Developmental Biology **251,** 142–156 (2002)
doi:10.1006/dbio.2002.0802

The Wnt Antagonist Frzb-1 Regulates Chondrocyte Maturation and Long Bone Development during Limb Skeletogenesis

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The Wnt antagonist Frzb-1 is expressed during limb skeletogenesis, but its roles in this complex multistep process are not fully understood. To address this issue, we determined Frzb-1 gene expression patterns during chick long bone development and carried out gain- and loss-of-function studies by misexpression of Frzb-1, Wnt-8 (a known Frzb-1 target), or different forms of the intracellular Wnt mediator LEF-1 in developing limbs and cultured chondrocytes. Frzb-1 expression was quite strong in mesenchymal prechondrogenic condensations and then characterized epiphyseal articular chondrocytes and prehypertrophic chondrocytes in growth plates. Virally driven Frzb-1 misexpression caused shortening of skeletal elements, joint fusion, and delayed chondrocyte maturation, with consequent inhibition of matrix mineralization, metalloprotease expression, and marrow/bone formation. In good agreement, misexpression of Frzb-1 or a dominant-negative form of LEF-1 in cultured chondrocytes maintained the cells at an immature stage. Instead, misexpression of Wnt-8 or a constitutively active LEF-1 strongly promoted chondrocyte maturation, hypertrophy, and calcification. Immunostaining revealed that the distribution of endogenous Wnt mediator β-catenin changes dramatically *in vivo* and *in vitro*, from largely cytoplasmic in immature proliferating and prehypertrophic chondrocytes to nuclear in hypertrophic mineralizing chondrocytes. Misexpression of Frzb-1 prevented β-catenin nuclear relocalization in chondrocytes *in vivo* or *in vitro*. The data demonstrate that Frzb-1 exerts a strong influence on limb skeletogenesis and is a powerful and direct modulator of chondrocyte maturation, phenotype, and function. Phases of skeletogenesis, such as terminal chondrocyte maturation and joint formation, appear to be particularly dependent on Wnt signaling and thus very sensitive to Frzb-1 antagonistic action.

Key Words: **Wnt signaling; Frzb-1; chondrocyte maturation; matrix mineralization; endochondral ossification; limb development.**

INTRODUCTION

Limb skeletogenesis remains the focus of much research activity, owing to its key role in limb morphogenesis and growth. It initiates with formation of mesenchymal cell condensations in the early limb bud (Hinchliffe and Johnson, 1983; Horton, 1993). The condensed cells then differentiate into chondrocytes that assemble recognizable cartilaginous long bone anlagen with specific anatomical characteristics. Chondrocytes located at each epiphyseal end acquire a permanent phenotype and give rise to articular cells, which remain functional and active and persist

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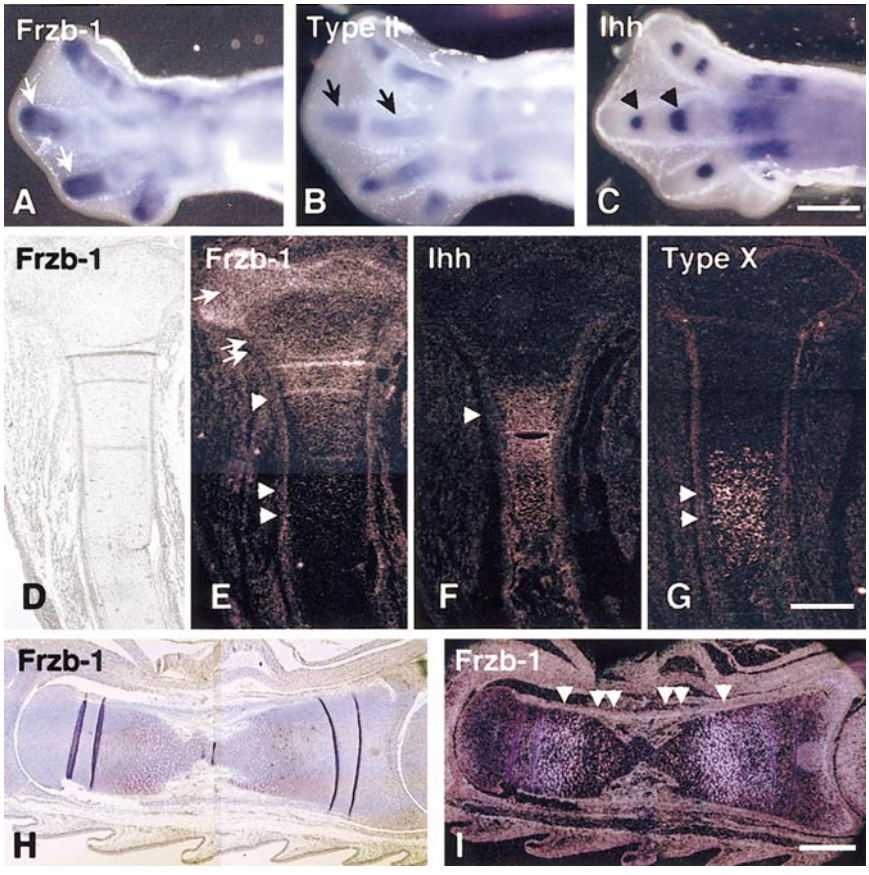

FIG. 1. Gene expression of Frzb-1 in the developing limb. Whole Day 6.5 chick embryo forelimbs (A–C) and longitudinal sections of Day 10 tiobiotarsus (D–G) and Day 13 phalanges (H–I) were processed for *in situ* hybridization to determine gene expression of: Frzb-1 (A, E, and I); type II collagen (B); Ihh (C and F), and type X collagen (G). (D, H) Bright fields of (E) and (I), respectively. See text for further details. Scale bars, (A–C) 1.5 mm; (D–G) 0.1 mm; (H, I) 0.2 mm.

throughout life in the joints (Pacifici *et al.*, 2000). Chondrocytes constituting the shaft and their progeny become organized into growth plates, undergo maturation, and are eventually replaced by bone cells. Chondrocyte maturation involves several steps and is required for the transition from cartilage to bone. During this process, resting immature chondrocytes first proliferate and then become prehypertrophic and matrix-producing cells. The prehypertrophic cells enlarge into hypertrophic type X collagen-expressing cells, which finally reach the posthypertrophic mineralizing stage (Horton, 1993; Hunziker, 1994). Mineralized hypertrophic cartilage is invaded by osteoprogenitor cells from surrounding tissues and replaced by endochondral bone. Invasion is aided by locally produced matrix proteases, including metalloprotease-9 (MMP-9), MMP-13, and MT-MMP1 (Vu *et al.*, 1998; Zhou *et al.*, 2000).

Limb skeletogenesis is regulated by a large number of factors, including members of the Wnt family. Wnt proteins are secreted signaling agents, are involved in a variety of developmental processes, and exert their action by binding to Frizzled receptors on the cell surface (Cadigan and Nusse, 1997; Moon *et al.*, 1997; Bhanot *et al.*, 1996; Hsieh *et al.*, 1999). Wnt binding and receptor activation result in stabilization of the effector cytoplasmic protein β-catenin and translocation to the nucleus (Willert and Nusse, 1998). β-Catenin forms complexes with resident transcription factors, such as members of the lymphoid enhancer factor/T-cell factor (TCF/LEF) family, and modulates expression of Wnt-responsive genes (Eastman and Grosschedl, 1999; Willert and Nusse, 1998). There is also evidence for an alternative signaling pathway in which Wnt binding to different Frizzled receptors would lead to increased cytoplasmic Ca^{2+} levels, activation of protein kinase C, and expression of Wnt-responsive genes (Sheldahl *et al.*, 1999). Regulation and specificity of Wnt signals have been shown to depend not only on the presence of different signaling receptors on target cells, but also on the action of proteins with

