Combinatorial signaling through BMP receptor IB and GDF5: shaping of the distal mouse limb and the genetics of distal limb diversity

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SUMMARY
In this study, we use a mouse insertional mutant to delineate gene activities that shape the distal limb skeleton. A recessive mutation that results in brachydactyly was found in a lineage of transgenic mice. Sequences flanking the transgene insertion site were cloned, mapped to chromosome 3, and used to identify the brachydactyly gene as the type IB bone morphogenetic protein receptor, BmprIB (ALK6). Expression analyses in wild-type mice revealed two major classes of BmprIB transcripts. Rather than representing unique coding RNAs generated by alternative splicing of a single pro-mRNA transcribed from one promoter, the distinct isoforms reflect evolution of two BmprIB promoters: one located distally, driving expression in the developing limb skeleton, and one situated proximally, initiating transcription in neural epithelium. The distal promoter is deleted in the insertional mutant, resulting in a regulatory allele (BmprIB\textsuperscript{Tg}) lacking cis-sequences necessary for limb BmprIB expression. Mutants fail to generate digit cartilage, indicating that BMPRIB is the physiologic transducer for the formation of digit cartilage from the skeletal blastema. Expansion of BmprIB expression into the limb through acquisition of these distal cis-regulatory sequences appears, therefore, to be an important genetic component driving morphological diversity in distal extremities. GDF5 is a BMP-related signal, which is also required for proper digit formation. Analyses incorporating both Gdf5 and BmprIB\textsuperscript{Tg} alleles revealed that BMPRIB regulates chondrogenesis and segmentation through both GDF5-dependent and -independent processes, and that, reciprocally, GDF5 acts through both IB and other type I receptors. Together, these findings provide in vivo support for the concept of combinatorial BMP signaling, in which distinct outcomes result both from a single receptor being triggered by different ligands and from a single ligand binding to different receptors.

Key words: Signaling, BMP receptor, GDF5, Apoptosis, Skeletal morphogenesis, Digit formation, Mouse

INTRODUCTION
The cartilage template through which the limb skeleton forms, results from the iteration of three basic processes: condensation of chondrogenic cells to form a focus, bifurcation of the focus to generate Y-shaped elements and axial segmentation to produce subelements. This program begins with a single de novo mesenchymal condensation – the anlagen of the humerus (or femur in the hindlimb) – and proceeds in a proximodistal direction. First, the condensation grows distally by appropriating nearby mesenchymal cells into the focus. The distal end then branches and segments to form the ulna and radius (tibia and fibula); these, in turn, elongate, branch and segment to form the proximal carpals (tarsals). The axis of development now switches from proximodistal to posteroanterior with the ulna giving rise to the perpendicularly oriented axis of the digital arch, comprising distal carpals and metacarpals (distal tarsals and metatarsals). Each metacarpal (metatarsal), also referred to as a digital ray, then segments axially to form the phalanges. Most elements of the limb are, therefore, thought to arise by ordered branching and segmentation of preexisting chondrogenic elements (Oster et al., 1988; Shubin and Alberch, 1986).

The de novo condensation within the early limb bud results from interactions between mesenchyme and ectoderm and involves a number of secreted molecules, including FGFs (Martin, 1998), Wnts (Kengaku et al., 1998; Parr et al., 1993; Tickle, 1995) and BMPs (Hogan, 1996; Kingsley, 1994). Surrounding mesenchyme is then recruited into the chondrogenic condensation in what appears to be an autocatalytic process – as the density of chondrogenic cells increases, further aggregation is enhanced, which in turn increases recruitment of chondrogenic cells (Shubin and Alberch, 1986). Aggregation favors the distal end of the element where the adjacent progress zone provides proliferating mesenchyme. In contrast, proximal and lateral recruitment is restricted by a more limited supply of mesenchyme, and by differentiation of the outerzone of the condensation into a tangentially stacked cell layer, the perichondrium, which separates the cartilage element from its
organized in both space and time. As the condensation elongates, it eventually reaches a size and stage where the more proximal region differentiates, while the distal domain continues to recruit chondrogenic cells. By this stage, the cartilage element is polarized, with the immature distal end acting as a stronger focus for condensation than the more differentiated proximal end. Together with tightly controlled cell death, the outcome is segmentation of the element between the more and less differentiated domains into two subelements; these subelements will go on to differentiate and articulate with each other in a synovial joint. Cartilage condensation and segmentation are, therefore, highly coordinated processes acting as a stronger focus for condensation than the more differentiated proximal end. By this stage, the cartilage element is polarized, with the immature distal end acting as a stronger focus for condensation than the more differentiated proximal end. Together with tightly controlled cell death, the outcome is segmentation of the element between the more and less differentiated domains into two subelements; these subelements will go on to differentiate and articulate with each other in a synovial joint. Cartilage condensation and segmentation are, therefore, highly coordinated processes organized in both space and time.

Secre ted signaling molecules of the TGFβ family, such as bone morphogenetic proteins (BMPs) and growth/ differentiation factors (GDFs), can affect the outcome of these linked processes of chondrogenesis and cleavage; the result being alterations in both bone shape and number (Hogan, 1996; Kingsley, 1994). BMPs were first discovered as proteins that induce ectopic endochondral bone formation (Hogan, 1996 and references therein). Compatible with such activity, BMP2-BMP7 were found to be expressed in discrete regions of the developing skeleton. GDF5-GDF7 were identified by their homology to BMPs and also show characteristic patterns of expression in the developing skeleton (Storm et al., 1994). BMPs and GDFs appear to bind similar receptors. These include various type I and type II transmembrane serine-threonine kinases (Yamashita et al., 1996). Type I receptors (TSR1= ALK1; ACTRI= ALK2; BMPRIA= ALK3, BRK1; BMPRIIB= ALK6, BRKII and RPKI) transmit the BMP or GDF signal to intracellular phosphorylation cascades involving members of the Smad family (Hoodless et al., 1996; Liu et al., 1996; Massague, 1996). Type II receptors (BMPRIIB= BRK3) act to facilitate both ligand binding and signal transmission through type I receptors. Recent data indicates that ligand binding can induce the formation of heterotetrameric complexes consisting of two type I receptors and two type II receptors (Yamashita et al., 1994). Thus, the repertoire of potential ligand-receptor interactions is considerable.

