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The CREB family of activators is required for endochondral bone development

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SUMMARY

We have evaluated the importance of the CREB family of transcriptional activators for endochondral bone formation by expressing a potent dominant negative CREB inhibitor (A-CREB) in growth plate chondrocytes of transgenic mice. A-CREB transgenic mice exhibited short-limbed dwarfism and died minutes after birth, apparently due to respiratory failure from a diminished rib cage circumference. Consistent with the robust Ser133 phosphorylation and, hence, activation of CREB in chondrocytes within the proliferative zone of wild-type cartilage during development, chondrocytes in A-CREB mutant cartilage exhibited a profound decrease in proliferative index and a delay in hypertrophy. Correspondingly, the expression of certain signaling molecules in cartilage, most notably the Indian hedgehog (Ihh) receptor patched (Ptch), was lower in A-CREB expressing versus wild-type chondrocytes. CREB appears to promote *Ptch* expression in proliferating chondrocytes via an Ihh-independent pathway; phospho-CREB levels were comparable in cartilage from *Ihh*$^{-/-}$ and wild-type mice. These results demonstrate the presence of a distinct signaling pathway in developing bone that potentiates Ihh signaling and regulates chondrocyte proliferation, at least in part, via the CREB family of activators.

Key words: CREB, Phosphorylation, Indian hedgehog, Mouse, Bone

INTRODUCTION

Endochondral bone formation is a multi-step process involving the establishment of a cartilage model that is later replaced by calcifying elements that comprise the mature bone. Mesenchymal cells in the primordial limb initially condense to form cartilage that contains uniformly arrayed chondrocytes (Hinchcliffe and Johnson, 1990). Later in development, chondrocytes at the ends of the cartilage model organize into a proliferative zone of cells. As cells exit this proliferative zone, they become hypertrophic, elaborate a distinct cartilage matrix, and eventually undergo programmed cell death (Poole, 1991).

The progression of chondrocytes through each developmental zone of the cartilage model is tightly regulated by a number of key signaling molecules. Indian hedgehog (Ihh), a secreted protein that is expressed predominantly in pre-hypertrophic and early hypertrophic chondrocytes, functions as a central organizer in this process (St-Jacques et al., 1999). Ihh controls the position of the hypertrophic layer within the developing cartilage element, in part, by stimulating expression of the gene for parathyroid hormone-related protein (PTHrP) (Vortkamp et al., 1996). Initially characterized as a humoral factor underlying malignancy-associated hypercalcemia (Broadus and Stewart, 1994), PTHrP keeps chondrocytes in the proliferative pool and delays chondrocyte hypertrophy via the PTHrP receptor (Chung et al., 1998; Karaplis et al., 1994; Lanske et al., 1996), a G-protein-coupled receptor that acts via the cAMP second messenger pathway.

The second messenger cAMP regulates cellular gene expression via the PKA-mediated phosphorylation of CREB (cAMP responsive element-binding protein) at Ser133 (Gonzalez and Montminy, 1989). Targeted disruption of the CREB gene results in neonatal lethality, owing to defects in lung maturation (Rudolph et al., 1998). Except for an impairment in T-cell development, other tissues develop normally in CREB-null mice, however, due in part to functional compensation by other CREB family members CREM (cAMP responsive element modulator) and ATF1 (activating transcription factor 1; Hummler et al., 1994). CREB and its paralogs ATF1 and CREM, contain a highly conserved kinase inducible domain (KID) that recruits the co-activator CREB binding protein (CBP) in a phosphorylation-dependent manner. The solution structure of the CREB:CBP complex reveals that KID undergoes a random coil to amphipathic helix transition upon binding to the KIX domain of CBP (Radhakrishnan et al., 1997); and this transition stabilizes the KID:KIX interaction via hydrophobic contacts with a shallow groove in the KIX domain. Consistent with the ability of each factor to promote cellular gene expression in response to cAMP, all of the hydrophobic contact residues in CREB are shared by ATF1 and CREM (Radhakrishnan et al., 1997).

In addition to cAMP, several growth factor signaling pathways, including insulin-like growth factor (IGF), epidermal growth factor (EGF), transforming growth factor β (TGFβ) fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF), have also been shown to promote the phosphorylation of CREB family members (Cesare and Sassone-Corsi, 2000; Montminy, 1997; Shaywitz and Greenberg, 1999), suggesting a potential role for these proteins in cell proliferation. In this regard, overexpression of a phosphorylation-defective (Ser133Ala) CREB polypeptide in somatotrophs of the anterior pituitary, for example, leads to congenital dwarfism with markedly reduced numbers of somatotrophs (Struthers et al., 1991). Proliferation of hepatocytes in response to partial hepatectomy, moreover, is delayed in CREM-null relative to wild-type mice (Servillo et al., 1998). Whether the CREB family of activators actually promotes cellular proliferation in vivo, however, has not been determined.

We have examine the importance of the CREB family for endochondral bone formation by expressing a potent dominant negative CREB inhibitor, referred to as A-CREB (Ahn et al., 1998), under control of the cartilage-specific collagen type II promoter/enhancer (Nakata et al., 1993; Schipani et al., 1997). The A-CREB polypeptide contains the CREB leucine zipper plus an acidic domain that extends the dimerization interface with CREB, ATF1 and CREM, and blocks the basic regions of the wild-type proteins from binding to DNA (Ahn et al., 1998). A-CREB is highly selective for CREB family members; this inhibitor does not associate detectably with other bZIP family members (Ahn et al., 1998).