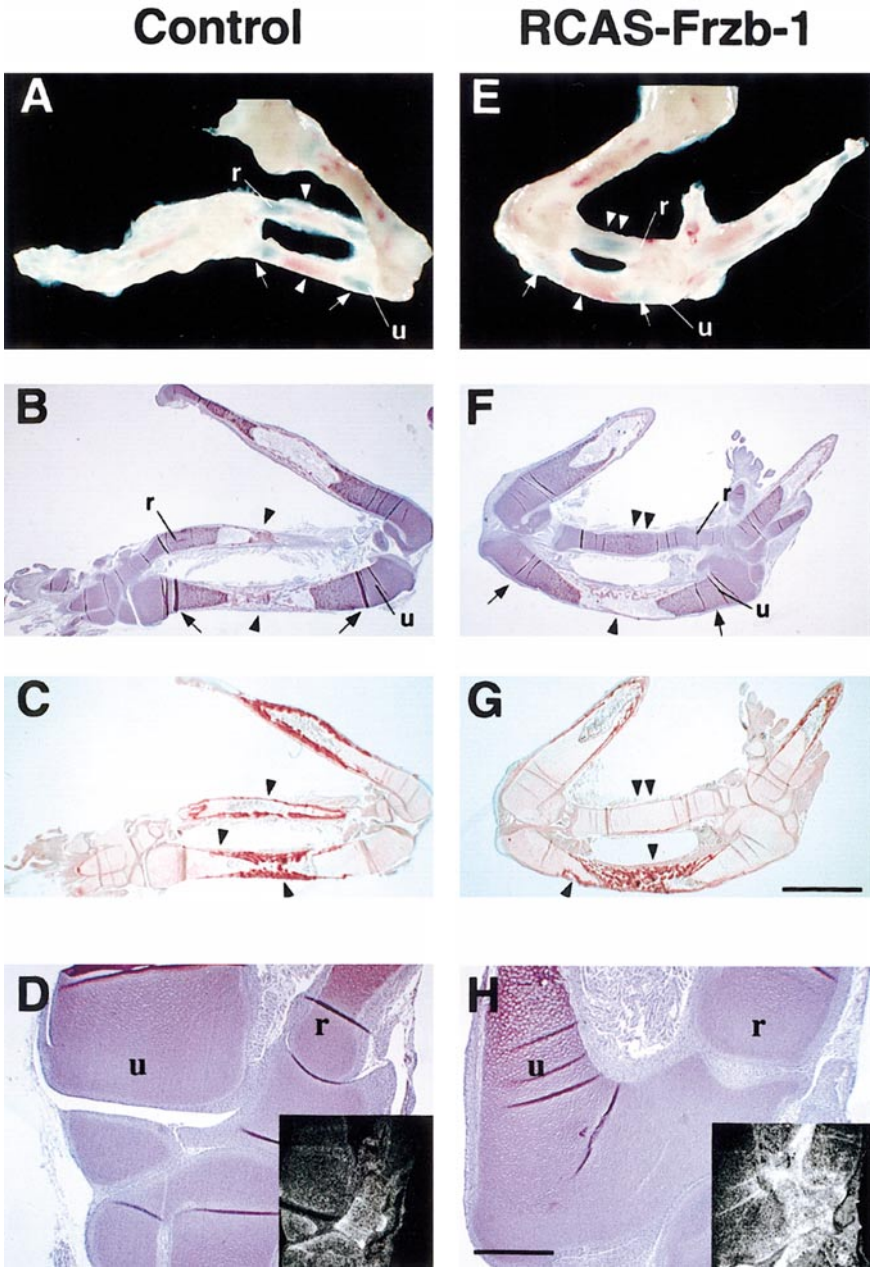

FIG. 2. Effects of Frzb-1 misexpression on long bone development. Control insert-less retroviral particles (A–D) or particles encoding Frzb-1 (E–H) were implanted in stage 22–23 chick embryo forelimb buds *in ovo*; embryos were reincubated and examined on Day 10. (A, E) Anatomical view of freshly dissected live limb skeletons; (B, F) Histological longitudinal sections stained with hematoxylin–eosin; (C, G) Longitudinal sections stained with alizarin red; (D, H) Higher magnification view of wrist region of sections (A) and (E), respectively. Insets in (D) and (H) show Frzb-1 *in situ* hybridization with a chick Frzb-1 probe. Note that the radius (r) in experimental limb is entirely cartilaginous (E–G, double arrowhead), whereas it contains cartilage (arrow) and bone with marrow (arrowhead) in control limb (A–C). Ulna (u) was normal in both limbs. Note also that the wrist region was abnormal in experimental embryo (H) compared with control (D). Scale bars, (A–C) and (E–G) 3 mm; (D, H) 0.5 mm.

Wnt antagonistic properties. Frzb-1 is the founding member of this protein group (Hoang *et al.*, 1996). Other proteins share structural similarities with Frzb-1, and the group is currently known as secreted frizzled-related proteins (Sfrps) (Finch *et al.*, 1997; Leyns *et al.*, 1997; Rattner *et al.*, 1997; Wang *et al.*, 1997a). All the Sfrp

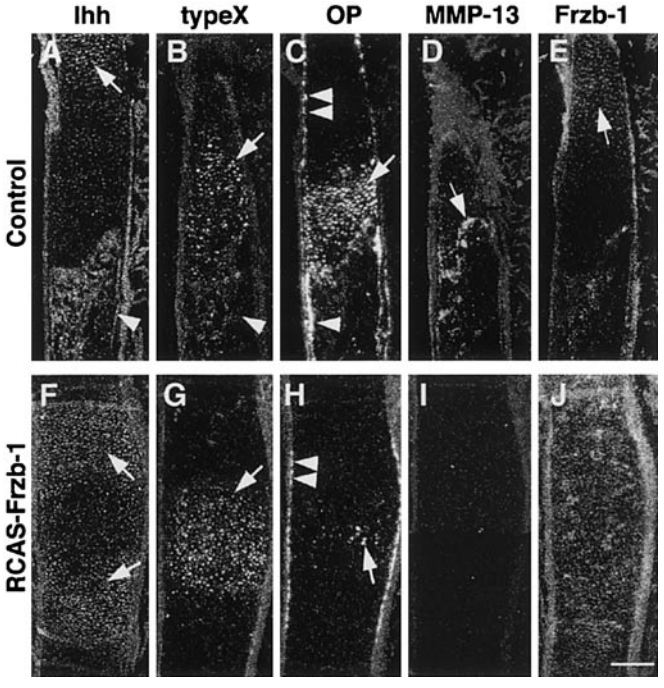

FIG. 3. *In situ* hybridization analysis of gene expression in longitudinal sections from Day 10 control (A–E) and Frzb-1-misexpressing (F–J) radii. (A, F) Ihh; (B, G) type X collagen; (C, H) osteopontin (OP); (D, I) MMP-13; and (E, J) Frzb-1 (chick Frzb-1 probe). In the Frzb-1-misexpressing radius, OP expression was severely reduced (H, arrow) and MMP-13 expression was absent (I) compared with control (C, D, arrows). Note also that OP expression was maintained in perichondrial tissue (C, H, double arrowhead), and that Frzb-1 transcripts were widely distributed in the radius (J) but restricted to the prehypertrophic zone in control (E, arrow). See text for further details. Scale bar, 0.2 mm.

proteins contain the Wnt-binding CRD ectodomain, but lack the transmembrane and intracellular signaling domains; thus, they essentially are soluble Frizzled receptors.

Several Wnt proteins as well as Frzb-1 are expressed during skeletogenesis. Wnt-3a, Wnt-5a, and Wnt-7a characterize the early limb bud and are thought to regulate skeletal patterning along the proximodistal and dorsoventral axes (Dealy *et al.*, 1993; Kengaku *et al.*, 1998; Parr and McMahon, 1995). Wnt expression is usually absent in mesenchymal prechondrogenic condensations, whereas Frzb-1 expression is specifically found in them (Baranski *et al.*, 2000; Duprez *et al.*, 1999; Hoang *et al.*, 1996; Wada *et al.*, 1999). This correlates well with the finding that misexpression of Wnt-1 and Wnt-7a inhibits chondrogenic cell differentiation (Rudnicki and Brown, 1997). At later developmental stages, Wnt-4 and Wnt-14 are expressed at sites of future joint development, Wnt-5a and Wnt-11 are expressed in perichondrium, Wnt-5b characterizes prehypertrophic chondrocytes in the growth plate (Hartmann and Tabin, 2000, 2001; Kawakami *et al.*, 1999; Ladher *et al.*, 2000; Lako *et al.*, 1998), and Frzb-1 is particularly prominent in epiphyseal prearticular chondrocytes (Hoang *et al.*, 1996). Recent evidence indicates also that Wnt-5a misexpression delays chondrocyte maturation and long bone development, whereas Wnt-4 misexpression accelerates it (Hartmann and Tabin, 2000; Kawakami *et al.*, 1999).

Clearly, limb skeletogenesis involves complex interplays between Wnt signaling and possible modulation by Sfrp proteins. This is in line with current thinking that Wnt roles in development depend on a fine balance between Wnt action and Sfrp antagonistic action (Duprez *et al.*, 1999; Finch *et al.*, 1997; Ladher *et al.*, 2000; Wada *et al.*, 1999). We are, however, far from having a complete and detailed understanding of Wnt roles in skeletogenesis. There is even less information on Sfrp roles. This is particularly regrettable given the intriguing strong expression of Frzb-1 during the early steps of chondrogenesis and at the epiphyseal end of long bone anlagen (Hoang *et al.*, 1996). This suggests that Frzb-1 modulates Wnt signaling at specific sites and times during skeletogenesis and may be involved in selective developmental events, including genesis of permanent articular chondrocytes. With these and other questions in mind, we carried out the present study in which we first determined in detail Frzb-1 gene expression patterns in developing chick long bones. To gain insights into Frzb-1 roles, we misexpressed it in the developing limb and in chondrocytes in culture and determined its effects on chondrocyte development, behavior, and function. The effects of Frzb-1 misexpression were contrasted to those elicited by misexpression of Wnt-8, a target of Frzb-1 antagonistic action expressed in cartilage, or constitutively active and dominant-negative forms of Wnt nuclear mediator LEF-1.

MATERIALS AND METHODS

In Situ Hybridization and Northern Blots

Whole-mount and tissue-section *in situ* hybridization was carried out by using digoxigenin-conjugated or ^{35}S -labeled riboprobes (Koyama *et al.*, 1995). Most of the cDNA clones used were described previously (Iwamoto *et al.*, 1993). A chick Ihh cDNA was a gift of Dr. C. Tabin (Harvard Medical School, MA); and a chick MMP13 clone (nucleotide 607-1238 of AF040748) was amplified by RT-PCT and subcloned into pGEM-T vector (Promega Corporation, Madison, WI). The chick FRZB clone was described elsewhere (Wada *et al.*, 1999). Whole cellular RNAs isolated from tissues and cultured cells (Smale and Sasse, 1992) were denatured by glyoxalation, electrophoresed on agarose gels, transferred to Hybond-N membrane (Amersham Life Science, Tokyo, Japan) by capillary blotting, and hybridized to ^{32}P-labeled cDNA probes (Iwamoto *et al.*, 1993).