BMPRIA and BMPRIIB are of particular interest for understanding the regulation of skeletogenesis. BmprIA mRNA can be detected in the limb progress zone, interdigital mesenchyme, periarticular perichondrium and differentiated hypertrophic chondrocytes (Zou et al., 1997). From misexpression studies, two different functions have been proposed for BMPRIA: regulation of chondrocyte differentiation (Zou et al., 1997) and signaling interdigital cell death (Yokouchi et al., 1996). In contrast to IA, BmprIB is expressed in the earliest chondrogenic condensations (Kawakami et al., 1996; Zou et al., 1997) and has been hypothesized to control mesenchyme aggregation and cartilage formation (Enomoto-Iwamoto et al., 1998; Kawakami et al., 1996; Merino et al., 1998; Zou et al., 1997). Although not normally expressed in interdigital mesenchyme, misexpression of a constitutively active IB receptor (caBMPRIB) in this region has also been shown capable of inducing cell death (Zou et al., 1996, 1997). While the misexpression experiments have been informative in defining a range of activities these receptors possess, the physiological functions for the IA and IB receptors remain to be defined.

Here we report a mouse insertional mutant in which cartilage condensation and segmentation of the distal limb are disrupted, thereby providing a unique opportunity to define gene activities critical to these processes. Through positional cloning, we show that this insertion mutation is a regulatory allele of the BMP receptor IB gene (BmprIBbp-J) and that this allele is null with respect to the limb. Our results show that BMPRIB is the physiological transducer mediating the development of digit cartilages and that this differentiation program is initiated through the binding of GDF5. While digit chondrogenesis appears to require this particular receptor-ligand pair, we show

Fig. 1. Transgene insertion uncovers brachydactyly locus. Adult forelimbs stained with Alizarin red. (A-C) Wild-type (WT) forefoot; (D,E) insertional mutant (Tg/Tg); (F,G) brachypod (Gdf5bp-J/bp-J). (A-E) Ventral forefoot. Insertional mutant comprises normal metacarpals but is missing the basal (P1) and medial (P2) phalanges. In the WT forefoot (A), sesamoid (S) bones can be seen at the metacarpophalangeal joints and the distal phalangeal joints (P2-P3). (B,C) Higher magnification of the joint regions shown in A. (D) Ventral foot. Insertional mutant comprises normal metacarpals but is missing the basal (P1) and medial (P2) phalanges. In the WT foot (A), sesamoid (S) bones can be seen at the metacarpophalangeal joints and the distal phalangeal joints (P2-P3). (B,C) Higher magnification of the joint regions shown in A. (D) In the insertional mutant, note the fused V-shaped metacarpophalangeal sesamoids, the absence of P1 and P2 phalanges, and the reduction in size of the distal sesamoid. (E) Higher magnification of an MC-P3 joint from D. (F) Ventral forefoot from Gdf5bp-J/bp-J mice. As reported elsewhere (Gruneberg and Lee, 1973; Storm et al., 1994), metacarpals are significantly reduced in length, rudimentary phalanges (P1/P2) replace P1 and P2. (G) Higher magnification of a distal dig showing fused metacarpophalangeal sesamoids and a rudimentary P1/P2 element.
that BMPRIB and GDF5 each regulate additional aspects of skeletal morphogenesis through other partners.

To evaluate the molecular nature of the $BmpriB^{Tg}$ allele, we have determined the structure of the mouse $BmprIB$ gene and have discovered that its transcription initiates from two distinct promoters: one that drives expression in neuroepithelium and one, located more than 20 kb upstream, which is required for expression in the developing limb skeleton. The generation of this distal regulatory element may have been a critical event in the genetic evolution of distal limb form.

**MATERIALS AND METHODS**

**Identification of the insertion locus**
To clone DNA flanking the transgene insertion, we synthesized

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**Fig. 2.** Transgene insertion on mouse chromosome 3 disrupts the $BmprIB$ gene. (A,B) Double labeling of wild-type metaphase chromosome spreads with insertion probe P13304 and a centromere 3-specific probe. (B) Higher magnification of the chromosome in A marked by an arrowhead. The insertion is located at a position that is 91% of the distance from the centromere to the telomere of chromosome 3, corresponding to band H2. By nucleotide sequence, insertion probe P13304 was syntenic to human chromosome 4q21. (C) Structure of the wild-type (wt) $BmprIB$ gene and the transgenic allele $BmprIB^{Tg}$. In $BmprIB^{Tg}$, a random transgene insertion was accompanied by a deletion including exon 1. Upper, arrangement of $BmprIB$ exons. Exons are depicted by black boxes, untranslated sequence by white boxes. The location of fragments used as probes in Southern blotting are shown below, and primers used in RT-PCR and 5' RACE are marked above. Exons were identified using published $BmprIB$ cDNA sequence as reference, along with the novel cDNAs identified by 5' RACE (diagrammed at figure bottom). The cDNA sequence has been renumbered to account for the newly identified 5' exons (1-3). Sequences corresponding to exon 1 are 1-283; exon 2, 284-437; exon 3, 438-521; exon 4, 522-681; exon 5, 682-786; exon 6, 787-887; exon 7, 888-985; exon 8, 986-1123; exon 9, 1124-1315; exon 10, 1317-1612; exon 11,1613-1790; exon 12,1797-1921; exon 13,1922-2345. Exon 1 is situated more than 20-kb upstream of exon 2. The extracellular domain is encoded in exons 4-6, the transmembrane domain in exon 7, the GS box in exon 8, and the kinase domain in exons 9-13. The entire $BmprIB$ gene is contained within a BAC contig of ~200-kb (depicted as overlapping black lines below the wt locus). 5' RACE identified two classes of $BmprIB$ transcripts: exon 1-containing mRNAs are designated form 1 and represent the only mRNAs isolated from embryonic limb; exon 2-containing mRNAs, designated form 2 (a and b), predominate in neuroepithelium. These two RNA classes are transcribed from two distinct promoters; a distal promoter immediately upstream from exon 1 (prom 1), and a proximal promoter, adjacent to exon 2 (prom 2). Lines indicate the splicing patterns for the three transcripts. (D) Southern blot analysis of genomic DNA showing loss of $BmprIB$ exon 1. DNA isolated from a wt (+/+) animal, $BmprIB^{Tg}$ homozygote (+/−), and $BmprIB^{Tg}$ heterozygote (+/−).
genomic libraries (Zap vectors, Stragetein) using kidney DNA isolated from transgenic animals. Primers derived from 3′ flankin DNA sequence were used to screen Pl and BAC mouse genomic libraries by PCR (Genome Systems). Primer sequences: SD140, CTACCAAGGCTTCC; SD142, GTGGTGTATTGGGACGG. P1 clone 13304 was used as a fluorescent in situ hybridization probe to localize the transgene insertion to mouse chromosome 3 (Genome Systems).

