Regulation of Rate of Cartilage Differentiation by Indian Hedgehog and PTH-Related Protein

Andrea Vortkamp,* Kaechoong Lee,* Beate Lanske, Gino V. Segre, Henry M. Kronenberg, Clifford J. Tabin†

Proper regulation of chondrocyte differentiation is necessary for the morphogenesis of skeletal elements, yet little is known about the molecular regulation of this process. A chicken homolog of Indian hedgehog (Ihh), a member of the conserved Hedgehog family of secreted proteins that is expressed during bone formation, has now been isolated. Ihh has biological properties similar to those of Sonic hedgehog (Shh), including the ability to regulate the conserved targets Patched (Ptc) and Gli. Ihh is expressed in the prehypertrophic chondrocytes of cartilage elements, where it regulates the rate of hypertrophic differentiation. Misexpression of Ihh prevents proliferating chondrocytes from initiating the hypertrophic differentiation process. The direct target of Ihh signaling is the perichondrium, where Gli and Ptc flank the expression domain of Ihh. Ihh induces the expression of a second signal, parathyroid hormone–related protein (PTHrP), in the periarticular perichondrium. Analysis of PTHrP (−/−) mutant mice indicated that the PTHrP protein signals to its receptor in the prehypertrophic chondrocytes, thereby blocking hypertrophic differentiation. In vitro application of Hedgehog or PTHrP protein to normal or PTHrP (−/−) limb explants demonstrated that PTHrP mediates the effects of Ihh through the formation of a negative feedback loop that modulates the rate of chondrocyte differentiation.

During vertebrate embryogenesis the first elements of the skeleton to be formed are cartilage templates that are subsequently replaced by bone tissue in a process called endochondral ossification (*1*, *2*). This process begins with the aggregation of undifferentiated mesenchyme. Cells in the core of these condensations differentiate into chondrocytes, whereas spindle-shaped cells at the periphery form a sheath around the cartilage, the perichondrium. These precursors of the skeletal elements elongate by proliferation of the chondrocytes and by matrix deposition. Shortly after the condensations form, chondrocytes in the central region of the cartilage elements cease proliferating, become hypertrophic, and alter their extracellular matrix. The changes in the extracellular matrix allow blood vessels to invade from the perichondrium. Bone marrow cells and osteoblasts appear in association with the vascularization and replace the cartilage with bone (ossification).

In this process, the chondrocytes serve both to drive the growth of the skeletal elements and to form a scaffold for the osteoblasts. Both the zone of hypertrophic cartilage and the ossified region subsequently expand toward the ends of the skeletal elements. Ultimately, the shaft of the bone is mostly mineralized, leaving growth plates—narrow bands of proliferating cartilage and transitional hypertrophic cartilage—at the ends of each bone. Premature ossification of the cartilage is prevented by

A. Vortkamp and C. J. Tabin are in the Department of Genetics, Harvard Medical School, Boston, MA 02115, USA. K. Lee, B. Lanske, G. V. Segre, and H. M. Kronenberg are in the Endocrine Unit, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed.

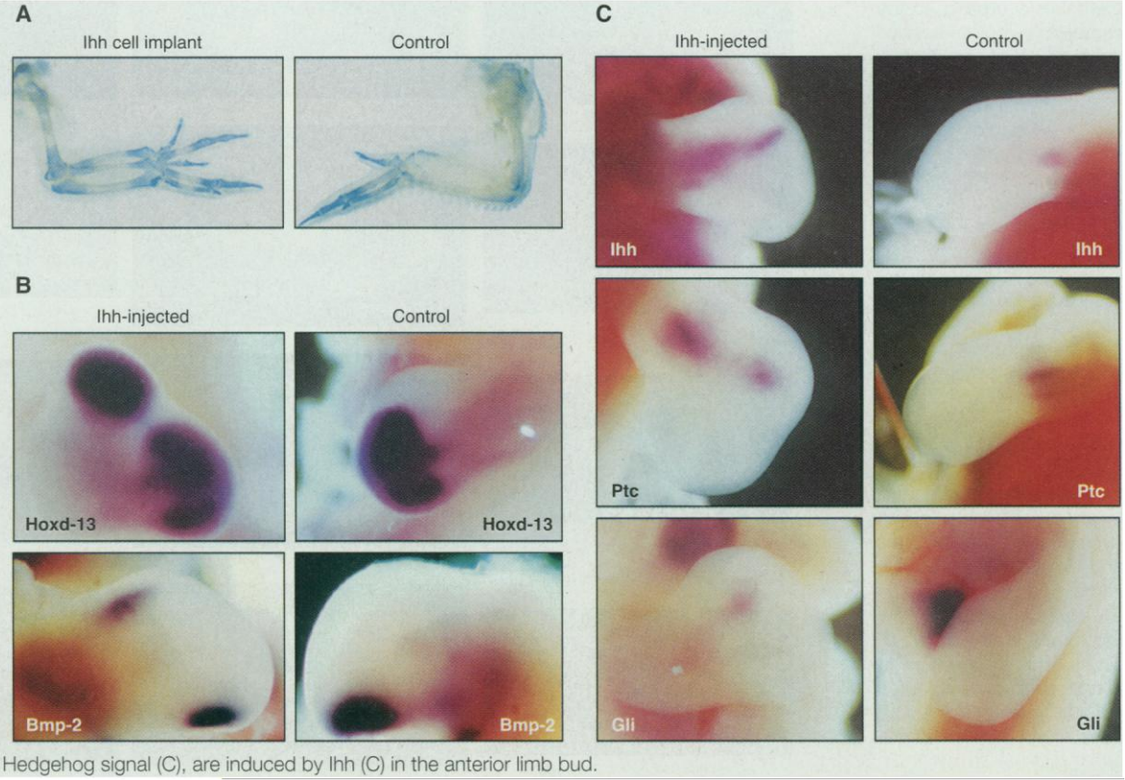

Fig. 1. Ihh activates the Shh signaling pathway. (**A**) Anterior misexpression of *Ihh* induces duplication of posterior limb structures. *Ihh*-expressing cells (*46*) were implanted into the anterior mesenchyme of a HH stage 21 chicken wing bud. After 7 days of incubation, Alcian blue staining of the limbs revealed anterior duplications of the radius and digits II and III. (**B** and **C**) Anterior misexpression of *Ihh* induces expression of Shh target genes. HH stage 21 chicken limb buds were infected with an *Ihh*-expressing replication-competent retrovirus and analyzed by whole-mount in situ hybridization 40 to 48 hours later (*46*, *48*). *Hoxd-13* and *Bmp-2*, genes thought to be involved in patterning (B), and *Ptc* and *Gli*, genes believed to transduce the Hedgehog signal (C), are induced by Ihh (C) in the anterior limb bud.

continued proliferation of the round chondrocytes at the ends of the bone and by careful control of the rate of their differentiation into hypertrophic chondrocytes. Regulation of the rate of endochondral ossification is critical to bone morphogenesis, because it plays a major role in determining the shape and length of the skeletal elements. Whereas the cellular steps in this process are well described (*1*, *2*), little is known about its regulation. Studies of mutant mice suggest roles for FGFs (*3*–*5*), IGFs (*6*), and PTHrP (*7*, *8*), but many other local and systemic factors are likely to be involved. Virtually nothing, however, is known about how the expression and action of these factors are regulated or about their specific functions in the coordination of skeletal formation.

In the mouse embryo, *Indian hedgehog* is expressed in the developing cartilage elements (*9*), suggesting that it might play a role in regulating bone formation. Hedgehog proteins constitute a conserved family of secreted molecules that provide key signals in embryonic patterning in many organisms. During *Drosophila* development, the segment polarity gene *hedgehog* (*hh*) regulates embryonic segmentation and anterior-posterior patterning of the imaginal disks (*10*–*12*). In higher vertebrates, there are at least three hedgehog genes: *Sonic hedgehog* (*Shh*), *Desert hedgehog* (*Dhh*), and *Indian hedgehog* (*Ihh*) (*13*–*15*). *Shh* has multiple functions during embryonic development, including the establishment of left-right asymmetry in the early embryo (*16*), the induction of ventral cell fates in the neural tube (*14*, *15*, *17*), the specification of sclerotomal cell fate in the somites (*18*, *19*), the mediation of epithelial-mesenchymal signaling in the gut (*20*), and the specification of the anterior-posterior limb axis (*13*). Dhh functions as a spermatocyte survival factor in the testes (*21*). In contrast, specific biological roles for *Ihh* have not yet been determined.

Whereas the various Hedgehog proteins have distinct biological functions, they appear to use the same signal transduction pathway. On the basis of genetic analysis, *patched* (*ptc*), which encodes a transmembrane protein (*22*, *23*), and *cubitus interruptus* (*ci*), which encodes a putative transcription factor (*24*), are two key genes in the *Drosophila* Hedgehog transduction pathway (*25*–*27*). Both are required for cellular responses to Hedgehog signaling. Their vertebrate homologs, *Patched* (*Ptc*) (*28*, *29*) and *Gli* (*30*, *31*), are also expressed in cells capable of responding to ectopic Hedgehog and are highly up-regulated in all tissues actively responding to Shh or Dhh (*21*, *28*, *29*, *31*, *32*). Transforming growth factor–β (TGF-β) family members can act as secondary signals downstream of Hedgehog proteins. In *Drosophila*, *decapentaplegic* (*dpp*) mediates aspects of Hedgehog signaling in the imaginal disks (*33*–*35*), whereas the vertebrate homologs, *Bmp-2* and *Bmp-4*, are induced in several tissues in response to Shh (*9*, *20*, *36*). However, in contrast to *ptc*/*Ptc* and *ci*/*Gli*, the TGF-β family members are not consistent targets of Hedgehog signaling in either *Drosophila* or vertebrates. Knowledge of these pathways, particularly of the apparently universal targets *Ptc* and *Gli*, is useful for evaluating the role of *Ihh* in developing bone.