Construction of Frzb-1, Wnt-8, Wnt-4, and LEF-1 Viral Vectors

To facilitate construction of recombinant retrovirus vectors, we inserted a polylinker into the *Cla*I site of RCASBP(A) and

RCASBP(B) vectors (Fekete and Cepko, 1993). Briefly, oligonucleotides 5′-CGATGCATCACGTGACTAGTTCGAAGCGGCCGC-3′ and 5′-CGGCGGCCGCTTCGAACTAGTCACGTGATGCAT-3′ were annealed, phosphorylated, and ligated into *Cla*I-digested RCASBP vectors. The resulting modified vectors [RCASBP(A) L-14 and RCASBP(B)L-14] have unique restriction sites in the following order: *Cla*I, *Nsi*I, *Spe*I, and *Not*I. The entire coding sequence of mouse Frzb-1 (sFRP3) (Rattner *et al.*, 1997) was amplified from E14 mouse limb bud total RNA by RT-PCR and subcloned into the *Eco*RV site of pBluescript SK+ vector (Stratagene, La Jolla, CA). The sequence was identical to that of a sFRP3 clone from a mouse cDNA library obtained from Dr. J. Nathans (Johns Hopkins University). After sequence verification, insert was released by *Cla*I/*Not*I digestion and subcloned into the *Cla*I/*Not*I site of RCASBP(A) L-14. Entire coding sequence of mouse Wnt-8A (Bouillet *et al.*, 1996) plus influenza virus hemagglutinin (HA) epitope tag (termed Wnt-8-HA) was generated by PCR with 5′-CATATCGATGGGACCATGGGACACTTGTTAATGCTG-3′ and 5′-CTAAGCATAGTCTGGGACATCATATGGATACCAGGCACTGTCCTTGC-3′ using mouse Wnt 8 cDNA clone Stra11 (kindly provided by Dr. P. Chambon, IGBMC, Strasbourg) as template. Amplified fragment was subcloned into pGEM-T vector and subjected to sequence verification. Wnt-8-HA was released by *Cla*I/*Not*I digestion and subcloned into RCASBP(B)L-14. We also subcloned mouse Wnt-8 without HA tag and chick Wnt-4 (Yoshioka *et al.*, 1994) into RCASBP(B)L-14 to make sure that the tag does not alter Wnt-8 biological activity. Sequences encoding LEF-DN, which lacks amino acids 17–264 of murine LEF-1, and LEF-CA, which includes amino acids 695–781 of β-catenin fused to the C terminus of LEF-DN, were subcloned into RCAS (A) vector. Both LEF constructs (Vleminckx *et al.*, 1999) contain sequences encoding HA tag. Recombinant viral particles were prepared in chick dermal fibroblast cultures and concentrated by centrifugation (Enomoto-Iwamoto *et al.*, 1998).

Cell Cultures and Viral Infection

Chondrocytes isolated from the caudal and cephalic portions of Day 17 chick embryo sterna (line M) (Nisseiken, Yamanashi Japan) were cultured in high-glucose DMEM containing 10% FBS (Pacifici *et al.*, 1991a). When indicated, freshly isolated chondrocytes were infected with concentrated viral preparations and subcultured at the density of about $6.0 \times 10^4/cm^2$ by trypsinization after 1 week, and maintained in complete medium containing 10 μg/ml of ascorbic acid. By passage one, more than 85% of the cells were routinely infected as revealed by immunocytochemistry of viral antigens (Enomoto-Iwamoto *et al.*, 1998); expression of introduced genes was confirmed by Western blot (see Fig. 8K for Frzb-1 and Wnt-8, see Figs. 9B–9H for LEF-DN and LEF-CA). For matrix mineralization, cultures were provided with 1 mM β-glycerophosphate (Iwamoto *et al.*, 1993). DNA and glycosaminoglycan (GAG) contents were determined as described (Enomoto-Iwamoto *et al.*, 1998). Alkaline phosphatase (APase) activity associated with the cell layer was measured by using p-nitrophenyl phosphate (pNP) as a substrate (Pacifici *et al.*, 1991b).

Wnt Expression

Whole cellular RNAs were isolated from Day 10 chick embryo cartilaginous tibiotarsus (containing small amounts of adjacent perichondrial tissues) and lower sternal chondrocyte cultures. RNAs were processed for RT-PCR (Enomoto-Iwamoto *et al.*, 1998) by using the following primer pairs: 5′-GAAACTCACCAGCCTGGGACTA-3′ and 5′-GTTTGGGTGCCAGTACCTGACT-3′ for chick MMP2 (Aimes *et al.*, 1994), generating a 435-bp fragment; 5′-CTTCGTGGAGAAATGCTGGTCTT-3′ and 5′-TCGCAGAACTCTGCTTTCCTCTA-3′ for chick MMP13 (Lei *et al.*, 1999), generating a 632-bp fragment; and 5′-GCATCGTGATTGGCGATGATGA-3′ and 5′-GTCGAGGGCGTATCCAACAACA-3′ for chick hypoxanthine guanine phosphoribosyl transferase (HPRT) (AJ132697), generating a 653-bp fragment. The Wnt primers were: 5′-AGAGGCGTCTGATCCGACAGAAC -3′ and 5′-GCTGGTGATGGCGAAGATGAAC-3′ for chick Wnt 1 (Bally-Cuif and Wassef, 1994), generating a 194-bp fragment; 5′-ATCCGTGAGTGCCAGTACCAATTC-3′ and 5′-TCGTGGGCTTCCTGAAGTTCTTG-3′ for chick Wnt 2b (Kawakami *et al.*, 2001), generating a 577-bp fragment; 5′-ATGGGATCTGCGTGGAAGAAGTG-3′ and 5′-AAGGCGGATTCCCTGGTAGCTTTG-3′ for chick Wnt 3a (Kawakami *et al.*, 2000), generating a 326-bp fragment; 5′-GCAACGTGCTGAAGGAGAAATTCG-3′ and 5′-ATGGCAGGGTTTGCACTTGACAG-3′ for Wnt 4 (Yoshioka *et al.*, 1994), generating 335-bp fragment; 5′-CAACAATGAGGCTGGGAGGAGAAC-3′ and 5′-GCACACAAACTGGTCCACGATCTC-3′ for chick Wnt 5a (Kawakami *et al.*, 1999), generating a 475-bp fragment; 5′-CCGCCGACATCAGATACGGAATAG-3′ and 5′-TGAGGAAGGTTGGACGCTTGTTGC-3′ for chick Wnt 7a (Kawakami *et al.*, 2000), generating a 300-bp fragment; 5′-GCATAAGCTGGAGATGGACAAGA-3′ and 5′-ATCAGGAGTGCTTCCAAGCAAC-3′ for chick Wnt8C (Hume and Dodd, 1993), generating a 739-bp fragment; 5′-TGTCAGCCACCAAGGTCGTTCATC-3′ and 5′-GACGAGGTGCTTTCATTTGCAGAC-3′ for chick Wnt 11 (Kawakami *et al.*, 1999), generating a 353-bp fragment; and 5′-CCTTCCTCTACGCCATTTCTTCTGC-3′ and 5′-GCTGCTTCCCTATTTCGTGGAATG-3′ for chick Wnt 14 (Bergstein *et al.*, 1997), generating a 360-bp fragment.

Immunoblot

Nuclear and cytoplasmic preparations were isolated by slight modification of previous methods (Kawabe *et al.*, 1999; Hsieh *et al.*, 1999). Proteins were separated by electrophoresis on 10.0% gels and transferred to PVDF membranes (Millipore Japan, Tokyo, Japan). After blocking, membranes were incubated with rat anti-HA epitope monoclonal antibody 3F10 (Roche Molecular Biochemicals, Mannheim, Germany), rabbit polyclonal antibodies raised against a synthetic peptide of human or mouse β-catenin (amino acids 768–781) (Sigma, St. Louis, MO), or goat antibodies to mouse sFRP-3 (Frzb-1) (R&D Systems, Inc, Minneapolis, MN). Bound antibodies were visualized by incubation with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) followed by peroxidase-conjugated Streptavidin (Jackson ImmunoResearch, West Grove, PA) and color development.

Histochemistry and Immunochemistry

Proteoglycan accumulation and APase activity in cell layer were visualized by histochemistry (Enomoto-Iwamoto *et al.*, 1998). For immunohistochemistry, paraffin sections of Day 15 chick embryo tibiotarsus were treated with 1500 units/ml bovine testis hyaluronidase in PBS containing 10% FBS (Sigma) for 3 h at 37°C and then with 0.1% pepsin in 0.02 N HCl for 15 min at 37°C. Sections were blocked with 10% goat serum and reacted with a 1:2000 dilution of the rabbit antibodies human or mouse β-catenin. For immunocytochemistry, chondrocyte cultures were fixed with 3.7% formalde-

hyde, permeabilized with 0.05% Triton X-100 in PBS, and incubated with rat anti-HA epitope monoclonal antibody (3F10) or a 1:200 dilution of rabbit polyclonal antibodies raised against a synthetic peptide of human β-catenin (amino acids 680–781) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Bound antibodies were visualized by incubation with biotinylated secondary antibodies (Vector Laboratories) followed by Cy3-conjugated Streptavidin (Jackson ImmunoResearch).