Structure of the BmprIB gene

The murine BmprIB cDNA (GenBank Z23143) was cloned from a 13.5 days post coitum (dpc) embryonic cDNA library (GIBCO BRL) by RT-PCR and used to screen BAC mouse genomic libraries by hybridization (Research Genetics). Positions of exons were determined directly, by nucleotide sequence (Biopolymer Facility, Howard Hughes Medical Institute, Harvard Medical School), or indirectly, by Southern blot analysis of BAC and genomic DNA. BmprIB region-specific radiolabeled probes were generated from cloned (TA Cloning Kit, Invitrogen) RT-PCR products. Primer sequences (F indicates forward; R, reverse) for probe 1 (Fig. 2C): F2, GATTTGGCTGAGCTTAGTAC; R25, A TA TTGTTGACTGGCAACAG; for probe 2: F-AUAP (GIBCO BRL, RACE anchor primer), GGCCACGCGTGTGTAGTAC; R25, ATATTGTGACCTGAGCTTCCC. End-specific probes were used to orient the BAC and P1 genomic clones and grossly delineate the deletion caused by the transgene insertion. Assembly of DNA sequences, conceptual translation and sequence alignments were performed using LaserGene Navigator (DNASTAR). Sequence comparisons with the GenBank database were performed using the BLAST network services.

Mice and genotyping

Genotyping for the 4917Tg (BmprIB+/-) allele was performed by Southern blot analysis of HindIII-digested yeast sac or tail DNA (Dymecki, 1996) probed with radiolabeled junctional fragments from insertion clone P13303. The Gdf5genJ allele occurred spontaneously in the A/J strain (Jackson Laboratory) and contains an insertion of a guanine residue. Gdf5genJ allele detection was by PCR analysis of tail DNA. Primer sequences: wild-type FS50, GCGGAAACGCGGGG; RS52, GTGGGAGCGCAAGGGG.

Transcript detection

Total RNA (RNasey, Qiagen) isolated from embryonic tissues at 13.5 dpc, was used for RACE PCR (GIBCO-BRL) to obtain 5′ ends of BmprIB cDNAs. BmprIB-reverse primers for cDNA syntheses: R20, AGACATCGCAGAGATAAGC (RACE); R34, CCATGATGAATTCCGCTGTTC (RT-PCR). BmprIB-reverse primers for nested RACE PCR were R14 and R25. Detection of BmprIB cDNAs by RT-PCR was performed using the region-specific primers: F9, GGCAGGACACGATCGGGCCATC; R5, TCTTCCAGGAAAGTCTGAACT; F2; R14. In situ hybridization on cryosections (20 μm) of embryonic tissue at 12.5-13.5 dpc with sense or antisense digoxigenin (Boehringer Mannheim)-labeled riboprobes was described as described (Bau and Cepko, 1997). Riboprobes were as described previously: BmprIB (Zou et al., 1997), BmprIBA (Zou et al., 1997), ColadII (Lee et al., 1996), Gdf5 (Strom et al., 1994), Gli1 (Hui et al., 1994), Gli2 (Hui et al., 1994), Gli3 (Hui et al., 1994), Ihh (Bitgood and McMahon, 1995).

Skeletal preparations and histology

Skeletons of adult mice were prepared in 2% (w/v) potassium hydroxide and stained with Alizarin red (Green, 1968). To quantitate bone length, metatarsals were imaged, measured and compared between gender-matched mutant and wild-type siblings using a paired Student’s t-test. For Alcian blue histology, mouse limbs (12.5-16.5 dpc) were fixed in 4% paraformaldehyde/PBS overnight at 4°C, dehydrated and embedded in paraffin. 6 μm sections were mounted on glass slides, deparaffinized, rehydrated and stained for 3 hours (1% Alcian blue, 3% acetic acid).

Proliferation and apoptosis assays

Pregnant mice were injected intraperitoneally with 50 μg BrdU/gm body weight 1.5 hours before killing. Limbs (11.5-14.5 dpc) were processed as previously described for paraffin. Immunocytochemical detection of BrdU (Amersham Pharmacia Biotech) was performed on 6 μm sections as described (Nowakowski et al., 1989) with the addition of a Trypsin/EDTA permeabilization step (St-Jacques et al., 1999). Positive cells were visualized through peroxidase staining using diaminobenzidine (DAB) and then counterstained with hematoxylin. To quantitate the rate of cell proliferation, serial images of the same digit were collected and BrdU-positive (black) and -negative (gray) cells in the phalangeal region (Fig. 7E,F) were counted in two wild-type and two Tg/Tg litterate embryos at 13.5 dpc. The significance of the percentages was determined using the Student’s t-test. Apoptosis was detected by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (Boehringer Mannheim). Fluorescent images of fluorescein-dUTP incorporation were captured prior to immunocytochemical detection. Peroxidase staining with DAB was then performed to facilitate quantitation of labeled cells as for the BrdU assay.

RESULTS

Brachydactyly in 4917Tg homozygotes

The 4917Tg mouse line was produced by microinjection of transgene DNA into fertilized eggs (Dymecki, 1996). Southern blot analyses showed that heterozygotes harbored two copies of the 7 kb transgene in a head-to-tail configuration (data not shown). Heterozygotes were normal and fertile, and transmitted the transgene in a Mendelian fashion. Homozygosity for the transgene insertion caused neither embryonic nor postnatal lethality, as the predicted Mendelian ratio of alleles was observed among adult mice. Pups appeared and behaved outwardly normal, with the exception that homozygotes displayed brachydactyly (short digits).

Skeleton preparations revealed that 4917Tg homozygotes show two highly penetrant phenotypes: complete loss of proximal (P1) and medial (P2) phalanges in all digits (I-V) of both the forelimb and hindlimb, and fusion of the small sesamoid bones that normally articulate with these missing elements (Fig. 1). Although not fully penetrant, on average 4917Tg /Tg metatarsals were approximately 90% (±5% s.d.) the length of wild-type (P<0.005, Student’s t-test, n=42). Most other bones in the forefoot and hindfoot appeared normal in size, shape and number, including the terminal phalanges (P3). The axial skeleton was unaffected. In the normal course of development, P1 and P2 phalanges arise by sequentially segmenting off the cartilaginous digital ray; in contrast, the P3 phalanges form directly from distal membrane rather than segmenting from more proximal elements (Gruneberg and Lee, 1993). 4917Tg homozygotes show a specific loss of all endochondral-derived phalanges.

Sesamoids arise as secondary cartilages induced during embryonic development, usually initiated and remodelled by changes in tension at a joint (Hinchliffe, 1994). In wild-type forefoot and hindfoot digits, sesamoid bones are found at the metacarpophalangeal (metatarsophalangeal) joints and at the distal phalangeal joints (P2-P3) (Fig. 1B,C). In 4917Tg...
homozygous forelimbs, each pair of metacarpophalangeal sesamoids were found to be fused at the distal ends, resulting in a forked configuration (Fig. 1E); the P2-P3 sesamoids were absent or reduced in overall size. In both the mutant forefoot and hindfoot, the absence of P1 and P2 phalanges generates a novel joint between P3, the fused sesamoids, and the metacarpal or metatarsal. Mechanical instability and tension associated with this unusual articulation likely induces these atypical sesamoid morphologies.