A-CREB transgenic mice show short-limbed dwarfism, owing to a defect in cellular proliferation and a subsequent delay in differentiation. Remarkably, the expression pattern of certain key signaling molecules in developing bones of A-CREB transgenic mice is markedly attenuated compared with that found in wild-type littermates. Taken together, these results provide the first evidence that the CREB family of transcriptional activators regulates cellular proliferation during development.

MATERIALS AND METHODS

Transgenic mice

The collagen type II:A-CREB expression vector was constructed by cloning the A-CREB cDNA into a col II expression vector containing an upstream collagen promoter/enhancer and downstream bovine growth hormone polyadenylation signal (generous gift from Y. Yamada, NIH). A-CREB transgenic animals were generated from FVB mice (Taconic). Transgenic mice were identified by PCR amplification of the A-CREB cDNA. Homozygosity of the transgene

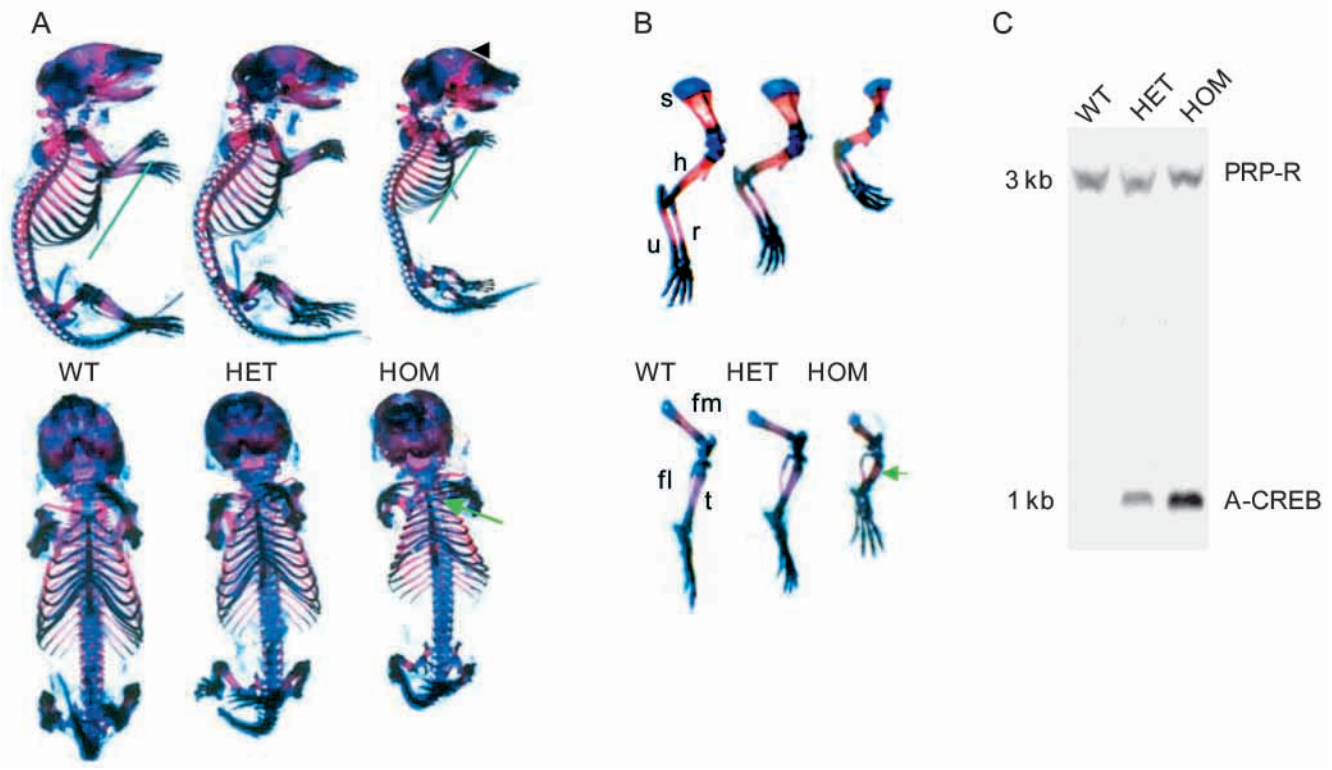

Fig. 1. Expression of dominant negative A-CREB inhibitor in chondrocytes results in short-limbed dwarfism and perinatal lethality, owing to a reduced rib cage circumference. (A) Whole skeleton preparations of 18.5 dpc littermates containing 0 (WT), 1 (HET) or 2 alleles (HOM) of the A-CREB transgene. When viewed from the side (upper panel), the rib cage (indicated by green lines) of the homozygous A-CREB mouse is considerably shorter than either wild-type or heterozygous littermates. The dome-shaped skull vault in homozygous A-CREB mouse is indicated with black arrowhead. The ventral view (lower panel) shows that upper portion of the homozygous mutant rib cage (arrow) is restricted. (B) Comparison of forelimbs (upper panel) and hindlimbs (lower panel) in same embryos showing progressive shortening of hind and fore limbs from wild-type to homozygous A-CREB embryos. Arrow indicates tibial bowing in homozygous A-CREB mice. fl, fibula; fm, femur; h, humerus; r, radius; s, scapula; t, tibia; u, ulna. (C) Representative Southern blot assay of mouse genomic DNA digested with *Hin*dIII. Position of 0.9kb A-CREB hybridizing band indicated. PTHrP receptor (PrP-R) shown as internal control.

was determined by genomic Southern blot hybridization. Mouse genomic DNA was digested with *Hin*dIII and Southern analyses carried out with A-CREB and PTHrP-receptor probes. Expression levels of the A-CREB transgene were evaluated by northern blot analyses using total RNA harvested from cultured rib cage chondrocytes.