Isolation and characterization of a chicken *Ihh* clone: Ihh and Shh have similar biological activities. To examine the role of Ihh in endochondral bone development, we isolated a cDNA clone containing the chicken *Ihh* coding sequence of 408 amino acids (*37*). Like the other Hedgehog proteins, the predicted amino acid sequence of Ihh contains a conserved COOH-terminal protease mo-

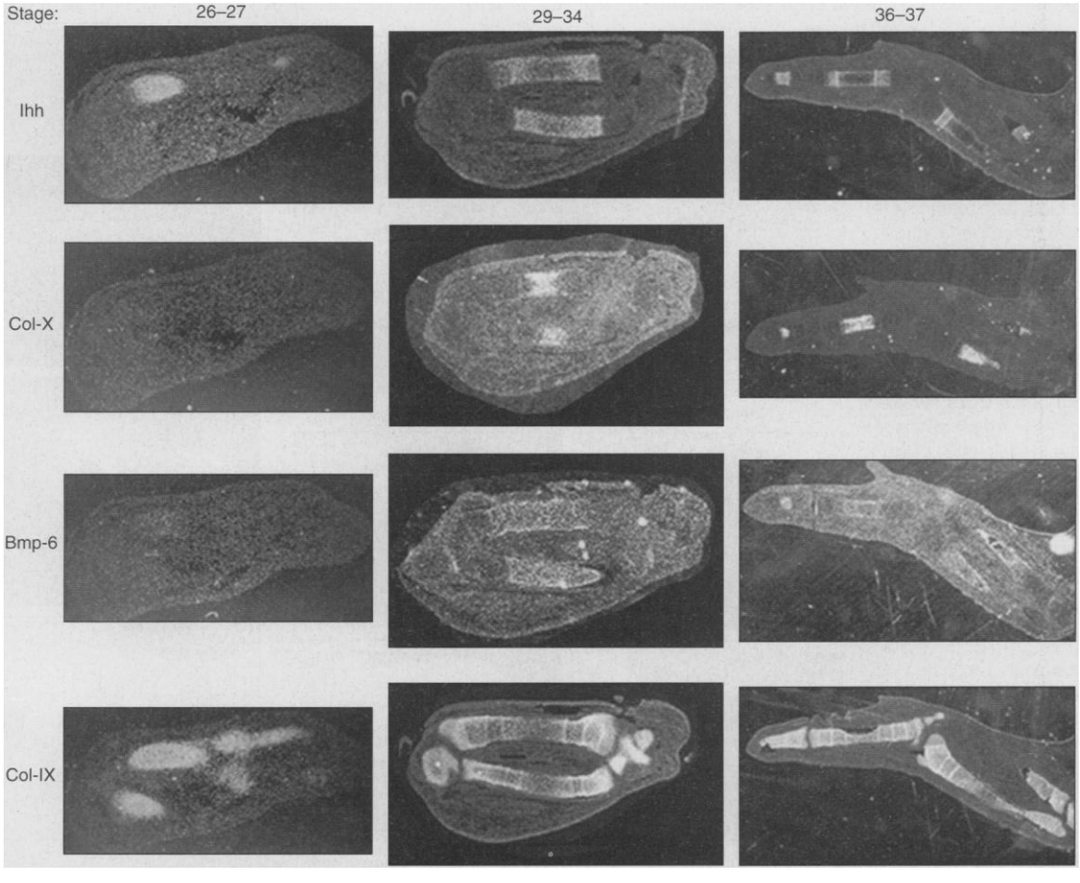

Fig. 2. Ihh expression in prehypertrophic chondrocytes. The expression of *Ihh* during cartilage development was compared to the expression of the specific cartilage markers *Col-IX*, *Col-X*, and *Bmp-6* (*51*). At all stages investigated, *Col-IX* is expressed throughout the entire cartilage element, whereas *Col-X* identifies hypertrophic chondrocytes. At stage 26, before the first chondrocytes become hypertrophic, *Ihh* is already expressed in the middle of the cartilage element overlapping the expression domain of *Bmp-6*. Between stages 29 and 37, the expression of *Ihh* expands toward the distal ends of the cartilage elements and fades in their center where the chondrocytes have differentiated into hypertrophic chondrocytes and express *Col-X*. *Bmp-6* expression at this stage overlaps both the *Ihh* and the *Col-X* expression domains. Radioactive in situ hybridization was carried out on serial sections of limb buds of the different stages (*48*).

tif, which has been shown to be responsible for autocatalytic cleavage of other Hedgehog proteins (*38–40*). Ihh also contains the conserved protease cleavage site, indicating that it is likely to be processed in a manner similar to that for Hedgehog and Shh. Whereas the COOH-terminal domain of Ihh is substantially divergent from that of Shh, the NH_2-terminal domains of the two proteins are 93% identical. Because the NH_2-terminal fragment encodes the functional Hedgehog signal in long- and short-range signaling (*41–43*), the high extent of NH_2-terminal conservation between Shh and Ihh suggests that they might have similar biological properties. However, this cannot be assumed because in zebrafish not all Hedgehog proteins have the same activity (*44*).

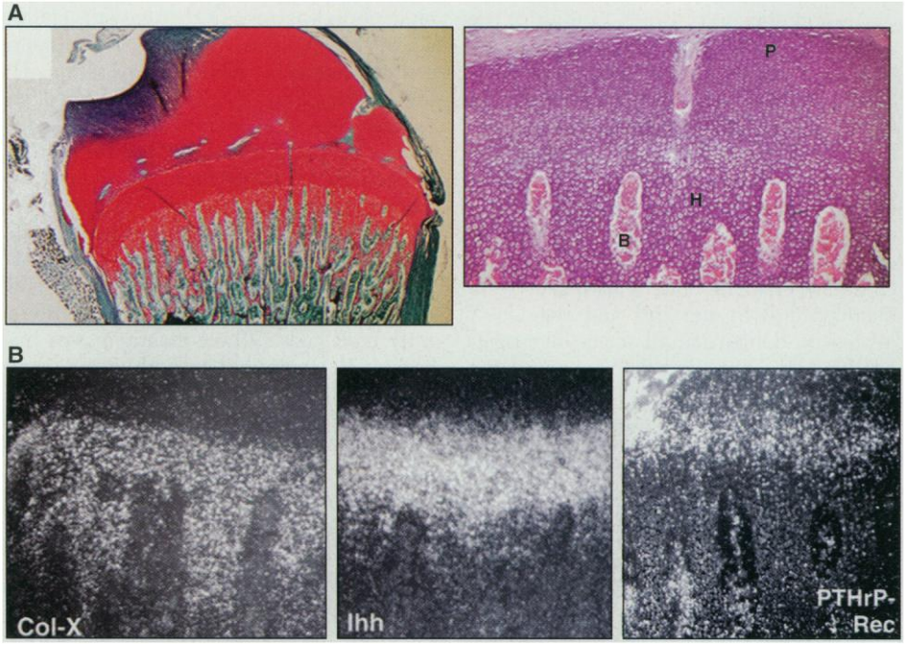

Fig. 3. Expression of *Ihh* in the growth plate. (**A**) Morphology of the growth plate of a chicken ulna, shown in low and high magnification. Longitudinal sections of an ulna from a 3-week-old chicken were stained with Weigert-Safranin to differentiate between cartilage (red) and bone (blue) and with H&E to visualize the morphology of the growth plate. B, bone and bone marrow; H, hypertrophic chondrocytes; P, proliferating chondrocytes. (**B**) Relative expression patterns of *Ihh*, *Col-X*, and *PTH/PTHrP receptor*. The three genes are expressed in overlapping domains, with the *PTH/PTHrP receptor* most distally located, followed by *Ihh* and *Col-X*. The *PTH/PTHrP receptor* and *Col-X* appear to be expressed in mutually exclusive domains, whereas the *Ihh* expression domain partially overlaps with both of them.

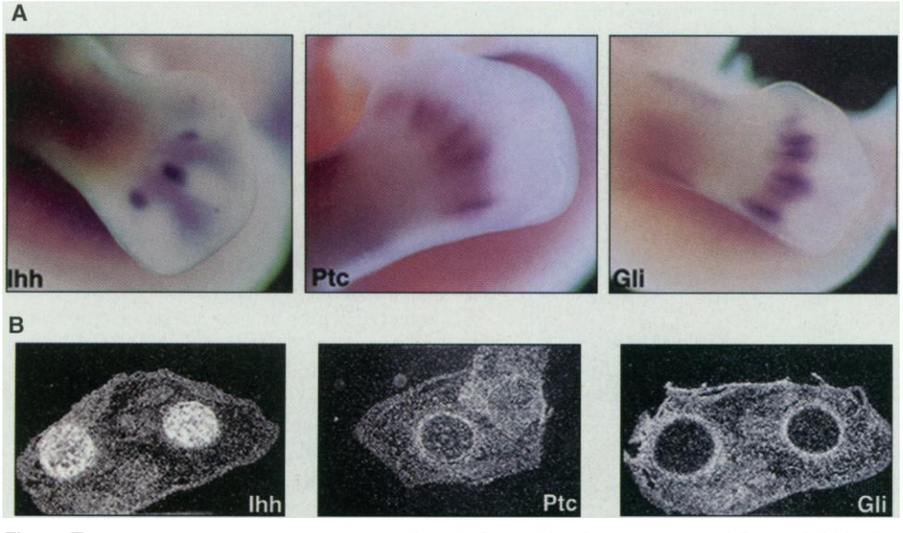

Fig. 4. The Ihh expression domain in the cartilage is flanked by the expression of Ptc and Gli in the perichondrium. Neither Ptc nor Gli is expressed in the cartilage core. (**A**) Whole-mount in situ hybridization with Ihh, Ptc, and Gli was carried out on HH stage 30 limb buds. (**B**) Radioactive in situ hybridization was carried out on sections of stage 34 chicken wings.