Zymography

When indicated, chondrocyte cultures were switched to serum-free medium and grown for 24 h. Medium conditioned during this period was harvested, clarified by centrifugation at 5000 rpm for 5 min, and stored at –70°C until further analysis. Aliquots were processed for zymography to detect MMP activity, using a commercial kit (Yagai Co., Yamagata, Japan). Briefly, samples were electrophoresed on polyacrylamide gradient gel containing 0.1% gelatin. Gels were incubated in reaction buffer at 37°C for 16 h and stained with Coomassie Brilliant Blue (CBB) (Fluka Chemie, Buchs, Switzerland) to reveal clear bands of proteolytic activity. Identity of MMP-2, pro MMP-2, and MMP-9 bands was based on standard samples included in the kit.

RESULTS

Frzb-1 Expression in the Developing Limb

We first determined the gene expression patterns of Frzb-1 during limb skeletogenesis. Whole-mount *in situ* hybridization on Day 6.5 chick embryos showed that Frzb-1 gene expression was particularly strong in prechondrogenic areas, such as the distal tip of digits (Fig. 1A, arrow) (Ladher *et al.*, 2000). Expression was lower in the cartilaginous portion of each phalange, which instead contained chondrocyte-characteristic type II collagen transcripts throughout their length (Fig. 1B, arrows) and Indian hedgehog (Ihh) transcripts in their diaphyseal areas (Fig. 1C, arrowheads). To determine Frzb-1 expression patterns during chondrocyte maturation, we processed longitudinal serial sections of Day 10 tibiotarsus for *in situ* hybridization. At this stage of development, the tibiotarsus contains a well-defined epiphyseal cap where resting prearticular chondrocytes reside, and a growth plate extending from metaphysis to diaphysis that contains proliferative, prehypertrophic, and hypertrophic zones of chondrocyte maturation (Fig. 1D) (Pacifici, 1995). Frzb-1 transcripts were abundant in epiphyseal articular cap (Fig. 1E, arrow) and were present in underlying proliferative (Fig. 1E, double arrow) and prehypertrophic zones (Fig. 1E, arrowhead). Interestingly, expression in hypertrophic zone was minimal (Fig. 1E, double arrowhead) (Hoang *et al.*, 1996). Frzb-1 expression in prehypertrophic zone partially overlapped that of Ihh (Fig. 1F, arrowhead), whereas there was minimal overlap with type X collagen expression characteristic of hypertrophic chondrocytes (Fig. 1G, double arrowhead). Similar overall Frzb-1 expression patterns were seen in other skeletal elements, such as Day 13 chick embryo phalanges (Figs. 1H–1I).

Effects of Frzb-1 Misexpression in Vivo

The restricted Frzb-1 expression patterns above suggest that it may have specific roles in chondrocyte behavior and maturation, possibly modulating action of locally produced Wnt proteins. To test this possibility, we misexpressed Frzb-1 in the developing chick limb, using the replication-competent viral vector RCAS (Fekete *et al.*, 1993). Viral particles encoding Frzb-1 or insert-less control particles were injected in the anterior middle portion of Day 3.5 (HH stage 22–23) chick embryo wing buds, aiming to infect the mesenchymal condensations of radius, ulna, or both. Embryos were reincubated and examined at later stages. Anatomical inspection of control Day 11 embryos showed that ulna and radius displayed normal length, shape, and organization (Fig. 2A). Their epiphyses and metaphyses were cartilaginous (Fig. 2A, arrows), and their diaphysis contained endochondral bone, marrow, and blood cells (Fig. 2A, arrowheads). In the embryo injected with Frzb-1-RCAS virus (Fig. 2E), the ulna had remained uninfected (see below), was similar to control, and contained a normal blood/marrow/bone-rich diaphysis (Fig. 2E, arrowhead). In contrast, the radius was infected, was entirely cartilaginous, contained no sign of endochondral ossification (Fig. 2E, double arrowhead), and was wider and about 25–30% shorter than control. It is of interest that shortening of the radius was accompanied by a significant bending of the ulna (Fig. 2E).

Histological and histochemical analyses confirmed that the diaphyses of ulna and radius in control embryo and the diaphysis of ulna in experimental embryo were undergoing normal ossification processes (Figs. 2B and 2F, arrowheads). They all contained endochondral bone and an intramembranous bone collar staining with alizarin red (Figs. 2C and 2G, arrowheads). In comparison, the diaphysis of Frzb-1-RCAS-infected radius was entirely cartilaginous (Fig. 2F, double arrowhead), lacked endochondral bone and marrow, and was surrounded by a much reduced bone collar barely staining with alizarin red (Fig. 2G, double arrowhead). The epiphyseal ends of Frzb-1-misexpressing skeletal elements were often abnormal (5/8), resulting in joint dismorphogenesis. For example, the wrist region in one infected embryo contained abnormally enlarged and fused cartilaginous skeletal elements (Fig. 2H) and drastically increased Frzb-1 transcripts (Fig. 2H, inset), reflecting endogenous plus virally driven expression; the wrist of control uninfected embryo displayed well-defined and smooth-contoured elements (Fig. 2D) and normal Frzb-1 expression (Fig. 2D, and inset).

To define the molecular phenotype of growth plate chondrocytes, serial longitudinal sections of Day 10 control and Frzb-1-misexpressing radii were hybridized with cDNA clones encoding: (A) Ihh; (B) collagen X; (C) osteopontin; (D) MMP-13; and (E) Frzb-1. In control tissues, transcripts for these proteins had typical patterns (Figs. 3A–3E). For example, osteopontin (OP) transcripts were abundant in hypertrophic and posthypertrophic chondrocytes (Fig. 3C,

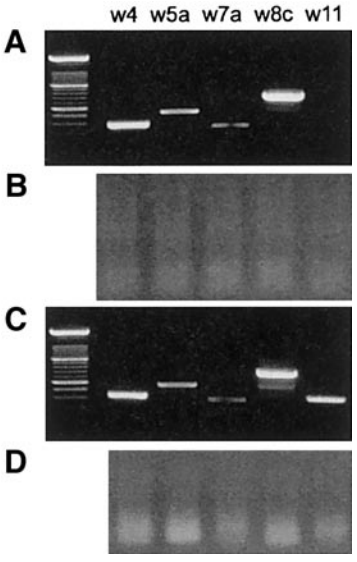

FIG. 4. Expression of various Wnts in cartilage and cultured chondrocytes. Total RNA from cultures of Day 17 chick embryo sternal chondrocytes isolated from the sterna (A, B) and Day 10 chick embryo tibiotarsus and associated perichondrium (C, D) was analyzed by RT-PCR (A and C), using primers for Wnt-4, -5a, -7a, -8c, and -11. No products were present in the absence of the reverse-transcription step (B, D). Left lanes of both panels are molecular weight markers.

arrow), diaphyseal endochondral bone (Fig. 3C, arrowhead), and intramembranous bone collar (Fig. 3C, double arrowhead); and MMP-13 transcripts were restricted to posthypertrophic chondrocytes (Fig. 3D, arrow). However, the diaphysis of Frzb-1-misexpressing radius, which had remained entirely cartilaginous, contained Ihh (Fig. 3F, arrows) and type X collagen transcripts (Fig. 3G, arrow) and markedly reduced levels of osteopontin transcripts, which were limited to a few diaphyseal chondrocytes (Fig. 3H, arrow) and a thin intramembranous bone collar (Fig. 3H, double arrowhead). There were no detectable MMP-13 transcripts (Fig. 3I). As to be expected, Frzb-1 RNA was very abundant and widely distributed, as a result of expression from cellular and viral genomes (Fig. 3J). Identical results were obtained when ulnas (rather than radii) were infected by Frzb-1-encoding virus. Clearly, Frzb-1 misexpression significantly delays both terminal chondrocyte maturation and ossification.

Opposite Effects of Frzb-1 and Wnt-8 on Chondrocyte Behavior in Vitro

To clarify the mechanisms underlying the latter phenomena and to establish that Frzb-1 action on chondrocytes is direct and not mediated by systemic changes, we carried out experiments with chondrocytes *in vitro*. Thus, we analyzed the responses of cultured chondrocytes to RCAS virus-driven misexpression of Frzb-1 and compared them with those elicited by misexpression of Wnt-4 and Wnt-8. We chose these Wnts because Wnt-4 stimulates endochon-