Null mutations in the \textit{Gdf5} gene (\textit{Gdf5\textsuperscript{bp-J}}) also result in brachydactyly. Although described in detail elsewhere (Gruneberg and Lee, 1973; Storm et al., 1994; Storm and Kingsley, 1996), \textit{Gdf5\textsuperscript{bp-J}} mutant limbs are presented for comparison (Fig. 1F,G). As reported, examination of the phalanges showed that P1 and P2 are replaced by a single rudimentary element (P1/P2) in \textit{Gdf5\textsuperscript{bp-J}} mutants. Rather than arising from the digital ray, these rudimentary elements are thought to derive from a cartilage template formed out of perichondrial-like dense mesenchyme located in the presumptive phalangeal region (Storm and Kingsley, 1999). This is in distinction to 4917\textsuperscript{Tg} homozygotes where we observed a complete loss of P1 and P2 elements. Both mutants display unusual sesamoid morphologies. In addition to the phalangeal defect resulting in brachydactyly, the most striking anomaly in \textit{Gdf5\textsuperscript{bp-J}} mutants is a severe reduction in the length of many of the long bones of both the forelimb and hindlimb (Gruneberg and Lee, 1973; Storm and Kingsley, 1996). \textit{Gdf5\textsuperscript{bp-J}} mutant metatarsals were approximately 44\% (±5\% s.d.) the length of wild-type (P<0.005, Student’s t-test, n=12). Because these defects encompass more than just the digits, the \textit{Gdf5} genomic site has been designated the \textit{brachypodium (bp)} locus. Despite the differences between 4917\textsuperscript{Tg} and \textit{Gdf5\textsuperscript{bp-J}} mutant limbs, the shared loss of digital ray-derived phalanges suggested that the 4917\textsuperscript{Tg} and \textit{Gdf5} loci may participate in common or parallel genetic pathways in vivo to pattern the distal limb.

\textbf{4917\textsuperscript{Tg} insertion disrupts \textit{BmprIB}}

To clone the mutant and corresponding wild-type loci and identify the brachydactyly gene, a genomic library was prepared from 4917\textsuperscript{Tg} mouse DNA and screened with a radiolabeled probe for the transgene. Clones containing both murine and transgene DNA were identified. Using the murine-radiolabeled probe for the transgene. Clones containing both loci (Fig. 2). Because of discrepancies between published \textit{BmprIB} cDNA sequence and the genomic sequence obtained from BAC insertion clones 384 and 246, we used RACE to examine the 5’ ends of \textit{BmprIB} transcripts found in different tissues. \textit{BmprIB} is expressed in a variety of embryonic tissues at midgestation (Dewulf et al., 1995), most prominent being the chondrogenic condensations in the limb and vertebral bodies, and the neural tube and olfactory neuroepithelium. From these tissues, we isolated two major classes of \textit{BmprIB} RNA (Fig. 2C). Those containing exon 1 were termed form 1 and were the only RNAs found in limb; those lacking exon 1, but containing exon 2, were called form 2 and were the predominant species identified in olfactory epithelium. Subcategories of form 2 RNAs have also been identified, differing by the absence (form 2a) or presence (form 2b) of exon 3. All three RNA species appear to differ only in the 5’-untranslated region. Rather than representing unique coding transcripts generated by alternative splicing of a single pro-
is located at a distance and is required for expression in the developing limb skeleton (promoter 1), and one which is situated proximal to the coding region and drives expression in neural epithelium (promoter 2). Details of the promoter sequence will be presented elsewhere.

**BmprIB^Tg** is a regulatory allele, missing cis-sequences required for limb expression

In situ hybridization to BmprIB^Tg/Tg embryos at 11.5-14.5 dpc showed only a subset of the wild-type BmprIB expression pattern. Most notable was the loss of BmprIB expression in the mesenchymal condensations of the developing limb (Fig. 3B) and vertebral column (Fig. 3D), indicating that the BmprIB^Tg allele is null with respect to the limb. These hybridization findings were confirmed by RT-PCR (Fig. 3E) using two different sets of BmprIB region-specific primers. Indeed, this lack of detectable BmprIB mRNA in mutant limb is consistent with the insertion mutation being a deletion of exon 1, which would preclude expression of the only BmprIB RNA species normally found in limb.

In contrast to the loss of transcription in mesenchyme, BmprIB mRNA was detectable in mutant olfactory epithelium (Fig. 3D,E, lane 1). 5' RACE on dissected BmprIB^Tg/Tg neuroepithelium identified form 2 transcripts only. This supports the notion that form 2 RNAs are transcribed off a promoter downstream from the 3' end of the transgene-induced deletion.

While the insertion mutation abolishes BmprIB expression in discrete tissues, no new sites of expression were detected. It is therefore unlikely that the insertional mutation either introduces new regulatory elements driving ectopic IB expression or removes silencer elements that would normally mask expression. The BmprIB^Tg allele is, therefore, a regulatory allele in which cis-sequences required for skeletal expression have been deleted.

**Digit cartilage fails to develop in BmprIB^Tg mutants**

Adult BmprIB^Tg/Tg mice show loss of proximal and medial phalanges and fusion of sesamoid bones that normally articulate with these missing elements. Because the BmprIB^Tg allele is null with respect to the developing limb, these findings indicate that BMPRIB is required for the formation of endochondral-derived phalanges (P1 and P2). To analyze where in the process of phalangeal development IB signaling is required, we examined mutant limbs using both histological and molecular markers, asking if BMPRIB is essential for the early specification, proliferation, or condensation of prechondrogenic mesenchyme, or if IB is necessary for the later steps of chondrocyte proliferation and differentiation.