Analyses of embryos and chondrocyte cultures

Chondrocyte proliferation in wild-type and A-CREB transgenic mice was evaluated by BrdU-labeling experiments. Bromodeoxy uridine (BrdU; 10 mg/ml) and fluorodeoxy uridine (FdU; 0.1 mg/ml) were injected intraperitoneally (100 μl/10 g body weight) into pregnant females 2 hours prior to sacrifice. Skin samples were collected from each embryo for genotyping; and limbs were fixed overnight at room temperature in 10% formalin buffered in 1×PBS, rinsed in 1×PBS and transferred to 70% ethanol. Fixed limbs were processed, embedded in paraffin and sectioned at 6 μm. BrdU detection was performed using a Zymed kit. ^{35}S in situ hybridization was performed as previously described (Schipani et al., 1997). Whole skeletons from embryos were prepared according to McLeod (McLeod, 1980). Chondrocytes were prepared based on de Crombrugghe, from the ventral half of rib cage of 16.5 dpc embryos (Lefebvre et al., 1994). Cells derived from each embryo were plated out independently and skin samples were collected for genotyping. Transient transfections on primary chondrocyte cultures were carried out using Fugene 6 reagent (Boehringer Mannheim). Chick chondrocytes were harvested from day 12 embryonic tibias, according to Kim and Conrad (Kim and Conrad, 1977). Cells were dissociated and nuclear extracts were prepared (Schreiber et al., 1989) in the presence of phosphatase inhibitors (okadaic acid).

RESULTS

To evaluate the role of CREB family members in endochondral bone formation, we developed transgenic mice expressing the dominant negative CREB inhibitor A-CREB under control of the rat collagen II promoter/enhancer. Two independent founder lines, each harboring one copy of the A-CREB transgene, were identified and maintained for further analyses.