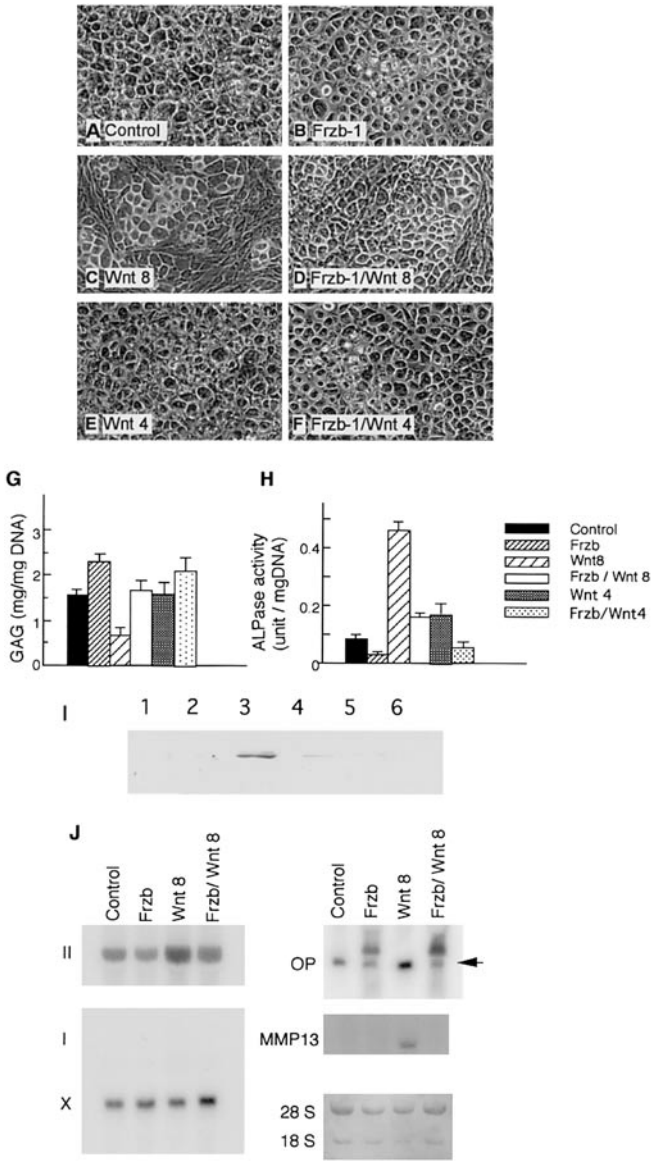

FIG. 5. Effects of Frzb-1, Wnt-4, and Wnt-8 on chondrocyte maturation in culture. (A–F) Phase micrographs of cultures infected with control (A), Frzb-1 (B), Wnt-8 (C), Frzb-1 plus Wnt-8 (D), Wnt-4 (E), or Frzb-1 plus Wnt-4 (F) viruses. (G, H) GAG content (G) and ALPase activity (H) in 2-week-old cultures infected with Frzb-1, Wnt 8, or both viruses. (I) Immunoblot analysis of β-catenin in nuclear fractions of 1-week-old cultures infected with control (lane 1), Frzb-1 (lane 2), Wnt-8 (lane 3), Frzb-1 plus Wnt-8 (lane 4), Wnt-4 (lane 5), or Frzb-1 plus Wnt-4 (lane 6). (J) Northern blot analysis of gene expression of type II collagen (II), type I collagen (I), type X collagen (X), osteopontin (OP), and MMP13 (MMP) in control and virus-infected cultures. Arrow points to position of osteopontin (OP) transcript.

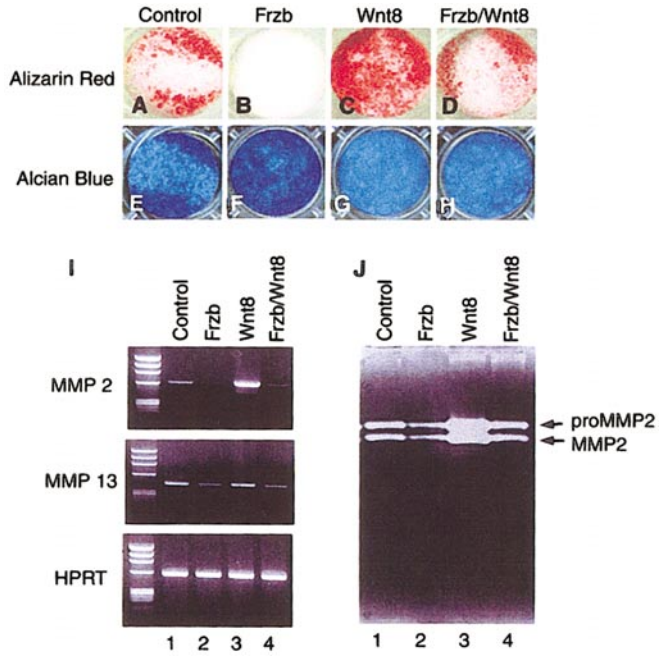

FIG. 6. Effects of Frzb-1 and Wnt 8 on matrix calcification and MMP activities. Alizarin red staining (A–D) and Alcian blue staining (E–H) to detect mineralization and proteoglycan accumulation in control cultures (A, E) and cultures infected with Frzb-1- (B, F), Wnt-8- (C, G), or Frzb-1 plus Wnt-8 (D, H) viruses. (I) RT-PCR analysis for MMP-2 and MMP-13 gene expression levels in similar cultures. (J) Zymogram to analyze MMPs activity. Note that compared with control, Wnt 8-infected cultures exhibited higher MMP-2 and MMP-13 activity, while Frzb-1-infected cultures had reduced activity.

dral ossification (Hartmann and Tabin, 2000) and Wnt-8 is a target of, and is antagonized by, Frzb-1 (Leyns *et al.*, 1997; Wang *et al.*, 1997a). In addition, RT-PCR revealed that both Wnts are expressed in chondrocytes *in vitro* (Fig. 4A) and cartilaginous skeletal elements and associated tissues *in vivo* (Fig. 4C); the group of expressed Wnts includes Wnt-4, -5a, -7a, -8c, and -11, but excludes Wnt-1, -2b, -3a, and -14.

Chondrocytes isolated from Day 17 chick embryo sterna, a convenient source of homogenous chondrocyte populations (Gibson and Flint, 1985), were immediately infected with viral particles encoding Frzb-1, Wnt-4, or Wnt-8; companion cultures were infected with insert-less control particles or were left uninfected. Microscopic analysis of 2-week-old caudal sternal cultures revealed that misexpression of Frzb-1 or Wnt-8 had affected cell shape and adhesion. Control cultures contained the expected mixtures of polygonal firmly adherent chondrocytes and oval–round chondrocytes (Fig. 5A). In contrast, Frzb-1-overexpressing chondrocytes were all round and had a highly refractile pericellular contour (Fig. 5B). Wnt-8-overexpressing chondrocytes were flatter and less refractile (Fig. 5C), and those overexpressing both Frzb-1 and Wnt-8 (encoded by subgroup A and B viruses) resembled control cells (Fig. 5D), suggesting that Frzb-1 had antagonized Wnt-8 action. Wnt-4-overexpressing chondrocytes were similar to control cells (Fig. 5E), and chondrocytes overexpressing both Frzb-1 and Wnt-4 resembled Frzb-1-overexpressing cells (Fig. 5F). The differences in cell appearance correlated well with differences in pericellular matrix proteoglycan content (Fig. 5G). Thus, compared with control cultures, Frzb-1 cultures contained higher proteoglycan levels, Wnt-8 cultures contained less, and Frzb-1/Wnt-8 cultures were similar to control (Fig. 5G). Proteoglycan content in Wnt-4 cultures was similar to control and that in Frzb-1/Wnt-4 cultures was similar to Frzb-1 cultures (Fig. 5G).

To investigate whether overexpression of Wnt-8 or Wnt-4 activates β-catenin-LEF/TCF pathway (Hsieh *et al.*, 1999; Papkoff and Aikawa, 1998), we examined the nuclear localization of β-catenin. Nuclear proteins extracted from 1-week cultures were analyzed by immunoblot (Fig. 5I). Nuclear fractions from Wnt-8 cultures contained a large amount of β-catenin (Fig. 5I, lane 3), whereas those from Frzb-1, Frzb-1/Wnt-8, and Wnt-4 cultures did not (Fig. 5I, lanes 1, 2, 4, and 5, respectively). This indicates that Wnt-8 activates β-catenin-LEF/TCF pathway and Frzb-1 antagonizes this activation in chondrocytes.

Next, we examined expression of phenotypic traits associated with the hypertrophic phase of chondrocyte maturation. Frzb-1 overexpression decreased APase activity, Wnt-8 overexpression strongly increased it, and Frzb-1/Wnt-8 coexpression led to intermediate APase levels (Fig. 5H). Wnt-4 also had a stimulatory effect on APase activity but the effect was mild (Fig. 5H), and APase activity in Wnt-4/Frzb-1 coinfected cultures was similar to that in Frzb-1 cultures (Fig. 5H). We also examined gene expression of collagen types X and I, osteopontin, and MMP-13. Frzb-1 overexpression led to a decrease in osteopontin gene expression (Fig. 5J). Wnt-8 overexpression had opposite effects and led to increased expression of both osteopontin and MMP-13 (Fig. 5J). The effects of Frzb-1 and Wnt-8 were largely, though not completely, neutralized by coexpression of Wnt-8 and Frzb-1 (Fig. 5J). Expression of collagen types II, I, and X was not dramatically affected by any condition tested (Fig. 5J).

Lastly, we examined whether Frzb-1 and Wnt-8 affect matrix mineralization, which is a terminal step required for replacement of cartilage with bone. Because of the well-established roles of metalloproteases in cartilage-to-bone transition (Vu *et al.*, 1998; Zhou *et al.*, 2000), activity of MMP-13 and MMP-2 was examined as well. Cephalic sternal chondrocytes infected with Frzb-1 virus, Wnt-8 virus, both viruses, or insert-less control virus were maintained in culture for 2 weeks. To allow matrix mineralization, cultures were provided with 1 mM β-glycerosphosphate, a phosphate donor, during the last 2 days of culture. Staining with alizarin red revealed that control cultures had deposited a substantial amount of alizarin red-positive mineral (Fig. 6A). However, there was no detectable mineral in Frzb-1 cultures (Fig. 6B), but Wnt-8 cultures had miner-