Each digit normally arises by sequential segmentation of a single chondrogenic condensation, the digital ray. This progressive process can be visualized histologically, using the general cartilage stain Alcian blue and, molecularly, using the cartilage differentiation markers Collagen II and Indian Hedgehog (Ihh). Collagen II, encoded by the Cola1(II) gene is an early chondrocyte-specific marker (Lee et al., 1996). Ihh, which encodes a member of the Hedgehog family of signaling molecules, is initially expressed by a subset of chondrocytes residing in the interior of early condensations. As the condensation matures, Ihh expression becomes progressively restricted to postmitotic prehypertrophic chondrocytes (Bitgood and McMahon, 1995).
In the wild-type animal, cartilaginous digital rays are clearly apparent in both the forelimb and hindlimb by 12.5 dpc (Fig. 4A). As the digital ray elongates, it eventually reaches a size and stage where the more proximal region differentiates and expresses *Ihh*, while the distal domain continues to aggregate mesenchyme from the progress zone. The outcome, by 13.5 dpc, is segmentation of the ray into two subelements: a proximal metacarpal and a distal phalanx (Fig. 4B). While each subelement stains strongly with Alcian blue (Fig. 4B-E) and expresses high levels of Collagen II (Fig. 5A, B), the intervening joint region does not. Differentiation progresses with *Ihh* detectable in both the metacarpal elements (Fig. 6E) and phalanges (Fig. 6E, G). By 14.5 dpc the phalanx has elongated and started to cleave such that, by 16.5 dpc, distinct P1 and P2 subelements can be recognized (Fig. 4K-O).

Although *BmprIB*^Tg/Tg^ and wild-type embryonic limbs are indistinguishable by Alcian blue staining at 12.5 dpc (Fig. 4A,F), a striking difference was observed by 13.5-14 dpc: mutant metacarpals (digital rays) failed to segment, despite being within a normal size range and expressing wild-type levels of Collagen II (Fig. 5C, D) and *Ihh* (Fig. 6F,H). A halo of mesenchyme surrounds the distal end of each mutant metacarpal giving the appearance of prechondrogenic cells attempting to condense and differentiate (Fig. 4H-J). This halo stained weakly with Alcian blue, but was negative for Collagen II, except for an outerzone of cells suggestive of perichondrium.
domain further restrict to a narrow band marking the presumptive metatarsophalangeal joint. By 14.5 dpc, this domain of approximately 15-20 cell diameters in the region of the cartilage. 24 hours later, expression is restricted to a domain of approximately 30-35 cell diameters distal to the presumptive joint interzone like Gli2 and Gli3. Gli1 mRNA localizes to the perichondrium (Hui et al., 1994; Fig. 6I). In BmprIB<sup>Tg/Tg</sup> mutant limbs, Gli1 transcripts are undetectable in the presumptive P1/P2 region (Fig. 6I), this is in contrast to the expanded domains of Gli2 and Gli3 (Fig. 6N,P). As described above, collagen II and Ihh RNA are similarly absent in the phalangeal region. Loss of these chondrocyte markers in the digital region of BmprIB<sup>Tg/Tg</sup> mice, together with persistence of prechondrogenic markers (Gdf5, Gli2 and Gli3), indicates that BMPRIB signaling is required for advancing from the prechondrogenic to the chondrogenic stage in digit formation.

Gli1 has been shown to be a target of Sonic hedgehog (Shh) signaling in the early limb bud (Hynes et al., 1997; Lee et al., 1997; Marigo et al., 1996). The coordinate loss of both Ihh and Gli1 in the phalangeal region of BmprIB<sup>Tg/Tg</sup> limbs suggests that in this later setting in the distal limb, Gli1 is likely to be a physiologic target of Indian hedgehog signaling.

Feedback regulation of Gdf5, Gli2 and Gli3 is disrupted in BmprIB<sup>Tg</sup> mutants

Since the failure to form digits appeared to be linked in BmprIB<sup>Tg/Tg</sup> mutants to a defect in segmentation, we examined molecular markers for joint development, including the TGFβ family member, Gdf5 (Storm et al., 1994), and two putative zinc finger transcription factors, Gli2 and Gli3 (Hui et al., 1994). Expression of Gdf5 has been reported to identify a mesenchyme domain of approximately 30-35 cell diameters distal to the growing 12.5 dpc digit ray (Storm and Kingsley, 1999); these marked cells appear specified to go on to condense and form cartilage. 24 hours later, expression is restricted to a domain of approximately 15-20 cell diameters in the region of the presumptive metatarsophalangeal joint. By 14.5 dpc, this domain further restricts to a narrow band marking the metacarpophalangeal articulation. Gli2 and Gli3 are expressed in a similar profile (Hui et al., 1994; Storm and Kingsley, 1999) and are considered, along with Gdf5, to first mark prechondrogenic mesenchyme and, later, mark joint interzones.

Analyses of IB mutant limbs showed expanded expression domains for Gdf5, Gli2 and Gli3 (Fig. 6L,N,P). Expression remained broad in the mutant even at 13.5 dpc, and failed to restrict to the presumptive metacarpophalangeal joint (Fig. 6K, arrowhead in wild-type limb). Thus, in the absence of IB function, there is an expansion of Gdf5, Gli2 and Gli3 to include nearly all distal mesenchyme. These observations suggest that prechondrogenic mesenchyme is present in the phalangeal region but that it fails to mature to form the P1/P2 anlagen. One explanation is that BMPRIB directs the differentiation of prechondrogenic mesenchymal cells into chondrocytes and is therefore necessary for further digit morphogenesis. In part, chondrocyte differentiation requires a threshold density of cells (Takahashi et al., 1998); the failure to form digits could therefore also reflect a defect in the condensation or proliferation process.

A similar expansion of Gdf5 and Gli3 expression is reported in mice homozygous for the frameshift null allele Gdf5<sup>bsp-1</sup>. This molecular phenocopy lends further support to the idea that GDF5 and BMPRIB may participate in common or parallel pathways in vivo, and that one common outcome is downregulation of Gdf5, Gli2 and Gli3.

BmprIB is required for Ihh and Gli1 expression in the phalangeal region

Gli1 expression is initially found throughout the condensing digit ray; however, rather than becoming restricted to the presumptive joint interzone like Gli2 and Gli3, Gli1 mRNA localizes to the perichondrium (Hui et al., 1994; Fig. 6I). In BmprIB<sup>Tg/Tg</sup> mutant limbs, Gli1 transcripts are undetectable in the presumptive P1/P2 region (Fig. 6I), this is in contrast to the expanded domains of Gli2 and Gli3 (Fig. 6N,P). As described above, collagen II and Ihh RNA are similarly absent in the phalangeal region. Loss of these chondrocyte markers in the digital region of BmprIB<sup>Tg/Tg</sup> mice, together with persistence of prechondrogenic markers (Gdf5, Gli2 and Gli3), indicates that BMPRIB signaling is required for advancing from the prechondrogenic to the chondrogenic stage in digit formation.

Gli1 has been shown to be a target of Sonic hedgehog (Shh) signaling in the early limb bud (Hynes et al., 1997; Lee et al., 1997; Marigo et al., 1996). The coordinate loss of both Ihh and Gli1 in the phalangeal region of BmprIB<sup>Tg/Tg</sup> limbs suggests that in this later setting in the distal limb, Gli1 is likely to be a physiologic target of Indian hedgehog signaling.