To test whether Ihh and Shh can function similarly, we compared their activities in the context of the early limb bud of the chick, a setting in which the role of *Shh* has been extensively characterized. Anterior misexpression of *Shh* at early limb stages induces mirror-image duplications of posterior limb structures (*13*). To test whether ectopic Ihh could induce similar morphological changes, we implanted *Ihh*-expressing cells in the anterior side of Hamburger Hamilton (HH) (*45*) stage 21 limb buds (*46*). After 7 days of incubation, the operated limbs showed duplications of the radius and digits II and III (Fig. 1A) similar to those induced by Shh in a parallel experiment (*47*). We also examined the ability of Ihh to replicate the effects of ectopic Shh on a molecular level after infection of early limb buds with an *Ihh*-expressing retrovirus (*46*). Using whole-mount in situ hybridization (*48*), we detected ectopic expression of limb-specific patterning genes located downstream of *Shh* (*36*), including *Hoxd-11*, *Hoxd-13*, and *Bmp-2* (Fig. 1B) (*47*), as well as expression of the universal Hedgehog response genes, *Ptc* and *Gli* (Fig. 1C). These results demonstrate that Ihh and Shh proteins have similar biological properties and can stimulate expression of the same downstream genes.

Expression of Ihh during bone development. Because Ihh and Shh appear to have identical signaling capabilities, their distinct embryonic roles are likely to be determined by spatial or temporal differences in their expression patterns. To investigate the developmental processes potentially regulated by *Ihh*, we analyzed its expression pattern by whole-mount in situ hybridization. Expression of *Ihh* was detected at two major sites in the embryo: in the endoderm of the developing midgut and lung (*47*) and in the cartilage of the developing long bones in the limbs (Fig. 2). No expression of *Ihh* was observed early in limb development (*47*), when *Shh* is expressed in the posterior mesenchyme, suggesting that *Ihh* plays a role distinct from that of *Shh*, acting later in limb development during the formation of the skeletal elements.

To determine the cell types expressing *Ihh* in the forming skeletal elements, we compared the expression pattern of *Ihh* to that of specific cartilage markers (Fig. 2) by in situ hybridization to parallel sections (*48*). During skeletal development, distinct

types of collagen are expressed at different stages of differentiation; thus, they serve as excellent markers for specific cell types. For example, *Col-IX* is expressed in all cartilage cells, whereas *Col-X* is specific for hypertrophic chondrocytes (*49*). In addition to *Col-X*, *Bmp-6* has also been described as a marker for hypertrophic chondrocytes (*50, 51*).

At stage 26/27, *Col-IX* was expressed in all cells of the developing cartilage elements of the wings. *Ihh* expression was detected in the middle of the *Col-IX* expression domain but was excluded from the distal portions of the cartilage elements. At this stage, the entire cartilage consisted of proliferating chondrocytes, and no expression of the hypertrophic cartilage marker *Col-X* could be detected. *Bmp-6*, however, was expressed in a region similar to that of the *Ihh* expression domain. During the subsequent stages, the expression of *Ihh* expanded toward the ends of the cartilage elements and was reduced in their central regions. At the same time, these central regions started to express *Col-X*, indicating their differentiation into hypertrophic chondrocytes. By stage 36, the *Ihh* expression domain had split into two regions, each distal to and slightly overlapping a central expression domain of *Col-X* in most of the skeletal elements of the wings (Fig. 2). Histological examination of hematoxylin and eosin (H&E)–stained sections verified that *Col-X* expression was restricted to morphologically hypertrophic cells, whereas Ihh was expressed in the region of transition from proliferating to hypertrophic chondrocytes and was excluded from hypertrophic cells (*47*). *Ihh* expression, therefore, preceded the hypertrophic state. *Bmp-6* expression overlapped both the Ihh and the *Col-X* expression domains at all stages investigated (Fig. 2). Thus, *Bmp-6* is expressed not only in hypertrophic cells (*50*) but also in the prehypertrophic, *Ihh*-expressing cells, possibly demarcating all cells that leave the proliferating zone.

The relative expression patterns of *Ihh* and *Col-X* are maintained as long as the bones are growing. At 3 weeks after hatching Ihh was expressed in the prehypertrophic chondrocytes in the growth plates, whereas *Col-X* was expressed in the adjacent hypertrophic chondrocytes (Fig. 3). *Ihh* is, therefore, expressed at a specific, critical stage of endochondral bone formation in cells undergoing the transition from proliferating to hypertrophic chondrocytes, suggesting that Ihh may play an important regulatory role in cartilage differentiation.

To identify the target tissue of *Ihh* signaling, we analyzed the expression of *Ptc* and *Gli*, which are ectopically induced by Ihh in early limb buds. Whole-mount in situ hybridization showed that both genes were expressed in the perichondrial region flanking the *Ihh* expression domain but that their expression extended slightly further toward the end of the cartilage elements (Fig. 4A). Hybridization to serial sections confirmed that both genes were expressed in the perichondrial region, and not in the cartilage core where *Ihh* is expressed (Fig. 4B). This expression pattern is consistent with *Gli* and *Ptc* being targets of Ihh signaling during bone development and strongly suggests that the perichondrium, and not the cartilage, is the natural target of the Ihh signal.

Phenotypic consequences of Ihh misexpression. To analyze the role of Ihh in the development of long bones, we misexpressed Ihh during cartilage formation with a replication-competent retroviral vector. We targeted our injections to restrict the infection to posterior and medial regions of HH stage 22 wing buds, resulting in a high level of infection of the posterior and medial cartilage elements including the ulnae and the humeri. Because the virus fails to spread within the cartilage tissue, infection was often excluded from anterior radii, which could therefore be used as internal controls (Fig. 5A).

To determine the morphological consequences of Ihh misexpression on the skeletal elements, we harvested and sectioned infected wings at late stages of embryonic development and treated them with stains that differentially demarcate bone and cartilage tissue (Weigert-Safranin) (*52*). On day 10 of development (stage 36), the centers of normal diaphyses consisted of hypertrophic cartilage that had not yet been invaded by blood vessels and was surrounded by a bone collar laid down by cells in the adjacent perichondrium. After this stage, the invasion of the blood vessels into the hypertrophic chondrocytes occurred, which

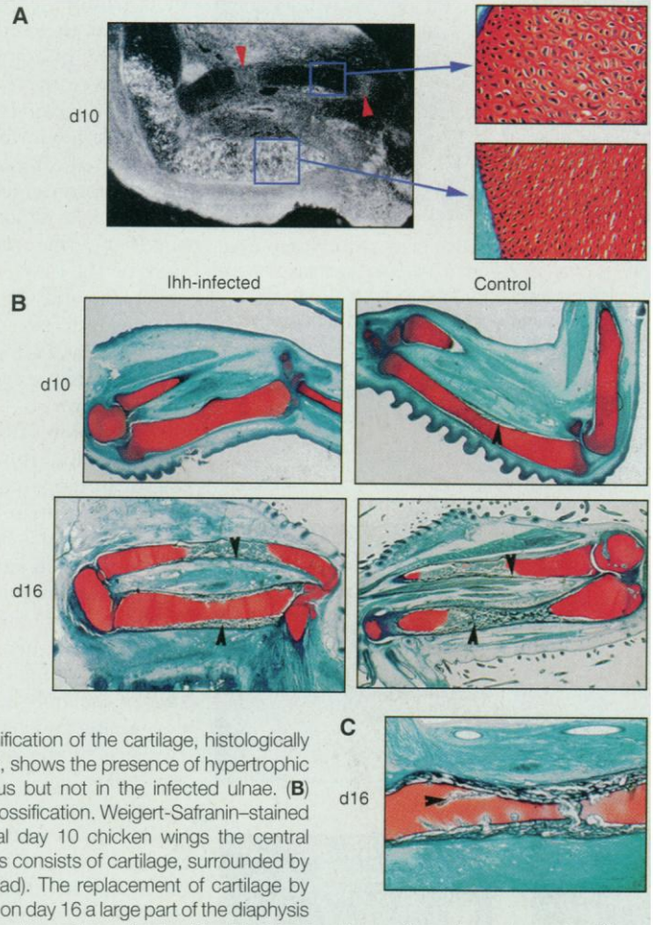

Fig. 5. Misexpression of Ihh disrupts endochondral bone development. An *Ihh*-expressing replication-competent virus (*46*) was used to infect the posterior and medial region of a HH stage 22 wing bud. Greater than 100 limbs were infected. In every instance, gross morphological alterations were observed. More than 30 of the infected limbs were sectioned and then stained or hybridized (or both) with probes as described, and every one displayed the described alterations. (**A**) On day 10 (d10) the ulna and the humerus are highly infected, whereas the radius is uninfected. Hybridization of a sectioned wing with an *Ihh* probe detects the viral transcript in ulna, humerus, and soft tissue and the endogenous *Ihh* expression (red arrowheads) in the radius above. High magnification of the cartilage, histologically stained with Weigert-Safranin, shows the presence of hypertrophic cells in the noninfected radius but not in the infected ulnae. (**B**) Infection with *Ihh* virus delays ossification. Weigert-Safranin–stained sections show that in normal day 10 chicken wings the central region of the skeletal elements consists of cartilage, surrounded by a bone collar (black arrowhead). The replacement of cartilage by bone starts in this region, and on day 16 a large part of the diaphysis consists of bone (arrowhead) with cartilage distally. After Ihh infection, the cartilage elements on day 10 are shorter and broader than those of noninfected controls. At day 16 the noninfected radius of an infected wing shows normal ossification similar to that of uninfected limbs, whereas the infected ulna is still characterized by a continuous nonhypertrophic cartilage core that is surrounded by a layer of membranous bone. (**C**) Irregular ossification after *Ihh* misexpression. Weigert-Safranin staining of an infected chicken wing (day 16) at higher magnification shows the continuous cartilage core surrounded by a thick layer of bone. A replacement of cartilage by bone takes place from the outside at various positions along the cartilage core. Red arrowheads demarcate endogenous Ihh expression, black arrowheads demarcate bone and bone marrow.

resulted in the replacement of cartilage by bone. By day 16 (HH stage 42), a large part of the diaphysis consisted of bone that was distally flanked by hypertrophic and proliferating cartilage (Fig. 5B).