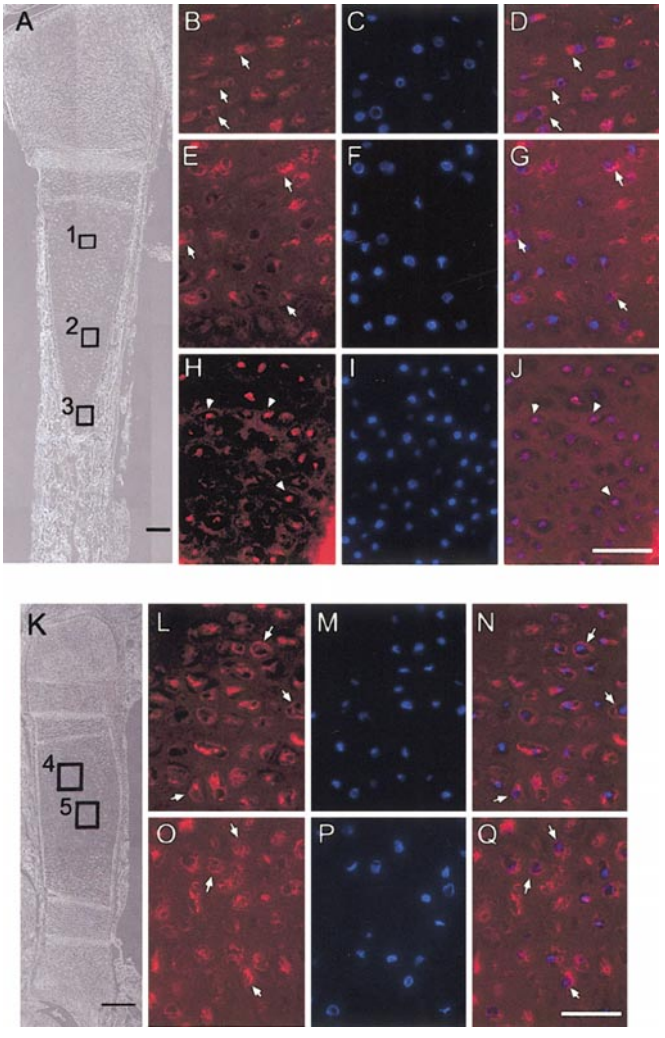

FIG. 7. Localization of β-catenin in the growth plate. Longitudinal sections of control and Frzb-1-misexpressing limbs (described in Fig. 2 legend) were processed for immunofluorescence with antibodies to the C terminus of β-catenin. (A–J) Radius in control limb; (B–D) Prehypertrophic zone (box 1 in A); (E–G) Nonmineralizing hypertrophic zone (box 2 in A); (G–J) Mineralizing hypertrophic zone (box 3 in A). (K–Q) Radius in Frzb-1-misexpressing limb; (L–N) Box 4 in (K); (O–Q) Box 5 in (K). (B, E, H, L, O) β-Catenin staining; (C, F, I, M, P) DAPI nuclear staining; (D, G, J, N, Q) Merged images of β-catenin and DAPI staining. Note that in control radius β-catenin was largely cytoplasmic in prehypertrophic (B–D, arrows) and nonmineralizing hypertrophic chondrocytes (E–G, arrows) but nuclear in mineralizing chondrocytes (H–J, arrowheads). In contrast, β-catenin was cytoplasmic throughout the Frzb-1-misexpressing radius (L–Q, arrows). Scale bars: (A, K) 100 μm; (B–J) and (L–Q) 25 μm

alized more than controls (Fig. 6C). Cultures overexpressing both Frzb-1 and Wnt-8 resembled control cultures (Fig. 6D). Staining with Alcian blue showed that proteoglycan content was inversely proportional to mineralization. Frzb-1 cultures (Fig. 6F) displayed much stronger Alcian blue staining than control and Wnt-8 cultures (Figs. 6E and 6G); cultures infected with both viruses had intermediate levels of staining (Fig. 6H). RT-PCR and zymography showed that Frzb-1 misexpression led to a significant decrease in MMP-13 and MMP-2 gene expression (Fig. 6I) and MMP-2 activity (Fig. 6J), whereas Wnt-8 misexpression increased these parameters (Figs. 6I–6J). Thus, Frzb-1 does inhibit the progression of chondrocytes toward terminal maturation, whereas Wnt-8 accelerates it.

Distribution of Wnt Mediator β-Catenin

Our results above show that β-catenin pathway mediates Wnt signaling in cultured chondrocytes. To determine whether this pathway operates in chondrocytes *in vivo*, serial sections of Day 12 chick embryo radius were processed for immunohistochemical detection of β-catenin distribution and histochemical nuclear Hoecht staining. Staining for β-catenin was observed in the prehypertrophic zone (Figs. 7A, box 1, and 7B), early hypertrophic zone (Figs. 7A, box 2, and 7E), and hypertrophic mineralizing zone (Figs. 7A, box 3, and 7H). Strikingly, while the bulk of β-catenin in prehypertrophic (Figs. 7B–7C, arrows) and early hypertrophic (Figs. 7E and 7F, arrows) chondrocytes was cytoplasmic, it was clearly nuclear in fully hypertrophic chondrocytes (Figs. 7H–7I, arrowheads). This shift was particularly evident in merged images (Figs. 7D, 7G, and 7J). It became obvious to ask next whether Frzb-1 misexpression would prevent β-catenin nuclear relocalization, in line with its ability to inhibit chondrocyte maturation. Indeed, sections from Frzb-1-misexpressing radius (Fig. 7K) showed that β-catenin remained largely cytoplasmic in every area and zone, including the diaphysis (Fig. 7K, boxes 4 and 5), which lacked fully mature hypertrophic mineralizing chondrocytes (Figs. 7L–7N and 7O–7Q).

To strengthen these observations, we determined whether a similar β-catenin nuclear relocalization occurs during chondrocyte maturation *in vitro*. Much of β-catenin was cytoplasmic in Day 5 proliferating and Day 15 matrix-synthesizing chondrocyte cultures (Figs. 8A–8C and 8D–8F, respectively), but was largely nuclear in Day 28 hypertrophic mineralizing cultures (Figs. 8G–8I). Immunoblot analysis of nuclear fractions from companion cultures revealed that nuclear β-catenin content gradually increased over time and maturation *in vitro* and became quite prominent at the terminal mineralization stage (Fig. 8J). We also asked whether modulation of Wnt signaling by overexpression of Frzb-1 or Wnt-8 would alter overall β-catenin content in cultured chondrocytes. Western blot analysis on Day 15 cultures showed that compared with control cells (Fig. 8K, lane 1), β-catenin content was low in Frzb-1-overexpressing chondrocytes (Fig. 8K, lane 2), high in Wnt-8-overexpressing chondrocytes (Fig. 8K, lane 3), and at intermediate levels in Frzb-1/Wnt-8 double-infected chondrocytes (Fig. 8K, lane 4). Parallel immunoblots with Frzb-1 and HA tag antibodies confirmed overexpression of Frzb-1 and/or Wnt-8 in the appropriate cultures (Fig. 8K).

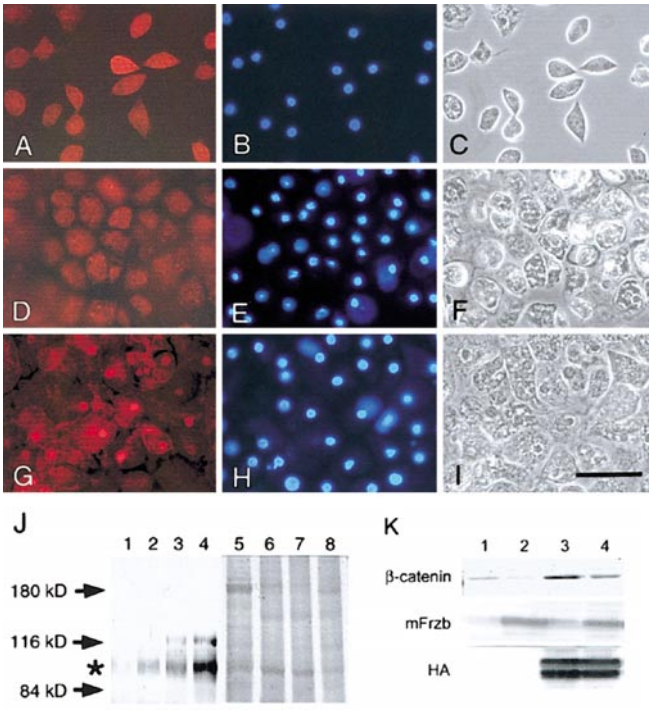

FIG. 8. Expression of β-catenin in cultured chondrocytes. (A–I) Localization of β-catenin in cultured chondrocytes. Day 5 (A–C), Day 15 (D–F), and Day 28 (G–I) cultures were processed for immunostaining with β-catenin antibodies (A, D, G), nuclear staining with DAPI (B, E, H), and phase microscopy (C, F, I). The same microscopic field is shown from each culture. Scale bar, 25 μm. (J) Nuclear fractions isolated from Day 5 (lanes 1 and 5), Day 15 (lanes 2 and 6), Day 21 (lanes 3 and 7), and Day 28 (lanes 4 and 8) cultures were separated by SDS–PAGE. One-half of the gel was immunoblotted with antibodies to β-catenin (lanes 1–4), and the other half was stained with Coomassie Blue (lanes 5–8) to verify equal protein loading/lane. Asterisk indicates position of β-catenin. Note the marked increase in nuclear β-catenin in mineralizing hypertrophic chondrocytes present in older cultures (G–I, J, lane 4). (K) β-Catenin, cytoplasmic fraction isolated from 15-day-old cultures infected with control (lane 1), Frzb-1 (lane 2), Wnt-8 (lane 3), or both (lane 4) viruses. Whole-cell proteins extracted from parallel cultures were examined by immunoblot with antibodies to mouse Frzb-1 (mFrzb) and HA antigen (HA).

