, in which the great majority (~80%) of the RA would be bound, buffering its release to the cells.) Thus, our data reporting inhibitory effects of RA on *Ihh* probably would occur physiologically, because in vivo retinoids are tightly bound to proteins. In contrast, the finding that RA stimulates *Ihh* expression [Yoshida et al., 2001], is difficult to reconcile with in vivo findings with normal exposure to vitamin A; rather, it may more properly model the skeletal disturbances seen when excessive levels of vitamin A are given [Wolbach and Hegsted, 1953; Pease, 1962; Vasan, 1975].

Recently Koyama et al. [1999] reported that the appearance of hypertrophic chondrocytes in avian embryo limbs is accompanied by a marked and selective upregulation of the expression of the RA receptor-γ gene. Because treatment with a RA antagonist blocked chondrocyte maturation at the *Ihh*-expressing stage, they concluded that retinoids act on *Ihh*-expressing prehypertrophic chondrocytes to promote their transition to the hypertrophic stage. They speculated that RA could directly or indirectly down-regulate *Ihh* and up-regulate RA receptor-γ to allow cells to overcome and escape the negative action of Ihh and PTHrP. Our finding that RA down-regulates the *Ihh* gene and further up-regulates *Shh* indicates that indeed RA would push the maturing chondrocytes toward the hypertrophic stage and favor bone formation. Thus, the finding of the different responses of the two hedgehog genes to RA helps clarify the complex interrelationships that exist between endogenous and exogenous factors during GP development and skeletal morphogenesis.

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