Reduced mesenchymal cell proliferation in mutant phalangeal region followed by excessive apoptosis

To assess whether lack of cartilage differentiation in the phalangeal region of BmprIB<sup>Tg/Tg</sup> mice involves a defect in cell proliferation, we analyzed bromodeoxyuridine (BrdU) incorporation into both early progress zone mesenchymal cells (11.5-12.5 dpc) and into later chondrogenic cells in the phalangeal regions (13.5-14.5 dpc). Though no differences were observed between mutant and wild-type at 11.5 and 12.5 dpc (Fig. 7A-D), we did detect a 6.4-fold reduction in the percentage of BrdU-positive nuclei in the digital region of mutant limbs at 13.5 dpc (P<0.005, Student’s t-test) (Fig. 7E,F). BMPRIB signaling is, therefore, not essential to maintain the high rate of proliferation observed in the early progress zone mesenchyme, but is required for proliferation of later prechondrogenic cells in the digit region.

In addition to exhibiting a decrease in cell proliferation rates, the mutant digit mesenchyme at 13.5 -14.5 dpc failed to condense and organize into tightly packed chondrocytes. Two morphological indicators of this differentiation process include the flattening of outer zone (preperichondrial) cells into concentric layers, and longitudinal stacking of inner zone (chondrogenic) cells. Both of these morphologic changes failed to occur in mutant digits (Fig. 7F,H).

By 14.5 dpc, mutant digital mesenchyme becomes even more disorganized, characterized by pyknotic cells and empty spaces starting at the presumptive metacarpophalangeal joint and extending well into the prechondrogenic aggregation. Because these changes in tissue integrity were suggestive of cell death, we analyzed mutant limbs for signs of apoptosis. Both wild-type and BmprIB<sup>Tg/Tg</sup> limbs at 11.5 and 12.5 dpc showed a low incidence of cell death in the progress zone, as assessed by end labeling of fragment DNA (data not shown).

Normal levels of apoptosis were, however, observed in interdigital mesenchyme of both mutant and wild-type embryos, indicating that the apoptotic process has not been more generally disrupted in the mutant. Together with the BrdU analysis, these results suggest that the supply of progress zone mesenchyme is normal in BmprIB<sup>Tg/Tg</sup> limbs, arguing...
against insufficient starting mesenchyme as causing the reduction in phalangeal elements.

In contrast to the normal appearing early stage limbs, later stage mutant limbs (13.5 and 14.5 dpc) show an approximate 5-fold increase in cell death as compared to wild-type limbs (P<0.005, Student’s t-test) (Fig. 8). The apoptotic area corresponds to the presumptive metacarpophalangeal joint space and extends distally over time to include most of the disorganized digit mesenchyme. Interestingly, this region of excessive cell death corresponds to the expanded expression domains of Gdf5, Gli2 and Gli3. Because expression of this gene set goes from marking prechondrogenic mesenchyme at early stages, to marking joints at later stages and because joint formation is associated with programmed cell death, it is possible that expanded joint development contributes to the brachydactyly phenotype. In summary, BMPRIB appears required to condense prechondrogenic digit mesenchyme into a proliferating cartilage focus. In the absence of this chondrogenic program and in the presence of prolonged expression of Gdf5, Gli2 and Gli3, excessive cell death occurs. The end result is complete loss of endochondral-derived phalanges.

**BmprIB**Tg; Gdf5bp-J double mutants indicate that GDF5 interacts with BMPRIB in vivo

GDF5 is likely to signal, at least in part, through BMPRIB. This is based on the observations that BmprIBTg and Gdf5bp-J homozygotes each show a similar loss of phalanges.
(Gruneberg and Lee, 1973; Storm et al., 1994; and reported here), and because in vitro studies show high-affinity binding of GDF5 to BMPRIB as compared to other type I receptors (Nishitoh et al., 1996). To investigate whether BMPRIB indeed transduces GDF5 signals in vivo, we compared skeletons isolated from adult BmprIB Tg ; Gdf5 bp-J double mutants, single mutants and compound heterozygotes. Skeletal preparations are shown in Fig. 9 alongside schematics illustrating the observed digit defects.

As described earlier, BmprIB Tg homozygotes display normal or slightly shortened metatarsals, each articulating directly with a terminal P3 phalanx; P1 and P2 phalanges are absent. Gdf5bp-J homozygotes show metatarsals that are markedly reduced in length, and loss of the digital-ray-derived phalanges P1 and P2; in place, is a rudimentary phalanx thought to be derived by appositional cartilage growth from perichondrial-like mesenchyme (Storm and Kingsley, 1999). In the digital region, BmprIB Tg ; Gdf5 bp-J double mutants appear similar to Gdf5 bp-J single mutants, indicating that GDF5 signals digit cartilage condensation and proliferation through BMPRIB. While normal digit cartilages failed to development in the double mutant, runted phalangeal elements, similar to those found in Gdf5 bp-J single mutants were observed (Fig. 9D,H).

In contrast to the digit region, synergistic malformations were found in the carpal and tarsal bones of double mutants: individual metacarpals (metatarsals) failed to segment properly from the carpal (tarsal) bones. This defect was especially striking in the hindfoot, between metatarsal III, cuneiform III and the navicular bone (Fig. 9, arrowhead). This synergistic phenotype indicates that BMPRIB and GDF5 serve redundant functions in regulating segmentation of the digital arch.

It is possible that some differences in cartilage growth
observed between BmprIB\textsuperscript{Tg} and Gdf5\textsuperscript{bp-J} mutant mice could, in part, stem from differences in the genetic background of the mutants: B6SJL for BmprIB\textsuperscript{Tg} versus AJ for Gdf5\textsuperscript{bp-J}. This is unlikely given that fully penetrant brachydactyly has been observed in IB mutants developed on other genetic backgrounds (129/Sv and a mixed 129/Sv × C57Bl/6; Yi, 2000), suggesting that the brachydactyly phenotype is not readily altered by strain-specific modifier genes.

**DISCUSSION**

We have shown that BmprIB\textsuperscript{Tg} mutants display a complete loss of endochondral-derived phalanges. Our analyses indicate that BMPRIB plays multiple roles in development of the distal limb skeleton; these include signaling cartilage condensation and differentiation from the digital blastema and regulating cartilage segmentation to yield subelements. In addition, we have further delineated GDF5 function in vivo, finding that GDF5 signals digit cartilage formation through IB, but regulates the overall length of skeletal elements largely through alternative receptors. These studies, in conjunction with previous analyses of Gdf5 null mice (Storm et al., 1994; Storm and Kingsley, 1999), indicate that IB and GDF5, acting together and separately, play pivotal roles in determining distal limb structure.