Role of β-Catenin-LEF/TCF Signaling

To obtain evidence that the β-catenin-LEF/TCF signaling pathway regulates chondrocyte behavior and function, we constructed two RCAS vectors encoding a dominant-negative LEF-1 (LEF-DN) and a constitutive active LEF-1 (LEF-CA) (Fig. 9A) (Vleminckx *et al.*, 1999). The resulting viral particles were used to infect freshly isolated chondrocytes, and each population was grown in monolayer for 2 weeks along with control cultures. Immunoblot analysis with HA tag antibodies showed that LEF-DN and LEF-CA were each expressed in their respective chondrocyte population, with LEF-DN and LEF-CA exhibiting apparent sizes of 25 and 46 kDa, respectively (Fig. 9B, lanes 3 and 4). Immunoblots with β-catenin antibodies revealed presence of a 46-kDa protein only in chondrocytes infected with LEF-CA virus (Fig. 9B, lane 8) and lack of strong immunopositive bands in control and LEF-DN cells (Fig. 9B, lanes 6 and 7). In good correlation with these data, immunocytochemistry showed that the nuclei of LEF-DN- and LEF-CA-misexpressing cells were strongly positive (Figs. 9E–9F and 9G–9H, respectively), while control cells were not stained significantly (Figs. 9C and 9D).

Misexpression of LEF-DN and LEF-CA caused phenotypic changes in chondrocytes which were quite similar to those seen after misexpression of Frzb-1 and Wnt-8 above. Thus, LEF-DN misexpression led to increased proteoglycan content (Figs. 9I and 10E) and decreases in: APase activity (Fig. 9J), osteopontin expression (Fig. 9K, lane 2), mineralization (Fig. 9B), and MMP-13 expression (Fig. 10G, lane 3). In contrast, LEF-CA misexpression led to decreased proteoglycan content (Figs. 9I and 10F) and increases in: APase activity (Fig. 9J), osteopontin expression (Fig. 9K, lane 3), mineralization (Fig. 10C), MMP-2 and MMP-13 gene expression (Fig. 10G, lane 4), and MMP-2 activity (Fig. 10I, lane 3). Expression of type II collagen gene was not affected significantly by LEF-CA and LEF-DN (Fig. 9K). In sum, LEF-DN inhibits chondrocyte maturation as Frzb-1 does, whereas LEF-CA promotes maturation as Wnt-8 does.

DISCUSSION

Our study provides clear evidence that Frzb-1 exerts strong influences on skeletogenesis and is a powerful and direct modulator of chondrocyte maturation, phenotype, and function. The data support and greatly extend previous proposals on the possible roles of Frzb-1 in chondrocytes (Hoang *et al.*, 1996) and stress the idea that Wnt signaling in developing cells and structures involves fine balances and interrelationships between positive and negative mechanisms (Duprez *et al.*, 1999; Finch *et al.*, 1997; Ladher *et al.*, 2000; Wada *et al.*, 1999).

Frzb-1 and Long Bone Development

As summarized above, long bone development occurs through multiple steps (Pacifici *et al.*, 2000). It entails mesenchymal cell condensation, chondrogenic cell differentiation, and development of articular and growth plate chondrocytes. We find that Frzb-1 is initially expressed in condensed mesenchymal cells and then in articular cap and prehypertrophic zone of growth plate. These strict spatiotemporal expression patterns indicate that Frzb-1 has multiple roles in limb skeletogenesis. Frzb-1 gene expression in mesenchymal condensations may initially be needed to counteract action of local inhibitory Wnt(s) and allow cytodifferentiation to occur (Rudnicki and Brown, 1997). Subsequent roles of Frzb-1 in long bone development are

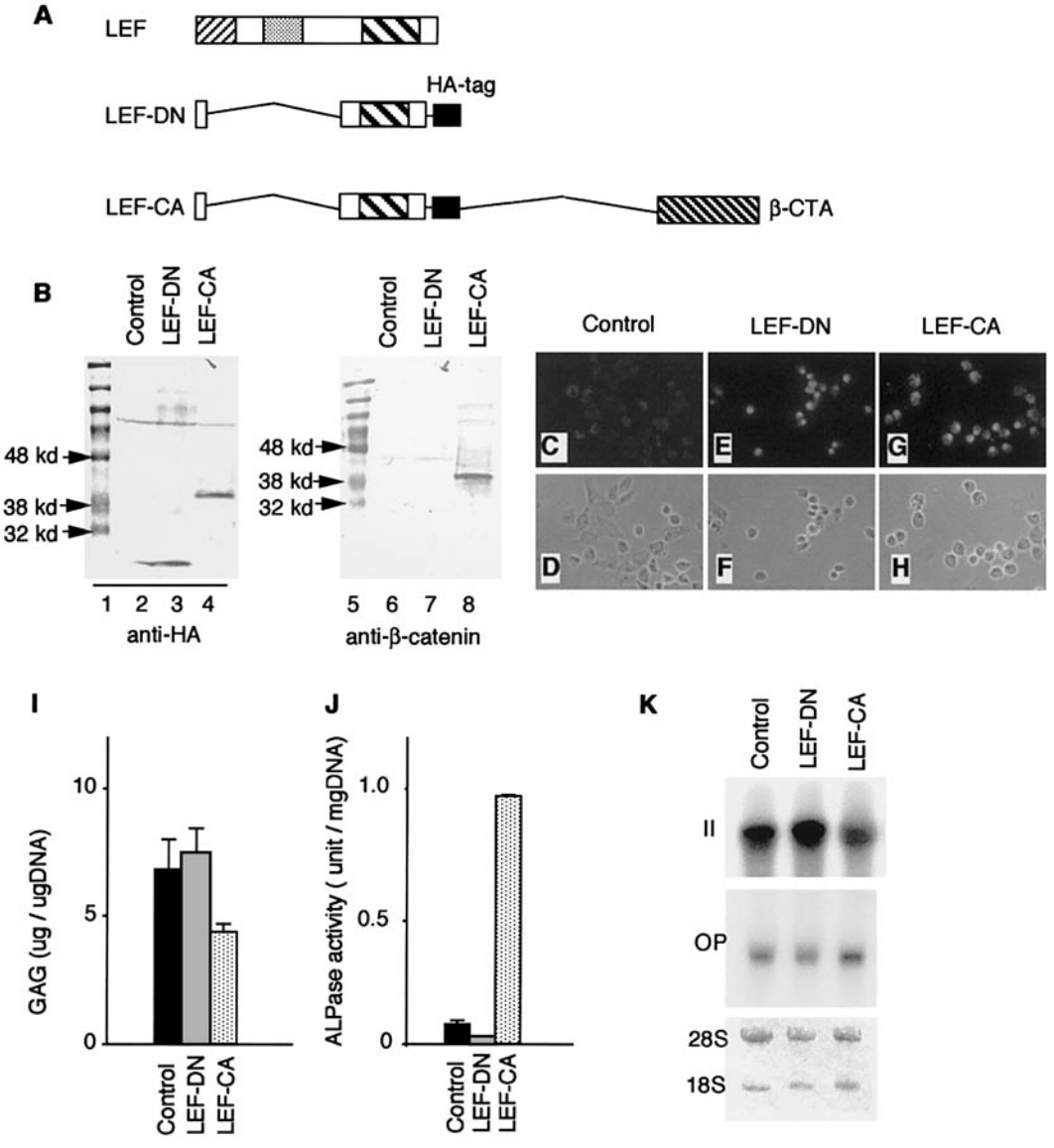

FIG. 9. Effects of LEF-DN and LEF-CA expression on chondrocyte maturation. (A) Schematic representation of LEF-DN and LEF-CA constructs compared with native LEF sequence. (B) Immunoblot analysis of LEF-CA and LEF-DN content in nuclei from control, LEF-CA- and LEF-DN-virus-infected cultures. LEF-DN (about 15 kDa) was detected in LEF-DN-virus-infected cultures (lane 2) with HA-tag antibodies; LEF-CA (about 35 kDa) was detected in LEF-CA-virus-infected cultures by using HA-tag (lane 4) or β-catenin (lane 8) antibodies. Lanes 1 and 5, Molecular weight markers. (C–H) Immunocytochemical localization of LEF-DN and LEF-CA in control and virus-infected cultures, using HA-tag antibodies. Note positive protein detection in nuclei of LEF-DN- and LEF-CA-virus-infected cultures (E, G) and negative staining of control culture (C). (D, F, and H) are phase micrographs of (C, E, and G), respectively. (I–K) GAG content (I), ALPase activity (J), and type II collagen and osteopontin transcript levels (K) in 2-week-old control and LEF-CA- or LEF-DN-virus-infected cultures. Northern blots (K) were prestained with methylene blue to mark the rRNA subunits.

suggested by our misexpression data. We show that forced Frzb-1 expression *in vivo* results in (1) altered epiphyseal morphogenesis and joint structure, (2) block of maturation at the early hypertrophic stage, and (3) prevention of cartilage invasion and endochondral ossification. In good agreement, Frzb-1 misexpression in cultured chondrocytes boosts expression of traits associated with the immature phenotype but inhibits traits associated with maturation. The Frzb-1-misexpressing chondrocytes display a round cell configuration, accumulate large quantities of pericellular proteoglycan-rich matrix, contain very low alkaline phosphatase activity, express low levels of metalloproteases, and

do not mineralize their matrix. It seems clear, therefore, that a primary role of Frzb-1 in limb chondrocytes is to limit their progression along the maturation pathway and contribute to determine behavior and function of chondrocytes in different portions of developing long bone anlagen. In the epiphysis, Frzb-1 would allow epiphyseal chondrocytes to maintain a functional, stable, and immature phenotype, organize the articular tissue and joint, and support joint function through life (Pacifici *et al.,* 2000) in cooperation with other secreted and intracellular factors, including the *ets* transcription factor C-1-1 (Iwamoto *et al.,* 2000). In the growth plate, Frzb-1 may cooperate with several other factors serving as negative modulators of maturation, including Ihh and members of the fibroblast growth factor family and their receptors (De Luca and Baron, 1999; Vortkamp *et al.,* 1996). Frzb-1 and these other inhibitors could counterbalance promaturation factors and molecules present or acting in the growth plate, such as IGF-1 and retinoids (Wang *et al.,* 1999; Koyama *et al.,* 1999), and would allow maturation and ossification to proceed at appropriate rates and occur at appropriate sites and times.