In most of the infected wings harvested on day 10 of development, the cartilage elements appeared broader and shorter than in the noninfected controls (Fig. 5B) and lacked hypertrophic cartilage throughout the cartilage core (Fig. 5A). The effect on bone morphology was more severe by day 16. A continuous cartilage core was still present in the infected limbs, whereas uninfected bones were largely ossified (Fig. 5B). Depending on the extent of infection, the radius was generally less affected and often contained hypertrophic cartilage (Fig. 5A)

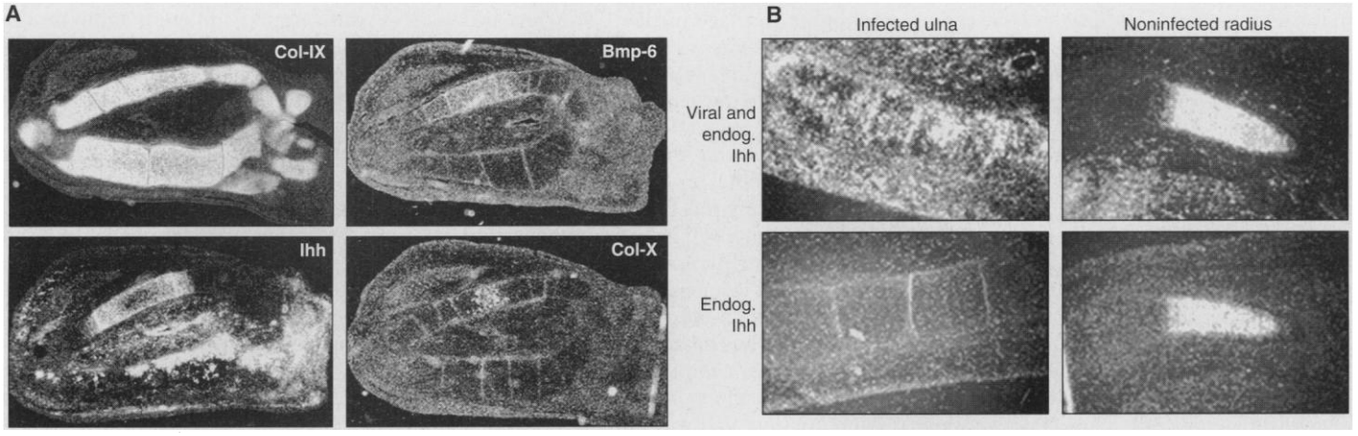

Fig. 6. *Ihh* regulates cartilage differentiation. (**A**) Markers of hypertrophic cartilage are suppressed by Ihh. The expression of the cartilage markers *Col-IX*, *Col-X*, and *Bmp-6* was analyzed in stage 34 chicken wings that had been infected with the *Ihh*-expressing retrovirus. Hybridization with an *Ihh* probe detects the viral transcript in the ulna and soft tissue and the endogenous *Ihh* expression domain in the noninfected radius. *Col-IX* is expressed in all cartilage cells, indicating that the infected cells are still viable. *Col-X*, a marker for hypertrophic cartilage, and *Bmp-6* are both expressed (as in noninjected control limbs) in the noninfected radius but not in the infected ulna. *Ihh* therefore represses the differentiation of hypertrophic cartilage. (**B**) *Ihh* misexpression prevents the expression of the endogenous *Ihh* gene. Serial sections of infected chicken wings of HH stage 30 were hybridized with an *Ihh* probe that detects both the endogenous and the viral gene transcripts and with an *Ihh* probe that is specific only for the endogenous *Ihh* transcript. The nonspecific probe detects a signal in the infected ulna and soft tissue and in the noninfected radius, whereas the endogenous *Ihh* transcript can be detected only in the radius and is absent from the infected ulna, indicating that misexpression of *Ihh* represses endogenous *Ihh* expression.

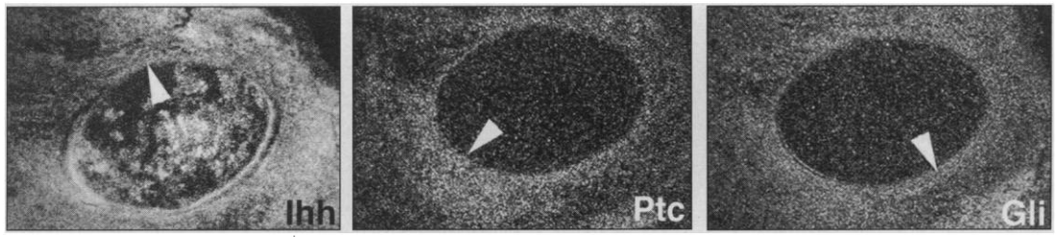

Fig. 7. Ptc and Gli are induced in response to Ihh misexpression. After Ihh infection, serial sections were prepared from a region of HH stage 34 wings where Ihh, Ptc, and Gli are normally not expressed. Hybridization with Ptc and Gli probes shows expression in the soft tissue and in the perichondrium of the ulna, but expression is excluded from the cartilage. In contrast, both soft tissue and cartilage express ectopic Ihh after infection. The perichondrium is demarcated by arrowheads.

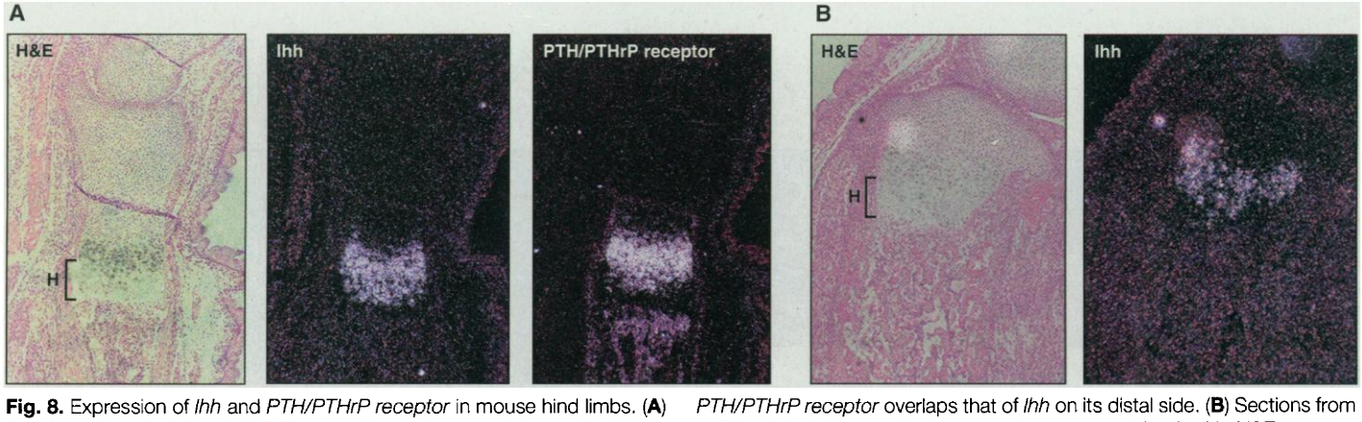

Fig. 8. Expression of *Ihh* and *PTH/PTHrP receptor* in mouse hind limbs. (**A**) Serial sections of wild-type E16.5 mouse hind limbs were stained with H&E to show cartilage histology or were hybridized with an *Ihh* or a *PTH/PTHrP receptor* probe. As in chicken limbs, the expression domain of the *PTH/PTHrP receptor* overlaps that of *Ihh* on its distal side. (**B**) Sections from a *PTHrP* (−/−) mutant mouse embryo were stained with H&E or were hybridized with an *Ihh* probe. Similar expression of *Ihh* can be observed in wild-type and mutant mouse limbs. H, hypertrophic cartilage.

and showed normal ossification (Fig. 5B).

Although on day 16 the cartilage was continuous along the length of most of the infected skeletal elements, an invasion of bone had begun. In contrast to normal development, however, this replacement was not initiated by the invasion of blood vessels at the center of the shaft which would result in the replacement of cartilage by bone tissue from the inside. Rather, the replacement occurred from the outside at multiple locations along the cartilage element, which appeared to be "eaten away" by the invading bone (Fig. 5C). The cartilage cells being replaced were morphologically quite large. However, in contrast to normal hypertrophic cartilage, these cells did not express either the specific hypertrophic marker *Col-X* or the pan-cartilage marker *Col-IX*, indicating that they may have been dead (*47*). At later stages the cartilage continued to be replaced by bone tissue, but the bones were abnormal in shape and did not have normal growth plates at the ends (*47*). Thus, misexpression of *Ihh* greatly disrupts the process of endochondral bone formation.

The role of *Ihh* in the regulation of cartilage differentiation. To identify the specific steps of bone development affected by *Ihh*, we analyzed serial sections for changes in the expression of molecular markers after *Ihh* misexpression. The effects of Ihh misexpression on cartilage differentiation were evident by HH stage 34. *Col-IX* was still expressed throughout the entire cartilage elements, indicating that the chondrocytes remained viable after Ihh infection. In contrast, the analysis of the hypertrophic cartilage marker *Col-X* revealed that, whereas *Col-X* was expressed in the noninfected radii, it was not expressed in highly infected ulnae and humeri (Fig. 6A). Histological analysis of the infected cartilage supported this interpretation. Hypertrophic chondrocytes could easily be identified in the central regions of uninfected cartilage elements. However, they were missing in highly infected cartilage elements, which throughout their length consisted of small chondrocytes, similar in appearance but less organized than those normally found in the proliferative zone (Fig. 5A). *Ihh*, therefore, appears to block the differentiation pathway from proliferating to *Col-X*–expressing, hypertrophic chondrocytes. In addition to repressing expression of the hypertrophic cartilage marker *Col-X*, we could not detect expression of *Bmp-6* in infected cartilage elements (Fig. 6A). Our observation that *Bmp-6* was expressed in both hypertrophic and prehypertrophic chondrocytes suggests that misexpressed Ihh might act before overt hypertrophic differentiation.

Because *Ihh* is expressed in cells undergoing differentiation, it could in principle act at either of two steps to repress the hypertrophic cell fate: Ihh could either block the terminal differentiation to the hypertrophic state, which would result in an accumulation of *Ihh*-expressing cells, or it could prevent proliferating cells from initiating the differentiation process, thus preventing the formation of the *Ihh*-expressing cell type itself. To distinguish between these possibilities, we analyzed the expression of the endogenous *Ihh* gene after viral misexpression. Hybridization with a probe specific for the endogenous *Ihh* transcript revealed that the expression of the gene was normal in noninfected radii. However, no expression was detected in infected cartilage elements, demonstrating that misexpression of *Ihh* suppressed the endogenous *Ihh* gene (Fig. 6B). Therefore, ectopic *Ihh* blocks chondrocyte differentiation before the transition to an *Ihh*-expressing state.