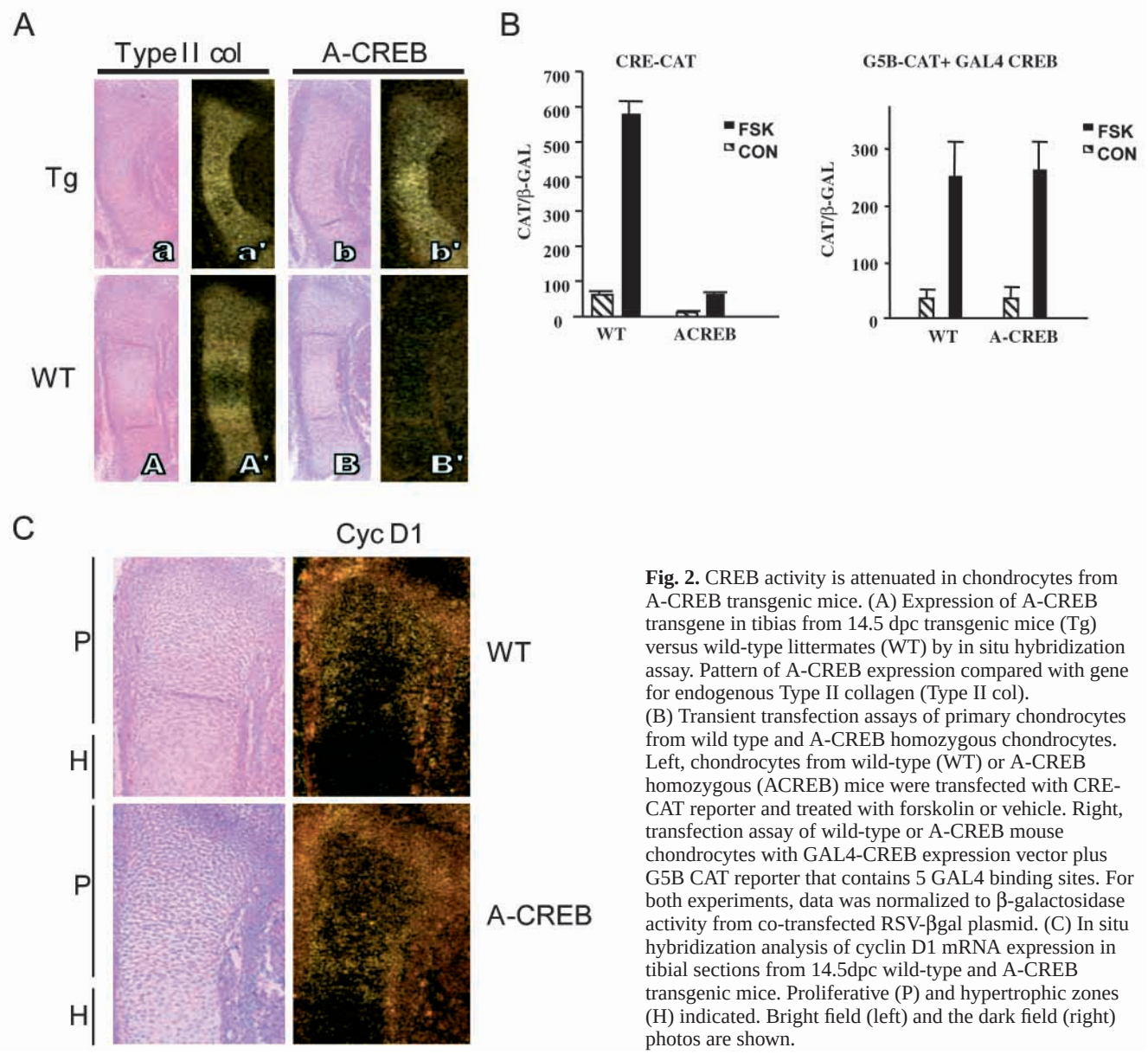

Fig. 2. CREB activity is attenuated in chondrocytes from A-CREB transgenic mice. (A) Expression of A-CREB transgene in tibias from 14.5 dpc transgenic mice (Tg) versus wild-type littermates (WT) by in situ hybridization assay. Pattern of A-CREB expression compared with gene for endogenous Type II collagen (Type II col).
(B) Transient transfection assays of primary chondrocytes from wild type and A-CREB homozygous chondrocytes. Left, chondrocytes from wild-type (WT) or A-CREB homozygous (ACREB) mice were transfected with CRE-CAT reporter and treated with forskolin or vehicle. Right, transfection assay of wild-type or A-CREB mouse chondrocytes with GAL4-CREB expression vector plus G5B CAT reporter that contains 5 GAL4 binding sites. For both experiments, data was normalized to β-galactosidase activity from co-transfected RSV-βgal plasmid. (C) In situ hybridization analysis of cyclin D1 mRNA expression in tibial sections from 14.5dpc wild-type and A-CREB transgenic mice. Proliferative (P) and hypertrophic zones (H) indicated. Bright field (left) and the dark field (right) photos are shown.