It is interesting to note that, in the growth plate of the Frzb-1-misexpressing anlagen, chondrocytes did reach the early hypertrophic type X collagen-expressing stage, but appeared unable to pass it and advance to the ultimate APase- and MMP-rich posthypertrophic stage. This is in line with our finding of lower MMP expression and activity in Frzb-1-misexpressing chondrocytes in culture. APase and metalloprotease activities are required for matrix degradation and mineralization and invasion of hypertrophic mineralized cartilage by surrounding osteoprogenitor and marrow mesenchymal cells (Inada *et al.,* 1999; Iwamoto *et al.,* 1993; Leboy *et al.,* 1989). It is thus possible that by reducing expression of these traits and possibly other traits, such as MMP-9 and MT-MMP1 (Vu *et al.,* 1998; Zhou *et al.,* 2000), Frzb-1 may have been able to effectively block endochondral ossification. An alternative but not mutually exclusive possibility is that Frzb-1 misexpression inhibits also production and release of angiogenic factors normally produced by late hypertrophic chondrocytes (Alini *et al.,* 1996; Carlevaro *et al.,* 2000). In the absence of these activities, mesenchymal and vascular cell recruitment and migration would be inhibited and hypertrophic cartilage would not be invaded, contributing to lack of endochondral ossification.

Wnt Roles

As summarized above, several Wnts are expressed in developing long bones. Wnt-4 and Wnt-14 are expressed at and around sites of joint formation, Wnt-5a and Wnt-11 are expressed in perichondrium, and Wnt-5b is expressed in prehypertrophic chondrocytes (Hartmann and Tabin, 2000, 2001; Kawakami *et al.,* 1999; Lako *et al.,* 1998). Our RT-PCR data now show that Wnt-7a and -8c are expressed as well. The fact that multiple Wnt proteins are expressed and display distinct patterns indicates that these signaling proteins have multiple roles in developing long bones. Long bone development might require subtle effects and local action by Wnt proteins at appropriate sites and times during limb skeletogenesis. In previous studies, Wnt-4 misexpression was found to promote maturation and endochondral ossification, while Wnt-5a inhibited it (Hartmann and Tabin, 2000; Kawakami *et al.,* 1999). Wnt-4 misexpression stimulated LEF-1 expression in perichondrium, but not in maturing chondrocytes themselves (Hartmann and Tabin, 2000). Our data now show that Wnt-8 is a very strong stimulator of chondrocyte maturation, Wnt-4 is a mild stimulator, and Frzb-1 is a strong inhibitor. In addition, Wnt-8 induces β-catenin nuclear translocation but Wnt-4 does not (Shimizu *et al.,* 1997). Previous functional interference studies have indicated that Wnt-8 is antagonized by Frzb-1, whereas Wnt-3a, -5a, and -11 are not (Wang *et al.,* 1997b). Taken together, our data indicate that Wnt-8 may be a direct stimulator of chondrocyte maturation and endochondral ossification, while Wnt-4 may exert similar positive influences through a direct action and mechanisms involving perichondrium.

In addition to Frzb-1, Wnt-8, and Wnt-4, other Wnts regulate skeletal development together with Frzb-1 and other Sfrps in positive or negative manners. For instance, by being expressed at and around incipient joints, Wnt-4 and Wnt-14 may be important for genesis and function of interzone or other joint structures (Hartmann and Tabin, 2000). The interzone is a mesenchymal structure which comes to subdivide the previously uninterrupted cartilaginous skeleton at sites of future joints and which participates in development of epiphyseal structures and articular chondrocytes (Hinchliffe and Johnson, 1983). The deleterious consequences seen after misexpression of Wnt-14 or Frzb-1 imply that normal spatiotemporal patterns of Wnt signaling are very important for joint development and that the joint is particularly sensitive to changes in signaling due to misexpression of a Wnt protein or Frzb-1. As another example, it is possible that perichondrium-derived Wnt-5a exerts its anti-maturation activity on chondrocytes together with such perichondrium-derived secreted factors as PTHrP (Lanske *et al.,* 1996) and in combination with growth plate inhibitors, including FGF-Rs (De Luca and Baron, 1999).

Mechanisms of Wnt Signaling

Wnt signaling is transduced via β-catenin and LEF/TCF proteins (Eastman and Grosschedl, 1999; Vleminckx *et al.,* 1999; Willert and Nusse, 1998) or activation of protein kinase C (Sheldahl *et al.,* 1999). Our results show that misexpression of dominant-negative or constitutively active LEF-1 constructs elicits phenotypic changes in cultured chondrocytes which are comparable with those elicited by Frzb-1 or Wnt-8, respectively. The data indicate that the β-catenin/LEF pathway mediates action of at least some Wnts in chondrocytes, as also concluded by others (Hartmann and Tabin, 2000). The misexpression data complement very well our observation that the distribution of

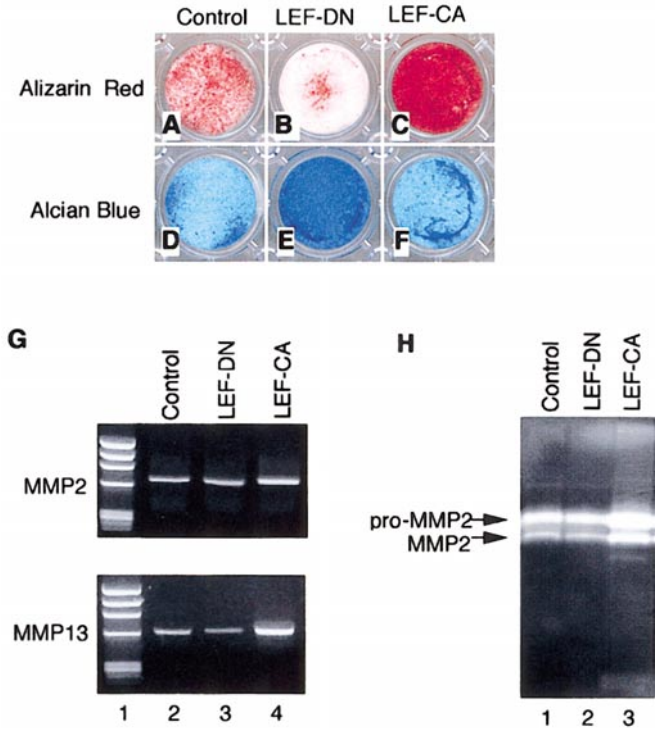

FIG. 10. Effects of LEF-DN and LEF-CA on matrix calcification and MMPs activities. Alizarin red staining (A–C) and Alcian blue staining (D–F) to detect mineralization and proteoglycan accumulation in control cultures (A, D), LEF-DN- (B, E), LEF-CA-virus-infected (C, F) cultures. (G) RT-PCR analysis of MMP2 and MMP13 gene expression in similar cultures. (I) Zymogram to analyze MMPs activity. Note that MMP expression and activities were higher in LEF-CA-virus-infected cultures and lower in LEF-DN virus-infected cultures compared with controls.

endogenous β-catenin changes dramatically during chondrocyte maturation. While most immunologically detectable β-catenin is cytoplasmic in the upper zones of control growth plate and in proliferating chondrocytes in culture, the bulk of it is nuclear in hypertrophic mineralizing chondrocytes. This change in β-catenin distribution is normally accompanied by a sharp drop in Frzb-1 endogenous expression in the hypertrophic zone and is prevented by Frzb-1 misexpression *in vivo* and vitro. Thus, it appears quite likely that the late hypertrophic stages of maturation require vigorous Wnt signaling and are particularly sensitive to Frzb-1 antagonistic action. We do not know whether β-catenin relocalization to the hypertrophic chondrocytes' nucleus involves inhibition of glycogen synthase kinase-3 (which phosphorylates β-catenin and targets it for degradation), activation of casein kinase I (which stabilizes β-catenin), or other Wnt signal transducing mechanisms identified in other systems (Peters *et al.*, 1999; Tago *et al.*, 2000; Yost *et al.*, 1998). It is likely, however, that such mechanisms operate in chondrocytes as well, given the conserved nature of Wnt signal transduction mechanisms.

ACKNOWLEDGMENTS

We thank Dr. J. Nathans (Johns Hopkins University) for providing murine sfrp3 cDNA, Dr. P. Chambon (IGBMC, Strasbourg) for mouse Wnt-8 cDNA, Dr. C. L. Cepko (Harvard Medical School) for RCASBP(A) and RCASBP(B) retroviral vectors, Dr. A. Hecht (Max Plank Institute of Immunology) for LEF mutant constructs, and Dr. C. Tabin (Harvard Medical School) for chick Ihh cDNA. This work was supported by grants-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan (to M.E.-I. and M.I.) and by NIH Grants AR46000 and AR47543 (to M.P.).

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Received for publication October 19, 2001
Revised May 1, 2002
Accepted July 31, 2002
Published online September 24, 2002