Although misexpression of *Ihh* has a profound effect on cartilage differentiation, the expression patterns of *Gli* and *Ptc* suggest that the perichondrium is the normal target of *Ihh* activity. To examine whether the morphological effects we observe in response to ectopic *Ihh* are also mediated by the perichondrium, we analyzed *Ptc* and *Gli* expression in infected limbs.

Both genes were strongly induced in the perichondrium as well as in muscle and soft tissue after Ihh infection (Fig. 7). However, their expression was clearly excluded from the cartilage, which was also infected (Fig. 7). Because there are no known examples in which a Hedgehog signal is mediated without the induction of *Ptc* and *Gli*, these misexpression studies imply that the effect of Ihh on the cartilage is indirect and mediated by the perichondrium.

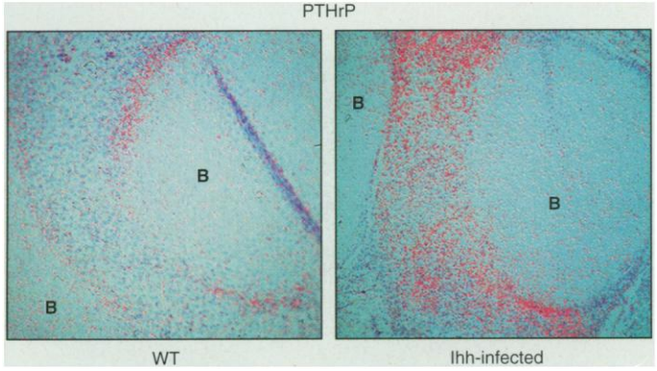

Fig. 9. *PTHrP* is induced in periarticular joint regions by Ihh. Serial sections of infected and noninfected control chicken wings of HH stage 30 were hybridized with *Ihh* and *PTHrP* probes. *PTHrP* is expressed in the perichondrium of the joint region of the developing cartilage elements and is strongly up-regulated in this area after infection of the wing buds with an *Ihh*-expressing virus. B, bone.

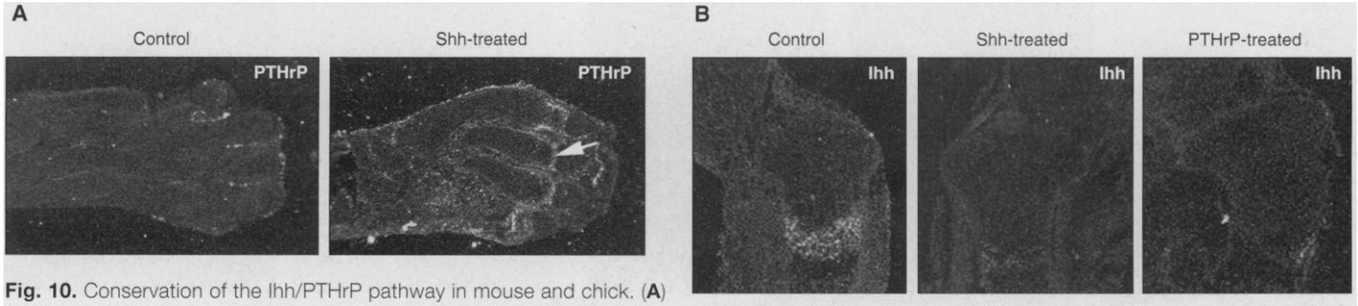

Fig. 10. Conservation of the Ihh/PTHrP pathway in mouse and chick. (**A**) Sections of wild-type mouse hind limb explants treated with control or Shh-containing medium were hybridized with the PTHrP probe. Whereas in the control limbs the PTHrP signal can barely be detected, an up-regulation of PTHrP expression (arrow) is seen in the periarticular perichondrium after Shh treatment. (**B**) Sections of wild-type mouse hind limbs treated with control or Shh- or PTHrP-containing medium were hybridized with an Ihh probe. Ihh expression is seen in the prehypertrophic chondrocytes of control limbs but is repressed by Shh and PTHrP.

Taken together, these results suggest that Ihh normally acts on the perichondrium to initiate a negative feedback loop that regulates the hypertrophic differentiation process. We propose that Ihh is produced by cells that become committed to the hypertrophic cell fate. The expression level of *Ihh* in this model may serve as a sensor within the skeletal element to regulate the number of cells committed to the process, where a certain threshold level of Ihh indirectly prevents additional cells from leaving the proliferative state. When *Ihh*-expressing cells undergo the final differentiation steps to become hypertrophic chondrocytes, they turn off the expression of *Ihh*, thereby attenuating the negative feedback loop and allowing more cells to commit to the differentiation pathway. By controlling the number of cells that differentiate at a given time, *Ihh* has a fundamental role in regulating the balance between growth and ossification of the developing bones.

PTHrP as a mediator of the Ihh signal. Our results suggest that the negative feedback loop initiated by *Ihh* is mediated by the perichondrium. *Parathyroid hormone–related protein* (*PTHrP*) (*53*, *54*) is expressed in the perichondrium of the developing cartilage elements of mouse and rat limbs, predominantly in the periarticular regions early in bone development (*55*, *56*), and appears to encode another signal that regulates the differentiation of cartilage. Targeted disruption of the *PTHrP* gene in mice has an effect on bone development opposite that produced by overexpression of *Ihh*: In PTHrP (−/−) mice the transition of chondrocytes from the proliferative to the hypertrophic phase is accelerated, which results in advanced, premature ossification (*7*, *8*). Although *PTHrP* itself is expressed at a considerable distance from the expression domain of *Ihh*, its receptor (*PTH/PTHrP receptor*) (*57*) is expressed in the prehypertrophic cartilage zone (as well as in osteoblasts) (*55*, *56*, *58*).

To investigate the possible interactions between *Ihh* and *PTH/PTHrP receptor*, we cloned a fragment of the chick *PTH/PTHrP receptor* (*59*) and compared its expression to that of *Ihh* in the prehypertrophic cartilage. In chicken, *PTH/PTHrP receptor* was strongly expressed in the osteoblastic precursors of the perichondrium from at least day 6, but no signal could be detected in the developing cartilage at this stage (*47*). However, at later stages of bone development (3 weeks after hatching), *PTH/PTHrP receptor* was expressed in the prehypertrophic cartilage in a domain distal to and slightly overlapping the *Ihh* expression domain (Fig. 3B), consistent with the pattern of rodent expression (*55*, *58*). In rodents, *PTH/PTHrP receptor* expression was detected at earlier stages of cartilage development (Fig. 8A), suggesting that in limbs of early stage chicken embryos its expression might be present but too weak to be detected. Once they are both detectable, the relative expression domains of *Ihh* and *PTH/PTHrP receptor* indicate that cells express *PTH/PTHrP receptor* before differentiating into an *Ihh*-expressing cell type. The differentiation block induced by Ihh appears to occur upstream of *Ihh* expression, which is precisely the putative target cell type for the Ihh feedback loop.

To test directly whether *PTHrP* is regulated by Ihh, we examined *PTHrP* expression in normal and *Ihh*-infected skeletal elements. As in rodents, *PTHrP* was normally expressed in the periarticular perichondrium adjacent to the developing chick cartilage elements. In *Ihh*-infected wings, increased *PTHrP* expression was de-