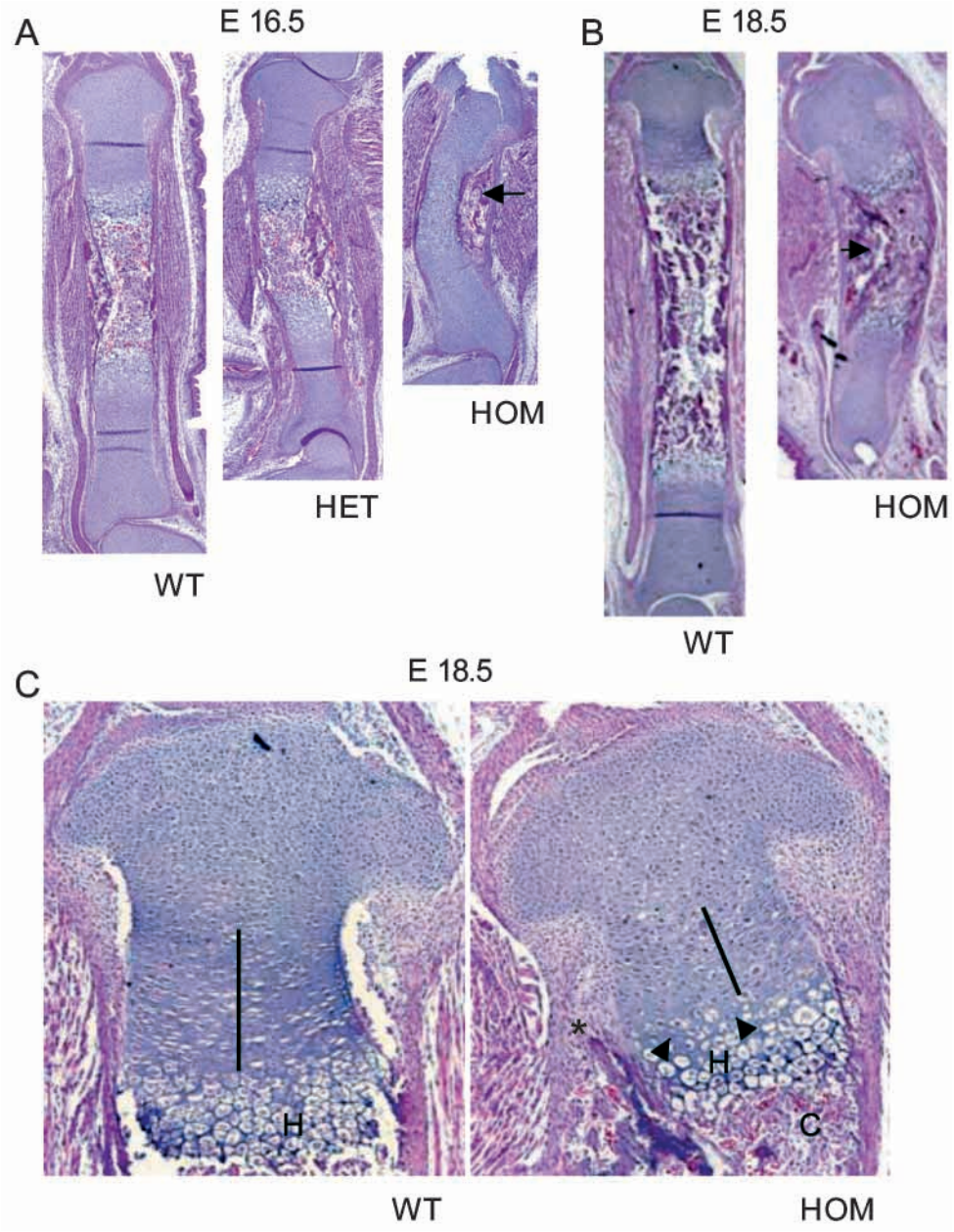

Fig. 3. Growth plate development is disrupted in A-CREB transgenic mice. Histological analyses of 16.5 dpc (A) and 18.5 dpc (B,C) embryos. Bowing phenotype and exuberant cortical bone formation on one side of the tibia visible in homozygous A-CREB (HOM) versus WT mice (arrows). Intermediate phenotype apparent in heterozygous A-CREB mice (HET) at 16.5 dpc. (C) Higher magnification of the proximal growth regions in tibias of 18.5 dpc mice showing cellular disorganization in the homozygous mutant (HOM) versus wild-type (WT) mice. Columnar organization of flat cells in wild-type tibia is replaced by disorganized round cells in the homozygous A-CREB embryos; equivalent regions demarcated by black lines. Arrowheads, small chondrocytes intermingled with hypertrophic cells (H) in the A-CREB mutant. Arrowheads in C, region of ectopic bone formation initiating beneath the perichondrium. C, bone marrow cavity.

Animals containing a single copy of the A-CREB transgene (A-CREB heterozygotes) appeared normal by visual inspection; but inbred mice harboring two copies of the transgene (A-CREB homozygotes) died minutes after birth. Compared with wild-type littermates, A-CREB homozygotes exhibited short-limbed dwarfism and a markedly reduced rib cage circumference that may underlie their perinatal lethality (Fig. 1A-C). Whole skeleton staining of 18.5 dpc embryos revealed that skeletal elements of A-CREB-expressing mice were shortened in a dose-dependent manner; A-CREB heterozygotes exhibited intermediate shortening compared with homozygotes (Fig. 1A,B). Consistent with a pronounced defect in growth plate development, tibias from transgenic embryos were also bowed anteriorly (Fig. 1B, lower panel).

The abnormal skeletal phenotype in A-CREB transgenic mice prompted us to examine the expression pattern of this inhibitor during development. In situ hybridization assays revealed that the A-CREB transgene was uniformly expressed in tibial chondrocytes of 14.5 dpc embryos, in a pattern resembling that of the gene for endogenous collagen II (Fig. 2A, compare a′ with b′). Levels of A-CREB protein in transgenic chondrocytes appear sufficient to disrupt CREB activity completely; in transient transfection assays of primary chondrocytes, addition of cAMP agonist induced CRE-CAT reporter activity seven- to eightfold in wild-type cells but had no effect on reporter activity in A-CREB transgenic cells (Fig. 2B). By contrast, the activity of a GAL4-CREB fusion protein (containing the GAL4 DNA-binding domain fused to the CREB trans-activation domain) on a GAL4 CAT reporter plasmid was comparable in wild-type and A-CREB cells stimulated with cAMP agonist, demonstrating that A-CREB specifically inhibits CREB DNA-binding activity without affecting its transcriptional potency (Fig. 2B).