In addition to delineating IB and GDF5 function in skeletal development, we have also determined the structure of the BmprIB gene and have identified two promoters, one of which is responsible for expanding BmprIB expression into the developing limb. Acquisition of this promoter may have implications for the evolutionary innovation of digits in the distal limb, and its utilization may contribute to the species-to-species variability found in distal limb architecture.

**BMPRIB is essential for differentiation of digit cartilage**

Previous misexpression studies performed in the chick (Kawakami et al., 1996; Zou et al., 1997; Merino et al., 1998) suggest that BMPRIB may act as a direct effector of chondrogenesis, consistent with its presence in cartilage primordia. In addition to promoting cartilage formation, a constitutively active IB receptor (caBMPrIB) has also been shown capable of inducing interdigital cell death (Zou et al., 1997). This latter result, however, is at odds with the absence of detectable BmprIB mRNA in interdigital tissue (this report, Fig. 6A; see also Zou et al., 1997). Should IB serve both chondrogenic and apoptotic functions, then a IB null mutation
would be predicted to result in a reduction in distal cartilage elements with a concomitant increase in interdigital tissue. Our analysis of BmprIB/g mutants shows that digit cartilages are lost without concomitant webbing; this finding supports the idea that IB signals chondrogenesis and argues against a physiological role for IB in signaling cell death.

Because distal pattern appeared to be specified in BmprIB/g/Tg mutant limbs (operationaly defined by the early expression of the prechondrogenic markers Gdf5, Gli2 and Gli3), we propose that the major function of IB is to signal condensation and differentiation of prechondrogenic cells, without which further digit morphogenesis cannot occur. It is, of course, possible that in the absence of normal cartilage condensation and differentiation, additional IB functions go unrealized and, therefore, cannot be excluded (e.g. maintenance of the differentiated phenotype).

While BmprIB expression is lost from a number of developing tissues in BmprIB/g/Tg embryos (vertebral condensations, gut, kidney and urogenital tract), the observed defects are restricted to distal limb (reported here) and distal genitals (to be described elsewhere). One explanation for the circumscribed phenotype is that other type I receptors can substitute for the loss of IB in these tissues. Such compensatory mechanisms are either insufficient or do not exist in distal limb and genitals, or are compromised by the accompanying misregulation of Gdf5.

**BMPRIB interacts genetically with GDF5**

Previous in vitro experiments showed that GDF5 could bind to different sets of type I receptors and that binding was most efficient to BMPRIB. Moreover, in cell culture, IB was shown to transduce a transcriptional activation signal following stimulation with GDF5 (Nishitoh et al., 1996). Examination of BmprIB/g; Gdf5bp-J double mutants indicates that, in vivo, BMPRIB does indeed mediate signals for GDF5, the outcome being formation of digit cartilage. Consistent with this genetic interaction, our finding that BMPRIB, like GDF5 (Storm and Kingsley, 1999), is required to downregulate Gdf5, Gli2 and Gli3 expression in the digit/joint region, but not in perichondrium. The phenotypic consequence of GDF5 signaling in these two regions (digit/joint versus perichondrium) is therefore different, suggesting that GDF5 may signal through distinct receptor complexes or trigger different downstream intracellular pathways depending on the spatial location.

Micromass limb cultures show that GDF5 can modulate the initial stages of chondrogenesis by increasing cell adhesion within a mesenchymal condensation (Francis-West et al., 1999; Hotten et al., 1996). Interestingly, prechondrogenic digit mesenchyme in BmprIB/g/Tg limbs appears unable to condense and organize into a cartilage element. Given this block in progressing from loosely aggregated prechondrogenic cells to compact chondroblasts, we propose that the physiological outcome of GDF5/BMPRIB signaling in the early digital ray is an increase in cell adhesion. The resultant condensation and high cell density then enables further cartilage proliferation and differentiation.

**Distinct and redundant roles for BMPRIB and GDF5 suggest combinatorial signaling in vivo**

While both BmprIB/g and Gdf5bp-J single mutants exhibit loss of endochondral-derived phalanges, the skeletons are nonetheless readily distinguishable due to a striking difference in bone length. BmprIB/g mutant limbs display near normal bone length, while Gdf5bp-J limbs are significantly shortened (Grueneberg and Lee, 1973; Storm et al., 1994). Double mutants show no further reduction. Thus, in contrast to GDF5, IB is not required to sustain normal longitudinal growth of more proximal limb elements.

The reciprocal phenotype of an increase in bone length has been reported following overexpression of Gdf5 in the chick limb (Francis-West et al., 1999; Merino et al., 1999). This has been interpreted within the context of the endogenous Gdf5 expression profile to suggest an additional role for GDF5. First, when Gdf5 is expressed in prechondrogenic cells, it promotes condensation by increasing cell adhesion. Later, when its expression is restricted to joints, it may signal to the epiphyses of adjacent skeletal elements to control chondrocyte proliferation and therefore overall bone length (Francis-West et al., 1999). Although IB is expressed in the developing cartilage epiphyses and has been hypothesized to signal chondrocyte proliferation, our loss-of-function analyses show that IB is not required to achieve normal bone length once the initial condensation has been established. This raises the possibility that, early on, GDF5 signals mesenchymal condensation through IB but, later, signals chondrocyte proliferation through additional type I receptors.

Although major differences in bone length were not observed between BmprIB/g; Gdf5bp-J double mutants and Gdf5bp-J single mutants, synergistic malformations were found in the carpal and tarsal bones of double mutants. BMPRIB and GDF5 therefore serve redundant functions in regulating segmentation of the digital arch, with IB mediating segmentation signals from other GDF/BMP ligands and GDF5 activating other receptors. Moreover, this analysis has revealed a function for BMPRIB in cartilage segmentation that was not uncovered through study of single mutants.

**Overexpression of Gdf5 in BmprIB/g mutants correlates with apoptosis**

The complete loss of P1 and P2 phalanges in BmprIB/g homozygotes would, at first glance, suggest that BMPRIB is required for development of digit cartilages, playing a role in two processes: the condensation and differentiation of digit cartilage from the digital ray, and the appositional growth of cartilage from perichondrium. In contrast, GDF5 appears to be required for the development of digit cartilage from the digital ray, but is nonessential for appositional-like growth (as rudimentary P1/P2 elements do develop). This would suggest that a different GDF or BMP signals appositional growth through IB. Surprisingly, rudimentary phalanges were found in the double mutant, suggesting that neither BMPRIB nor GDF5 are required for appositional growth. This seemingly contradictory finding makes sense when consideration is given to the genes that we see affected in BmprIB/g homozygotes.