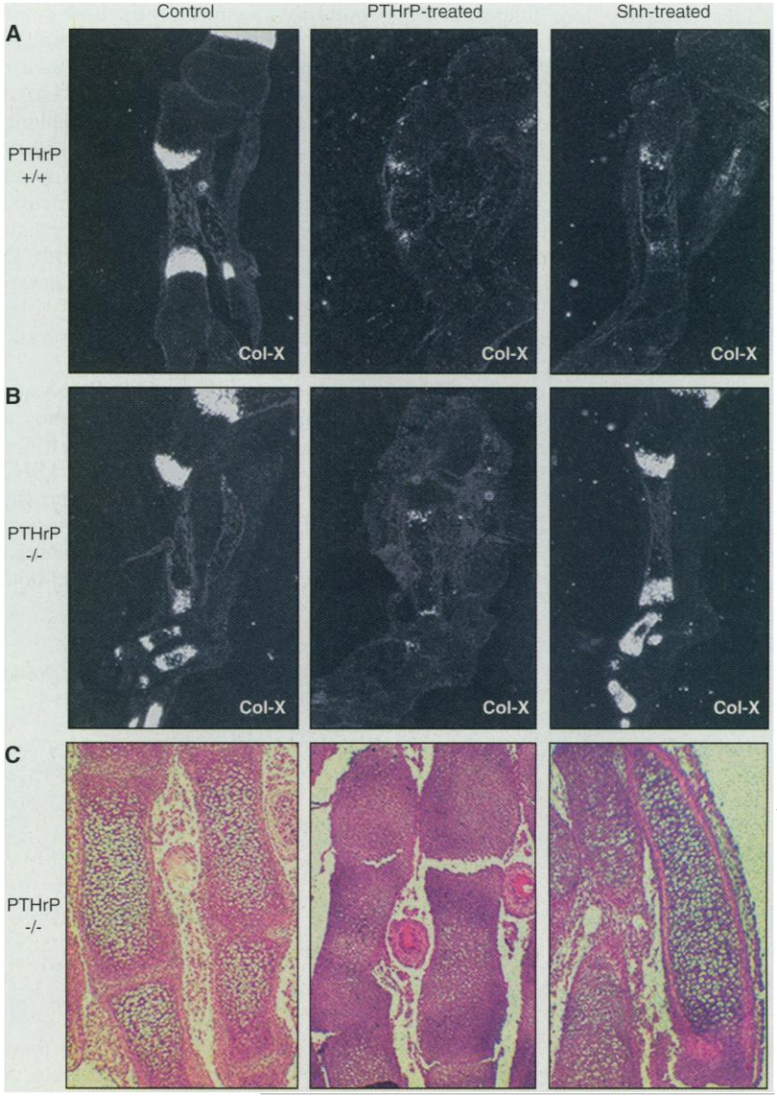

Fig. 11. *Shh* acts upstream of *PTHrP*. E16.5 mouse hind limbs from *PTHrP* (+/+) and *PTHrP* (−/−) mice were cultured for 4 days in control, PTHrP-, or Shh-containing medium. (**A**) *Col-X* expression is repressed after PTHrP and Shh treatment in *PTHrP* (+/+) hind limbs. In situ hybridization with a *Col-X* probe to sections of tibias from the control animals shows a repression of the hypertrophic cartilage marker *Col-X* after PTHrP or Shh treatment, relative to untreated limbs. (**B** and **C**) PTHrP rescues the *PTHrP* (−/−) phenotype, whereas Shh has no effect on cartilage. In *PTHrP* (−/−) animals the hypertrophy of the cartilage is advanced in all the bones including the tibia (B) and the digits (C), as shown by *Col-X* expression (B) and H&E staining (C). Treatment of the cultures with PTHrP not only rescues the wild-type phenotype but also induces the same repression of hypertrophic cartilage as observed in PTHrP treatment of normal limbs: *Col-X* expression is repressed (B), and morphologically no hypertrophic chondrocytes form during culture (C). In contrast to the rescue of the *PTHrP* (−/−) phenotype by PTHrP, Shh treatment does not change the phenotype of the cartilage elements; *Col-X* expression is still advanced (B), and H&E staining shows the premature hypertrophic cartilage (C).

tected throughout the periarticular perichondrium (Fig. 9). In contrast to the induction of *Gli* and *Ptc*, however, the Ihh-induced *PTHrP* expression was restricted to the perichondrium in the periarticular regions of infected limbs, indicating that either additional signals, required in concert with Ihh, are present only at the ends of the skeletal elements or that only a specific periarticular cell type is capable of expressing *PTHrP* in response to Ihh.

These results place *PTHrP* downstream of *Ihh* in regulating cartilage differentiation. To verify this epistatic relation, we compared the expression of Ihh in limbs of wild-type and *PTHrP* (−/−) mice. In wild-type limbs, *Ihh* was expressed overlapping with and medial to the *PTH/PTHrP receptor* (Fig. 8A), as in the chicken, and a similar level of *Ihh* expression was seen in *PTHrP* (−/−) limbs (Fig. 8B), excluding the possibility that *Ihh* expression is reciprocally regulated by *PTHrP*.

We conclude that both Ihh and PTHrP (on the basis of the murine mutant phenotype) repress hypertrophic cartilage differentiation, and that Ihh can induce *PTHrP*. This raises the question of whether the induction of PTHrP mediates the effect of Ihh. Assuming that the same signals regulate bone development in mice and in chicken, the existence of *PTHrP* (−/−) mutant mice provides the opportunity to definitively address this question. Because there is no convenient way to introduce ectopic Ihh into embryonic mouse limbs in utero, we cultured day 16.5 fetal mouse limb explants in vitro (60). Purified Ihh protein was not available for these studies. However, as shown above, Shh has the same biological activities as Ihh in early limb buds, activating the same target genes. Moreover, infection with a *Shh*-expressing retrovirus has the same effect as *Ihh* on chondrocyte differentiation and bone growth in chicken (47). We therefore used recombinant Shh protein in the mouse hind limb cultures. To verify that the PTHrP/Ihh feedback loop operates equivalently in the murine and chick systems, we examined PTHrP and Ihh expression in wild-type limb explants treated with control or Shh-containing medium. As in the chick, PTHrP was up-regulated and Ihh was repressed in response to Hedgehog protein in mouse limb explants (Fig. 10, A and B). Consistent with Ihh and PTHrP being in the same regulatory pathway, PTHrP treatment of limb explants also resulted in repression of Ihh expression (Fig. 10B).

To test whether PTHrP is required for cartilage to respond to Hedgehog protein, we placed explants of *PTHrP* (+/+) hind limbs [embryonic day 16.5 (E16.5)] in serum-free culture, treated them with control, PTHrP-containing, or Shh-containing media, and assessed the differentiation of hypertrophic cartilage. Morphologically (47) and as judged by *Col-X* expression (Fig. 11A), both PTHrP and Shh repressed hypertrophic differentiation in wild-type limbs in vitro, consistent with their effects in vivo. Explants of E16.5 *PTHrP* (−/−) mutant hind limbs placed in similar culture conditions showed extensive premature hypertrophy in vitro (Fig. 11, B and C), as they did in vivo. For example, hypertrophic changes in the cartilage of the feet progressed over the culture period and became completely hypertrophic after 4 days in culture (Fig. 11C). As expected, this premature hypertrophy could be prevented by addition of PTHrP to the media, which, moreover, blocked even normal hypertrophic differentiation, as it did after addition to wild-type limbs (Fig. 11, B and C). In contrast, Shh, which also blocked hypertrophy in wild-type limbs, had no effect on *PTHrP* (−/−) limbs. Shh-treated mutant explants were indistinguishable, both morphologically (Fig. 11C) and in their pattern of *Col-X* expression (Fig. 11B), from untreated mutant limbs. Because ectopic Hedgehog can induce *PTHrP* expression both in chick limbs and in mouse limb explants, and because Shh has no observable effect on *PTHrP* (−/−) mutant explants, we conclude that the effect of Hedgehog protein on cartilage differentiation is mediated by the PTHrP pathway. This further substantiates our inference that the perichondrium and not the cartilage is the direct target of Ihh activity and confirms that Ihh acts via the induction of *PTHrP*. In the feedback loop, PTHrP must act on cells before their activation of *Ihh*, because this regulation loop prevents *Ihh* expression. Consistent with this model, we have shown that one receptor of PTHrP, the *PTH/PTHrP receptor*, is specifically expressed in cells just distal to those expressing *Ihh*. In an accompanying report (58), we provide functional data demonstrating that not only is the PTH/PTHrP receptor in the right place, but it is absolutely necessary for Ihh and PTHrP to affect chondrocyte differentiation in the mouse. Because the PTH/PTHrP receptor is expressed at a considerable distance from PTHrP itself, this ligand must act across multiple cell diameters.

We therefore extend our understanding of the feedback regulation of chondrocyte differentiation as follows (Fig. 12): As proliferating chondrocytes decide to undergo hypertrophy, they express high levels of the *PTH/PTHrP receptor*. When they subsequently become committed to this pathway, they transiently express *Ihh*, until they become fully hypertrophic. The Ihh signal acts on the perichondrium adjacent to the prehypertrophic zone (*Ptc/Gli*-expressing cells) and (directly or indirectly) on the more distant periarticular perichondrium,

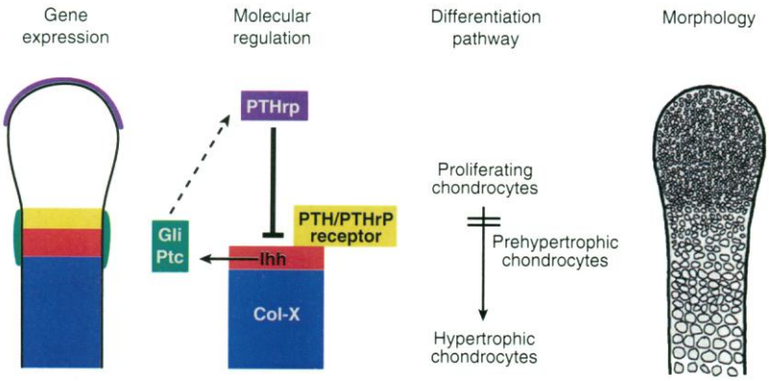

Fig. 12. Molecular regulation of the rate of cartilage differentiation. During cartilage development chondrocytes differentiate from proliferating chondrocytes (white) through an intermediate, prehypertrophic cell type to hypertrophic chondrocytes (expressing Col-X, blue). With the initiation of this differentiation pathway, the prehypertrophic chondrocytes express *PTH/PTHrP receptor* (yellow) and *Ihh* (red). *PTH/PTHrP receptor* is expressed in cells before the onset of Ihh expression. For clarity, the expression domains of the two genes (yellow and red) are shown as distinct regions. However, they actually overlap, both in chick and mouse. The target of the secreted Ihh signal is the perichondrium, which responds by expressing *Gli* and *Ptc* (green). This Ihh response in the perichondrium directly or indirectly results in the expression of *PTHrP* in the periarticular perichondrium (purple). PTHrP then signals back to the *PTH/PTHrP receptor* in the prehypertrophic cells, preventing additional chondrocytes from moving down the differentiation pathway and from expressing *Ihh* (73). When the prehypertrophic chondrocytes have fully differentiated into hypertrophic chondrocytes, they turn off Ihh expression. This reduction of the Ihh level attenuates the negative feedback loop and allows new cells to initiate the differentiation pathway. The Ihh level produced by the prehypertrophic chondrocytes, therefore, reflects the number of cells committed to the hypertrophic pathway, providing a mechanism for regulating the rate of hypertrophic differentiation.

ultimately inducing the expression of *PTHrP*. PTHrP then signals back to chondrocytes expressing the *PTH/PTHrP receptor*, thereby preventing nondifferentiated chondrocytes from moving down the hypertrophic pathway.

Ihh and PTHrP function in a common feedback loop that regulates the rate of chondrocyte differentiation and thereby balances the growth and ossification of long bones. The elucidation of this feedback loop provides insight into the molecular mechanisms regulating the formation of long bones and forms a basis for further analysis of these processes.