Consistent with the selectivity of the A-CREB inhibitor for

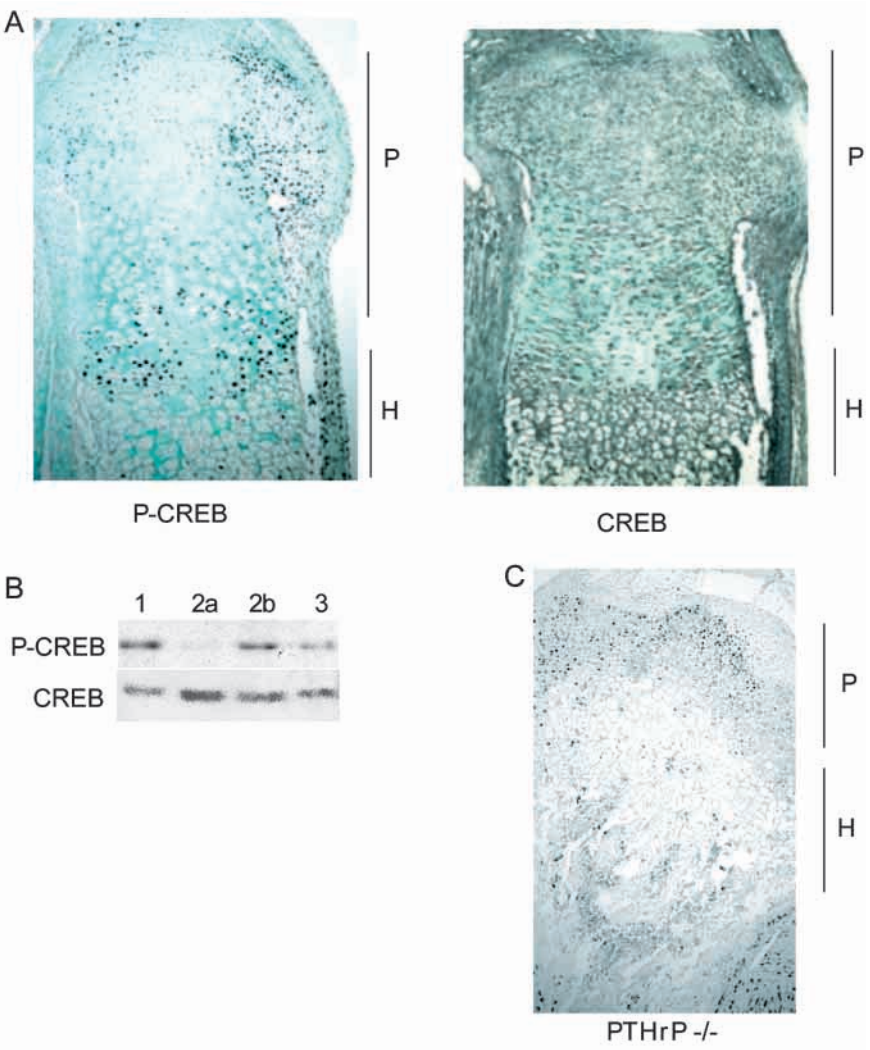

Fig. 4. Phosphorylation of CREB at Ser133 occurs in discrete cell populations of the developing growth plate.
(A) Immunocytochemical analysis of phospho (Ser133) CREB in tibial growth plates from wild-type (left) 16 dpc mice compared with total CREB (right) using phospho-specific (5322) and non-discriminating (244) CREB antisera, respectively. 5322 antiserum recognizes Ser 133 phosphorylated forms of CREB, CREM and ATF1. (B) Western blot analysis of total and phospho (Ser133) CREB levels in nuclear extracts from day 12 chick embryo chondrocytes. Cells were harvested from proliferative (zone 1; P), pre-hypertrophic (zone 2a, 2b) and hypertrophic (zone 3; H) regions of the tibia. (C) CREB activity in developing cartilage is regulated via a PTHrP-independent mechanism. Immunocytochemical analysis of phospho-CREB-positive cells in tibial sections from 16 dpc PTHrP-null mice.

CREB family members (Ahn et al., 1998), expression of the gene for cyclin D1, a target gene for the bZIP factor ATF2 in developing chondrocytes (Beier et al., 1999), was unaffected in transgenic compared with wild-type animals (Fig. 2C). Taken together, these results indicate that the effect of A-CREB on endochondral bone development reflects specific loss of CREB DNA-binding activity in transgenic chondrocytes.

To evaluate the cellular basis for the observed limb defects in A-CREB mice, we performed histological studies on 16.5 dpc embryos. Consistent with results from whole skeleton staining experiments, dose-dependent shortening and deformation of the tibia was readily apparent in A-CREB transgenic mice (Fig. 3A). Bowing of the tibia in homozygous embryos was accompanied by asymmetric deposition of cortical bone beneath the perichondrium. Such changes were apparent at 16.5 dpc (Fig. 3A) and continued at 18.5 dpc (Fig. 3B).

The organization of growth plate chondrocytes in transgenic mice was noticeably disrupted (Fig. 3C). For example, a large number of chondrocytes in the wild-type growth plate assume a flat morphology and become organized in columns prior to hypertrophy. By contrast, tibial chondrocytes from homozygous A-CREB mice adopt a rounded morphology in the same region and are typically disorganized (Fig. 3C).

To identify cell populations in which the CREB family is likely to be active, we performed immunocytochemical studies with phospho-specific antiserum 5322, an antibody that recognizes the Ser133 phosphorylated, and hence activated form of CREB, CREM and ATF1 (Hagiwara et al., 1993; Michael et al., 2000). Phospho-CREB proteins were unevenly distributed in growth plate chondrocytes; highest levels were noted in proliferative and pre-hypertrophic zones of the tibial growth plate at 16dpc (Fig. 4A). Within the proliferative zone, peri-articular chondrocytes were most positive for phospho-Ser133 CREB, whereas chondrocytes within the hypertrophic zone were largely negative (Fig. 4A). These results suggest that signals that regulate CREB family activity are confined to discreet regions of the developing growth plate.

The pattern of CREB phosphorylation in the developing growth plate appears to be well conserved; tibial chondrocytes in the proliferative and pre-hypertrophic zones of day 12 chick embryos contained highest levels of phospho-CREB by western blot assay (zones 1 and 2b, respectively; Fig. 4B), with lower levels of phospho-CREB in hypertrophic chondrocytes (zone 3, Fig. 4B).

The ability of PTHrP to regulate chondrocyte proliferation via a cAMP-dependent mechanism (Schipani et al., 1997)

prompted us to evaluate the importance of this growth factor for CREB Ser133 phosphorylation in the developing growth plate. Surprisingly, the number of phospho (Ser133) CREB-positive cells in tibial sections from PTHrP-null mice was comparable with that of wild-type littermates (Fig. 4C). These results indicate that the activation of CREB family members in developing chondrocytes proceeds primarily via a PTHrP-independent pathway.

The presence of phospho-CREB-positive cells in the proliferative zone of the developing growth plate prompted us to compare the proliferative status of chondrocytes in A-CREB homozygous and wild-type embryos. The proliferative zone, as defined by the region of BrdU-positive cells, was severely shortened in A-CREB transgenic compared with wild-type growth plates (Fig. 5A). Within this region, the proportion of BrdU-labeled cells was reduced threefold in homozygous A-CREB mice (18% in wild-type versus 6% in A-CREB mice) (Fig. 5B). Although most pronounced at 14.5 dpc (Fig. 5A), these differences between A-CREB transgenic and wild-type littermates were observed at all stages, indicating that CREB activity is necessary for cell cycle progression in developing chondrocytes (Fig. 5C,D).