Although the primary defect in BmprIB/g homozygotes is loss of BmprIB expression in the limb, this loss is accompanied by expanded and prolonged expression of Gdf5, Gli2 and Gli3 in the digital region (Fig. 6). The complete failure to develop digit cartilage in BmprIB/g homozygotes therefore results from the combined effects of both genetic alterations: loss of IB and gain of Gdf5, Gli2 and Gli3. The initial developmental defect in BmprIB/g homozygotes (observed at 13.5 dpc) appears to
be a failure to condense and differentiate prechondrogenic cells into digit chondroblasts. In the absence of this IB-dependent differentiation step, prechondrogenic mesenchyme undergoes apoptosis in the region in which Gdf5, Gli2 and Gli3 are overexpressed. This tissue loss (readily apparent by 14.5 dpc; Figs 7H, 8) precludes later cartilage development through ap positional mechanisms. Within this context, the simplest interpretation of the double mutant phenotype is that, in the absence of functional GDF5, the excessive apoptosis seen in BmprIBTg single mutants does not occur, thereby allowing rudimentary phalanges to form by apposition. From this, we deduce that GDF5 signals the aberrant cell death observed in BmprIBTg single mutants. Thus, the brachydactyly observed in BmprIBTg limbs likely results from the combined effects of loss of IB (failure to condense and differentiate digit cartilage) and overexpression of Gdf5 (later apoptotic loss of prechondrogenic mesenchyme).

Because the apoptosis observed in BmprIBTg single mutant digits begins at the metacarpophalangeal joint and then extends distally, following the expanded Gdf5 expression domain, we propose that GDF5 may signal the programmed cell death intrinsic to digit segmentation and joint formation; indeed, this would be consistent with one of the putative functions for GDF5 put forth by Storm and Kingsley (1999). In our model, GDF5 would signal apoptosis through an alternative type I receptor, as the observed cell death occurred in the absence of BMPRIIB. In the chick (Francis-West et al., 1999; Hotten et al., 1996; Merino et al., 1999) and in mouse limb culture (Storm and Kingsley, 1999), exogenous GDF5 protein has been shown to stimulate ectopic cartilage development, but not ectopic subdivisions in skeletal elements. Consequently, GDF5 would be necessary but not sufficient to induce cell death and joint formation. It is also possible that the cell death observed in BmprIBTg single mutants results from abnormally high levels of GDF5 protein inappropriately activating (or even antagonizing) other type I receptors.

Together, the above genetic studies suggest a number of important conclusions regarding BMP signaling and skeletal development: (1) GDF5 signals digit formation through BMPRIIB but regulates element length through an alternative type I receptor, (2) GDF5 and IB each exert segmentation functions, but, at least in part, do so independently, and (3) appositional cartilage growth is likely induced by other BMPs and receptors, since it occurs independent of both GDF5 and IB. Our analyses, not only identify both distinct and redundant functions for BMPRIIB and GDF5 in skeletal development, but provide evidence that combinatorial BMP signaling occurs in vivo.

**BMPRIIB signaling and Gli proteins**

In addition to regulating Gdf5 expression, we show that BMPRIIB activity is necessary to downregulate Gli2 and Gli3, and is required for subsequent Ihh and Gli1 expression. The BmprIBTg mutation therefore genetically separates the activities of Gli2 and Gli3 from Gli1, consistent with gene inactivation experiments showing that Gli2 and Gli3 serve redundant functions during skeletal development (Mo et al., 1997). This in vivo distinction supports recent in vitro structure-function studies that divide the Gli proteins into two categories: Gli1 appears to function primarily as a transcriptional activator and primary mediator of Hh function; conversely, Gli2 and Gli3 exhibit strong repressor activity, antagonizing both Hh and Gli1 function (Ruiz i Altaba, 1999; Sasaki et al., 1999). Indeed, Ihh and Gli1 are coordinately lost in the BmprIBTg mutant limb suggesting that Gli1 is a physiologic target of Ihh signaling.

Genetic and biochemical studies have suggested that Gli proteins interact with the BMP-triggered signaling cascade. In vitro studies show that carboxy-terminally truncated Gli3 proteins (but not Gli1) complex with Smad proteins, and that the complexes are disrupted by BMP signaling (Liu et al., 1998). Moreover, genetic studies in mice suggest an interaction between BMP4 and Gli3 (Dunn et al., 1997), and human syndromes associated with Gli3 mutations, such as Pallister-Hall syndrome (Kang et al., 1997) and polydactyly type A (Radhakrishna et al., 1997), each display skeletal defects similar to those resulting from aberrant BMP signaling. Here we show that Gli3 expression is downregulated by IB. Thus, BMPRIIB signaling likely regulates Gli3 activity at two levels: by altering Gli3-Smad protein complexes and by regulating Gli3 transcription.

**Use of an alternative distal promoter expands Bmpr1b expression into the developing limb**

Our analyses of the Bmpr1b gene structure and mRNA isoforms in wild-type and mutant mice have identified a second, more distal promoter required for expressing Bmpr1b in the developing limb skeleton. Because expression of IB is uniquely required for digit formation, acquisition of this novel cis-regulatory region could represent a pivotal genetic step for driving morphological diversity in distal extremities, exemplified by the formation of digits during the fin to limb transition. A primary example of evolutionary recruitment of gene function to generate new distal limb structure, comes from the identification of a digit regulatory element driving expression of Hoxd-10, Hoxd-11, Hoxd-12 and Hoxd-13 in the autopod (van der Hoeven et al., 1996). Given the functional equivalence of many of the Hox gene products, it has been proposed that novel morphogenetic variants can arise only through major regulatory changes such as this global digit enhancer (Zakany and Duboule, 1999). Given the overlapping activities reported for the different type I BMP receptors, the same logic could hold here: generation of phalanges requires acquisition of novel cis-regulatory elements to expand type I receptor expression into the distal limb. This parallel between Bmpr1b and Hox gene regulation extends into the development of the distal urogenital tract. The same regulatory element that promotes Hox gene expression in digits, simultaneously promotes expression in the genital tubercle (van der Hoeven et al., 1996). Similarly, the same cis-regulatory region required to express Bmpr1b in the limb also drives expression in mesenchyme of the tubercle. Moreover, Hoxa13<sup>+/−</sup>; Hoxd13<sup>−/−</sup> compound mutants display improper separation of the vagina from the urogenital sinus (Warot et al., 1997); although incompletely penetrant, BmprIBTg mutants show a defect in vaginal development (S. T. B. and S. M. D., unpublished data) in addition to the fully penetrant brachydactyly described here. As put forth for the Hoxd genes, perhaps acquisition of this distal Bmpr1b cis-regulatory region has, in part, simultaneously shaped both the distal limb and distal genital tract.
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BMPRIB signaling and digit formation