REFERENCES AND NOTES

1. J. R. Hinchliffe and D. R. Johnson, in *The Development of the Vertebrate Limb* (Oxford Univ. Press, New York, 1980), pp. 76–83.
2. A. Erlebacher, E. H. Filvaroff, S. E. Gitelman, R. Derynck, *Cell* **80**, 371 (1995).
3. F. Rousseau *et al.*, *Nature* **371**, 252 (1994).
4. R. Shiang *et al.*, *Cell* **78**, 335 (1994).
5. C. Deng, A. Wynshaw-Boris, F. Zhou, A. Kuo, P. Leder, *ibid.* **84**, 911 (1996).
6. J. Baker, J. P. Liu, E. J. Robertson, A. Efstratiadis, *ibid.* **75**, 73 (1993).
7. A. C. Karaplis *et al.*, *Genes Dev.* **8**, 277 (1994).
8. N. Amizuka, H. Warshawsky, J. E. Henderson, D. Goltzman, A. C. Karaplis, *J. Cell Biol.* **126**, 1611 (1994).
9. M. J. Bitgood and A. P. McMahon, *Dev. Biol.* **172**, 126 (1995).
10. C. Nüsslein-Volhard and E. Wieschaus, *Nature* **287**, 795 (1980).
11. J. J. Lee, K. D. Von, S. Parks, P. A. Beachy, *Cell* **71**, 33 (1992).
12. J. Mohler and K. Vani, *Development* **115**, 957 (1992).
13. R. D. Riddle, R. L. Johnson, E. Laufer, C. Tabin, *Cell* **75**, 1401 (1993).
14. Y. Echelard *et al.*, *ibid.*, p. 1417.
15. S. Krauss, J.-P. Concordet, P. W. Ingham, *ibid.*, p. 1431.
16. M. Levin, R. L. Johnson, C. D. Stern, M. Kuehn, C. Tabin, *ibid.* **82**, 803 (1995).
17. H. Roelink *et al.*, *ibid.* **76**, 761 (1994).
18. R. L. Johnson, E. Laufer, R. D. Riddle, C. Tabin, *ibid.* **79**, 1165 (1994).
19. C. M. Fan and M. Tessier-Lavigne, *ibid.*, p. 1175.
20. D. J. Roberts *et al.*, *Development* **121**, 3163 (1995).
21. M. Bitgood, L. Shen, A. McMahon, *Curr. Biol.* **6**, 298 (1996).
22. J. E. Hooper and M. P. Scott, *Cell* **59**, 751 (1989).
23. Y. Nakano *et al.*, *Nature* **341**, 508 (1989).
24. T. Orenic, D. C. Slusarski, K. L. Kroll, R. A. Holmgren, *Genes Dev.* **124**, 50 (1990).
25. P. W. Ingham, A. M. Taylor, Y. Nakano, *Nature* **353**, 184 (1991).
26. J. Capdevila, M. P. Estrada, E. Sánchez-Herrero, I. Guerrero, *EMBO J.* **13**, 71 (1994).
27. A. J. Forbes, Y. Nakano, A. M. Taylor, P. W. Ingham, *Dev. Suppl.* 115 (1993).
28. V. Marigo, M. P. Scott, R. L. Johnson, L. V. Goodrich, C. J. Tabin, *Development* **122**, 1225 (1996).
29. L. V. Goodrich, R. L. Johnson, L. Milenkovic, J. A. McMahon, M. P. Scott, *Genes Dev.* **10**, 301 (1996).
30. J. M. Ruppert *et al.*, *Mol. Cell. Biol.* **8**, 3104 (1988).
31. V. Marigo and C. J. Tabin, unpublished results.
32. _____, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
33. K. Basler and G. Struhl, *Nature* **368**, 208 (1994).
34. J. Capdevila and I. Guerrero, *EMBO J.* **13**, 4459 (1994).
35. R. Tabata and T. Kornberg, *Cell* **76**, 89 (1994).
36. E. Laufer, C. E. Nelson, R. L. Johnson, B. A. Morgan, C. J. Tabin, *ibid.* **79**, 993 (1994).
37. Cloning of the chicken Ihh cDNA: Unless otherwise noted, all molecular procedures followed published protocols (*61*). A random primed cDNA library from embryonic day 7 chicken limbs was constructed in the λ ZapII vector (Stratagene) with the use of Eco RI–Not I linkers. Clones (1×10^6) of the unamplified library were plated and transferred to duplicate sets of nylon filters (Colony Plaque Screen, New England Nuclear). A genomic *Ihh* DNA probe, pIhhex3, homologous to base pairs (bp) 908 to 1180 of the *Shh* cDNA and containing 400 bp of 3′ untranslated sequence, and the *Shh* cDNA probe pHH2 (*13*), were mixed and used as a hybridization probe. Hybridization was carried out in 0.5 M phosphate buffer, 7% SDS, 1 mM EDTA, and herring sperm DNA (100 μg/ml) at 65°C overnight. Filters were washed in 50 mM phosphate buffer, with 1% SDS at 65°C as the final stringency (*62*), and exposed to Kodak XAR-5 films for 16 to 72 hours. After two rounds of subcloning, three positive clones were identified that hybridized strongly to pIhhex3 and less strongly to pHH2. Nucleotide sequences of the three clones were determined with Sequenase 2.0 (US Biochemicals) and sequence-specific oligonucleotides as sequencing primers. DNA and amino acid sequences were analyzed with Genetics computer Group (Devreux) and DNA star software. A cDNA contig was assembled, consisting of 1958 bp with an open reading frame encoding a protein of 408 amino acids, flanked by 0.3 kb of untranslated cDNA on the 5′ side and 0.4 kb on the 3′ side. Comparison of the predicted amino acid sequence with the previously cloned vertebrate Hedgehog proteins showed highest conservation with the predicted mouse (73%) and human (75%) Ihh proteins and less but substantial conservation with the other Hedgehog proteins (55 to 61%), indicating that the isolated cDNA represents the chicken *Ihh* gene (GenBank accession number U58511).
38. J. J. Lee *et al.*, *Science* **266**, 1528 (1994).
39. J. A. Porter *et al.*, *Nature* **374**, 363 (1995).
40. D. A. Bumcrot, R. Takada, A. P. McMahon, *Mol. Cell. Biol.* **15**, 2294 (1995).
41. C. M. Fan *et al.*, *Cell* **81**, 457 (1995).
42. E. Marti, D. A. Bumcrot, R. Takada, A. P. McMahon, *Nature* **375**, 322 (1995).
43. H. Roelink *et al.*, *Cell* **81**, 445 (1995).
44. P. Currie and P. Ingham, *Nature*, in press.
45. V. Hamburger and H. L. Hamilton, *J. Exp. Morphol.* **88**, 49 (1951).
46. Misexpression of *Ihh*: The complete *Ihh* coding region and part of the 3′ untranslated cDNA [nucleotides (nt) 328 to 1691] were cloned into replication-competent retroviral vectors (RCAS) with various host ranges with the Slacks13 shuttle vector (*13*, *63–65*). *Ihh*-expressing RCAS(E)-type virus, which has a limited host range, was used to infect susceptible chicken primary fibroblast, prepared from chick line 15b. The *Ihh*-expressing cells were pelleted and transplanted into the anterior limb mesenchyme of a virally resistant outbred host strain, which resulted in a localized expression of *Ihh* and prevented the spread of the virus into the surrounding tissue. All other misexpression experiments were carried out with *Ihh*-expressing RCAS(A)-type viruses, which directly infected the outbred chicken host strain and spread throughout the surrounding tissue after infection (*13*), resulting in high *Ihh* expression in the infected limb tissue. Outbred specific pathogen-free eggs were obtained from SPAFAS (Norwich, CT).
47. A. Vortkamp and C. J. Tabin, unpublished results.
48. In situ hybridization: Whole-mount in situ hybridization was done as described (*13*), except that embryos older than stage 25 were treated with fourfold higher concentration of proteinase K than in the original protocol. In situ hybridization was done with ^{35}S-uridine 5′-triphosphate (UTP) or ^{33}P-UTP–labeled probes on 6-μm sections as described (*66*). Hybridization was done at 52° to 70°C in 50% formamid, and posthybridization washes were carried out at a final stringency of 55°C in 50% formamid and 2× SSC (sodium chloride/sodium citrate). For serial sections, three continuous sections were successively collected on six alternating slides. Probes used for hybridizations were as follows: *Ihh*: plhh-R1-4sac contains nt 1 to 557 of the cloned *Ihh* sequence and corresponds to the 5′ untranslated region and to exon 1. The probe detects the endogenous and the viral Ihh transcript. Endogenous *Ihh*: plhh-R1-4AE contains nt 1 to 276 of the *Ihh* cDNA, which correspond to the 5′ untranslated Ihh message and are not included in the viral construct. This probe detects only the endogenous Ihh transcript. Human *Ihh* (*67*), *Gli* (*31*), *Shh* (*13*), *Hoxd-11*, *Hoxd-13*, and *Bmp2* (*36*), *Ptc* (*28*), *Col-X* (*68*), *Col-IX* (*69*), *Bmp6* (B. Houston), *PTHrP* (*70*), *PTH/PTHrP receptor* (*59*), and rat *PTH/PTHrP receptor* (*71*).
49. T. F. Linsenmayer *et al.*, *Development* **111**, 191 (1991).
50. D. M. Kingsley, *Trends Genet.* **10**, 16 (1994).
51. BMP expression: Because members of the *Bmps/dpp* family are often induced by Hedgehog proteins, we also looked at the expression of *Bmp-2*, *Bmp-4*, *Bmp-5*, and *Bmp-7*. All were expressed in the perichondrium as described (*50*); however, none of their expression domains correlated with the domain of *Ihh* expression.
52. Weigert-Safranin staining: 16-μm paraffin sections of chicken wings were fixed in Bouins fixative and stained with a series of Weigert hematoxylin, fast green, and safranin O.
53. L. J. Suva *et al.*, *Science* **237**, 893 (1987).
54. A. Broadus and A. Stewart, in *The Parathyroids*, J. Bilezikiaan, M. Levine, R. Marcus, Eds. (Raven, New York, 1994), pp. 259–294.
55. K. Lee, J. D. Deeds, G. V. Segre, *Endocrinology* **136**, 453 (1995).
56. K. Lee *et al.*, in preparation.
57. H. Juppner *et al.*, *Science* **254**, 1024 (1991).
58. B. Lanske *et al.*, *ibid.* **273**, 663 (1996).
59. *PTH/PTHrP receptor* probe: pRP-R1/4, corresponding to amino acids 219 to 454 of *PTH/PTHrP receptor*, was amplified from reverse-transcribed RNA of day 8 chicken limbs and subcloned into pBluescript SK-. Degenerate primers [GACGGATCC(CA)GIAA (CT)TA(CT)AT(CTA)CA(CT)ATGCA and GACGAATTC(TC)TC(AG)TA(AG)TGCAT(TC)TGIAC(TC)TGCCA] were based on the human, mouse, rat, pig, opossum, and *Xenopus* cDNA sequences. Polymerase chain reaction (PCR) conditions: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C, followed by a final extension of 5 min at 72°C. Sequence analysis revealed that the resulting probe pRP-R1/4 shows highest homology to the opossum *PTH/PTHrP receptor* cDNA.
60. Mouse hind limb cultures: Hind limbs of E16.5 fetuses were severed at mid-femur and stripped of skin. They were then placed on a filter paper (pore size, 0.8 μm) on a wire mesh in a Falcon organ tissue culture dish, and 1 ml of BGJ_b (Gibco BRL) medium was added. The hind limbs, which lay at the air-fluid interface, were cultured at 37°C in a humidified atmosphere of 95% air–5% CO_2 with daily changes of medium. The hind limbs were treated with either 10 to 7 M human PTHrP (1–34), recombinant mouse Shh (5 μg/ml), or vehicle (BGJ_b medium containing 0.1% bovine serum albumin) alone from day 2 for 4 days. The samples were never exposed to serum. At the termination of culture, the hind limbs were fixed by 10% formalin in phosphate-buffered saline.
61. F. M. Ausubel *et al.*, *Current Protocols in Molecular Biology* (Greene and Wiley Interscience, New York, 1989).
62. G. M. Church and W. Gilbert, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1991 (1984).
63. D. M. Fekete and C. L. Cepko, *Mol. Cell. Biol.* **13**, 2604 (1993).
64. _____, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 2350 (1993).
65. S. H. Hughes, J. J. Greenhouse, C. J. Petropoulos, P. Sutrave, *J. Virol.* **61**, 3004 (1987).
66. L. Tessarollo, L. Nagarajan, L. F. Parada, *Development* **115**, 11 (1992).
67. V. Marigo *et al.*, *Genomics* **28**, 44 (1995).
68. P. LuValle, Y. Ninomiya, N. D. Rosenblum, B. R. Olsen, *J. Biol. Chem.* **263**, 18378 (1988).
69. I. Nishimura, Y. Muragaki, B. R. Olsen, *ibid.* **264**, 20033 (1989).
70. M. A. Thiede and S. J. Rutledge, *Nucleic Acids Res.* **18**, 3062 (1990).
71. A. B. Abou-Samra *et al.*, *Adv. Nephrol. Necker Hosp.* **23**, 247 (1994).