As they exit the cell cycle, growth plate chondrocytes become hypertrophic and elaborate a distinct collagen matrix (Linsenmayer et al., 1991). Compared with wild-type littermates, the hypertrophy process in early stage A-CREB

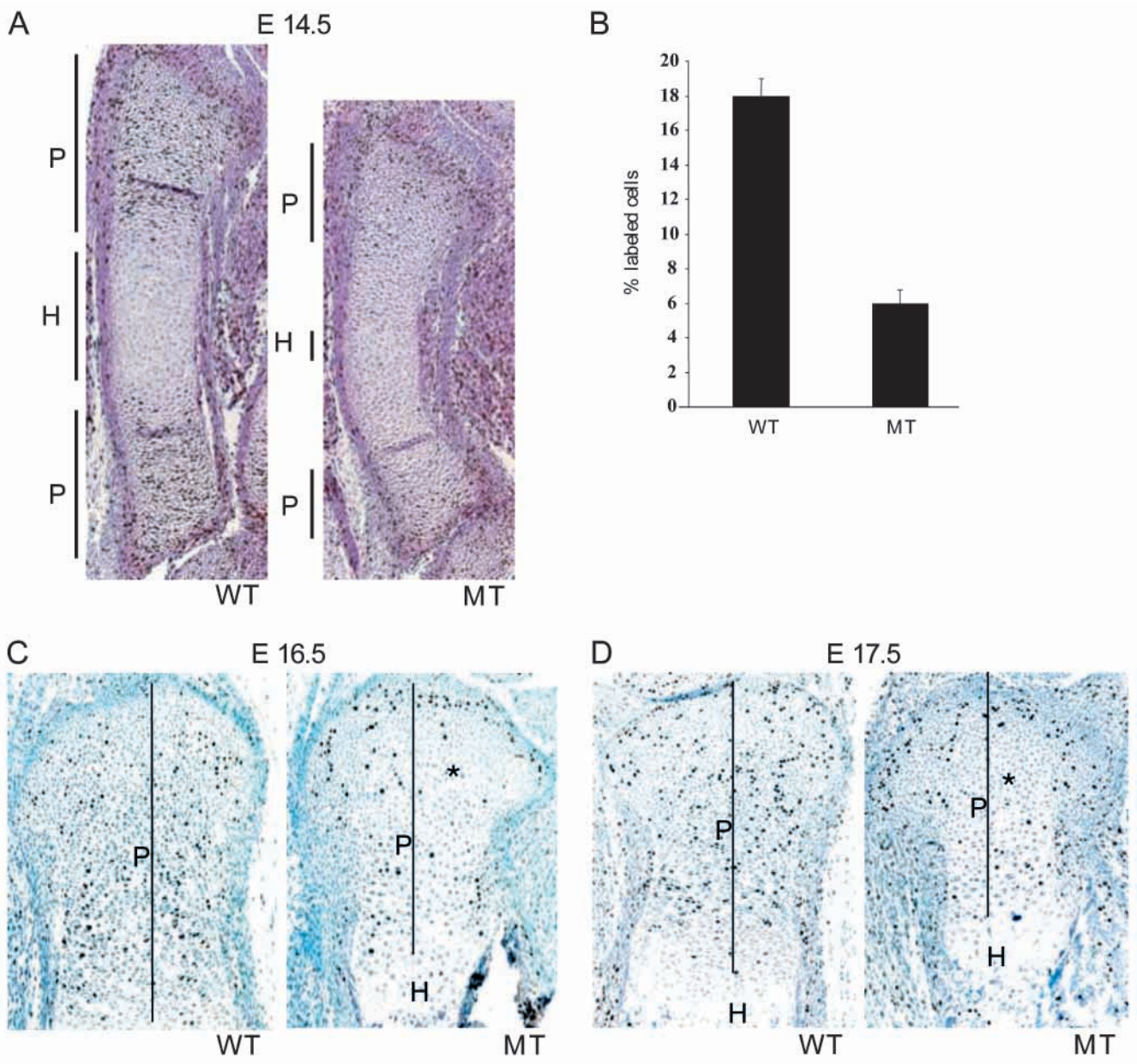

Fig. 5. Chondrocyte proliferation is disrupted in A-CREB mutant cartilage. BrdU labeling of wild-type (WT) and homozygous mutant embryos (MT) at 14.5 dpc (A,B), 16.5 dpc (C) and 17.5 dpc (D). B represents BrdU labeling indices in 14.5 dpc embryos. H, hypertrophy zone; P, proliferative zone. (C,D) Higher magnification showing proximal growth regions in 16.5 and 17.5 dpc mice, respectively. Areas within the proliferative zones devoid of BrdU labeling indicated by an asterisk.