72. A. Vortkamp *et al.*, data not shown.
73. Our model suggests that PTHrP represses chondrocyte differentiation, acting on prehypertrophic cells just before the onset of Ihh expression. However, the inhibitory PTHrP signal may also act, to some extent, on the proliferating chondrocytes before the prehypertrophic stage. In the mouse, both antibodies to the receptor and PTH-binding studies suggest that a very low level of PTH/PTHrP receptor may be present on the surface of proliferating cartilage cells even though the levels of PTH/PTHrP receptor mRNA are too low to be detected (*72*).
74. We thank R. L. Johnson for the genomic Ihh probes and sequence information; V. Marigo for the probes for Gli and Ptc; B. R. Olsen for the Col-IX and Col-X probes; B. Houston for the Bmp-6 probes; and T. Woolf, L. Wang, and Ontogeny for providing Sonic hedgehog protein, prepared from an unpublished clone provided by H. Roelink. We are grateful to V. Marigo, J. Capdevilla, E. Laufer, and M. Belliveau for critical discussion of the manuscript and to the members of the Tabin lab for technical advice. A.V. was supported by a fellowship of the Human Frontiers Science Program (HFSP) (LT246/94). Supported by a grant from the HFSP (to C.J.T.) and by National Institutes of Health grants DK47038 (to H.M.K.) and DK4723 to (G.V.S.).

10 April 1996; accepted 20 June 1996

Support for the Prion Hypothesis for Inheritance of a Phenotypic Trait in Yeast

Maria M. Patino, Jia-Jia Liu, John R. Glover, Susan Lindquist*

A cytoplasmically inherited genetic element in yeast, [*PSI*$^+$], was confirmed to be a prionlike aggregate of the cellular protein Sup35 by differential centrifugation analysis and microscopic localization of a Sup35–green fluorescent protein fusion. Aggregation depended on the intracellular concentration and functional state of the chaperone protein Hsp104 in the same manner as did [*PSI*$^+$] inheritance. The amino-terminal and carboxy-terminal domains of Sup35 contributed to the unusual behavior of [*PSI*$^+$]. [*PSI*$^+$] altered the conformational state of newly synthesized prion proteins, inducing them to aggregate as well, thus fulfilling a major tenet of the prion hypothesis.

Mammalian prions cause devastating neurodegenerative disorders (*1*). Unlike conventional pathogens, they are thought to consist entirely of protein—specifically, a normal nuclear-encoded protein, PrP^C, with an altered "scrapie" conformation, PrP^{Sc} (*1*). The key to prion pathology is thought to be the ability of PrP^{Sc} to induce new PrP^C molecules to adopt the altered structure, producing a protein-conformation cascade that causes the disease and gives rise to new infectious PrP^{Sc}.

A similar explanation can account for the otherwise baffling behavior of two genetic factors in yeast, [*PSI*$^+$] and [*URE3*] (*2*). The [*PSI*$^+$] factor increases translational read-through of all three nonsense codons, and is monitored in the laboratory by omnipotent suppression of nonsense mutations (*3*). Although unlinked to any known nucleic acid, [*PSI*$^+$] behaves as a dominant, cytoplasmically inherited genetic element. It bears an unusual relation to the nuclear-encoded protein Sup35 that is reminiscent of the relation between mammalian prions and nuclear-encoded PrP^C (*1–4*).

Normally, Sup35 is a subunit of the translation-release factor that causes ribosomes to terminate translation at nonsense codons. Release activity maps to the COOH-terminal domain (*5*), which is essential for growth (*6*). Sup35's NH_2-terminal domain is not essential and is required only for the propagation of [*PSI*$^+$] (*6*). Mutations in Sup35 can also cause omnipotent nonsense suppression, but unlike [*PSI*$^+$], the mutant phenotypes exhibit Mendelian inheritance (*3*). Remarkably, transient overexpression of Sup35, or just its NH_2-terminal domain, can induce de novo heritable [*PSI*$^+$] elements (*2*, *6*). Moreover, transient overexpression of the chaperone Hsp104 can restore translational fidelity, heritably converting cells from [*PSI*$^+$] to [*psi*$^-$] (*4*).

These observations argue that [*PSI*$^+$] represents the inheritance of a self-perpetuating alteration in the conformation of Sup35, which is initiated by the NH_2-terminal domain and impairs the ability of the COOH-terminal domain to function in translation. Although this mechanism successfully explains many perplexing genetic observations (*2*, *3*), such a revolutionary model for the inheritance of a phenotypic trait demands the support of direct physical evidence, which we provide here.

Insolubility of Sup35 in [*PSI*$^+$] cells. Isogenic [*psi*$^-$] and [*PSI*$^+$] strains of two different genetic backgrounds (*7*) contained the same quantity of Sup35 and Sup45 (Fig. 1A), the other subunit of the translation-release factor (*8*). Thus, the read-through of nonsense codons in [*PSI*$^+$] cells was not due to reduced accumulation of the termination-factor subunits. Nor was it likely due to posttranslational modification. The migration of the Sup35 and Sup45 proteins from [*PSI*$^+$] cells on high resolution two-dimensional gels was identical to that of the proteins from [*psi*$^-$] cells (*9*).

In contrast, the solubility of Sup35 was very different in [*PSI*$^+$] and [*psi*$^-$] cells. Most Sup35 protein in [*PSI*$^+$] lysates pelleted after centrifugation at 12,000*g*; most remained in the supernatant of [*psi*$^-$] lysates. In [*psi*$^-$] lysates, a substantial fraction of Sup35 remained soluble after centrifugation at 100,000*g*; none remained soluble in [*PSI*$^+$] lysates (Fig. 1B). Similar differences in the solubility of Sup35 in [*PSI*$^+$] and [*psi*$^-$] cells were obtained in the early, mid-, and late log phases of growth as well as in cells in the stationary phase (Fig. 1C) (*10*). No difference in the sedimentation properties of total proteins was detected by Coomassie blue staining, nor did immunostaining show any difference in the sedimentation of Sup45, ribosomal protein L3, or the chaperone proteins Hsp70, Hsp90, and Hsp104 (Fig. 1, B and C) (*10*). High salt (1 M KCl), EDTA (50 mM), and ribonuclease A (400 μg/ml) treatments did not reduce the quantity of Sup35 found in the pellet of [*PSI*$^+$] cells, nor did treatments with nonionic detergent (1% Triton X-100) (*10*). Moreover, like PrP^{Sc} (*1*), the Sup35 protein found in these aggregates was resistant to proteolysis (*11*).

Role of the chaperone Hsp104 in Sup35 aggregation. Overexpression of Hsp104, a protein that promotes the resolubilization and reactivation of heat-damaged proteins (*12*), converts cells from [*PSI*$^+$] to [*psi*$^-$] (*6*). If aggregates of Sup35 reflect the presence of [*PSI*$^+$], Sup35 should return to the soluble state after this conversion. When cells were transformed with a centromeric vector expressing Hsp104 from its own promoter, this was indeed the case (Fig. 1D). [In this and all experiments reported here, the [*PSI*$^+$] and [*psi*$^-$] states were confirmed by plating assays on selective media (Fig. 2) (*13*).] A stronger test of the relation between Sup35 aggregates and [*PSI*$^+$] derives from the ability of transient Hsp104 overexpression to heritably cure

The authors are at the Howard Hughes Medical Institute and the Department of Molecular Genetics and Cell Biology, University of Chicago, 5841 South Maryland Avenue, Chicago, IL 60637, USA.

*To whom correspondence should be addressed.