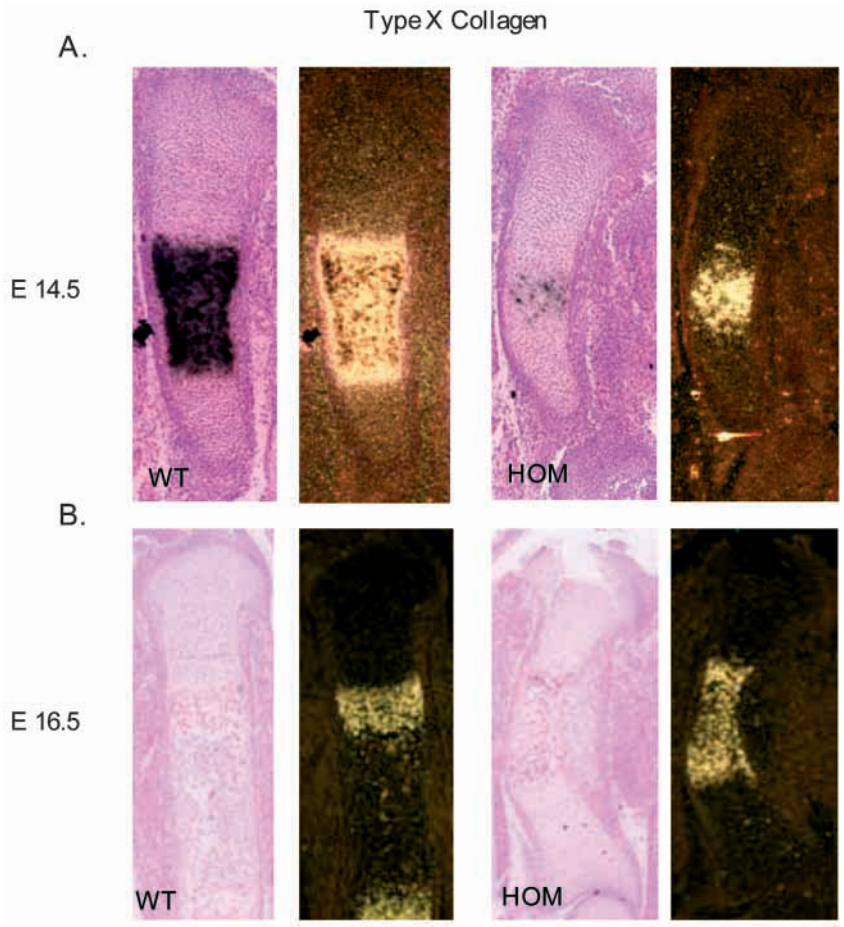

Fig. 6. Marked delay of chondrocyte hypertrophy in A-CREB (HOM) mutant compared with wild-type (WT) embryos. In situ hybridization analysis of tibial sections from 14.5 dpc (A) and 16.5 dpc (B) embryos using ^{35}S-labeled collagen type X antisense RNA probe. Bright field (left) and the dark field (right) photos are shown.

embryos was noticeably delayed (Fig. 5A). This delay in chondrocyte hypertrophy, coupled with the reduced width of the proliferative zone, resulted in formation of an extended postmitotic but nonhypertrophic region that is unique to A-CREB mutants (Fig. 5A).

To evaluate chondrocyte differentiation more accurately, we examined the expression of the Type X collagen gene, a marker for chondrocyte hypertrophy (Linsenmayer et al., 1991), by in situ hybridization assay. At 14.5 dpc, type X expression was readily detected in central hypertrophic cells of the wild-type tibia (Fig. 6A); but Type X expression was far lower in A-CREB mutant sections. Indeed, the boundaries of type X expression were disorganized in A-CREB transgenic compared with wild-type mice, confirming the loss of cell synchrony during progression from the proliferative to hypertrophic zones. Following vascularization of the hypertrophy zone, the bone marrow cavity is well established by 16.5dpc in wild-type mice (Fig. 6B). In A-CREB mutant embryos, however, the middle portion of the cartilage remained unvascularized, containing only a continuum of hypertrophic chondrocytes (Fig. 6B). These results are consistent with immunocytochemical data showing that pre-hypertrophic chondrocytes contain phospho Ser133 CREB; and they support the notion that, in addition to its effects on proliferation, CREB promotes chondrocyte differentiation.

To determine the molecular basis for the A-CREB phenotype, we examined the expression patterns of several key signaling molecules in endochondral bone development, including Ihh and its receptor *Ptch*, as well as PTHrP and the PTHrP receptor (PTHrP-R). *Ihh* is most abundantly expressed in so-called pre-hypertrophic cells immediately adjacent to the hypertrophic zone (Vortkamp et al., 1996). Consistent with the delayed expression of collagen type X noted above, the zone of *Ihh*-expressing cells in A-CREB transgenic tibia was reduced; but the expression levels of *Ihh* mRNA in individual cells appeared more comparable with those in wild-type littermates (Fig. 7a′,A′). Ihh regulates chondrocyte hypertrophy during development, at least in part, by inducing expression of PTHrP in periarticular cells. PTHrP mRNA levels in this region were comparable between wild-type and A-CREB transgenic, suggesting that CREB activity is not required for Ihh to induce PTHrP expression (Fig. 7b′,B′).

PTHrP keeps chondrocytes in the proliferative pool and delays their differentiation into hypertrophic chondrocytes (Karaplis et al., 1994; Lanske et al., 1996) via the PTHrP receptor, a G protein coupled receptor that mediates PTHrP action, at least in part, via the cAMP pathway. Expression of PTHrP receptor (PTHrP-R) is normally concentrated in the pre-hypertrophic region, a domain that also contains Ihh-producing chondrocytes (Lanske et al., 1996). Consistent with the delay in differentiation, tibias from 14.5 dpc A-CREB embryos contained fewer PHTrP-R-positive cells compared with wild-type littermates, and levels of PTHrP-R mRNA were also somewhat reduced in individual cells (Fig. 7c′,C′).

Previous studies showing that *Ptch* is also a transcriptional

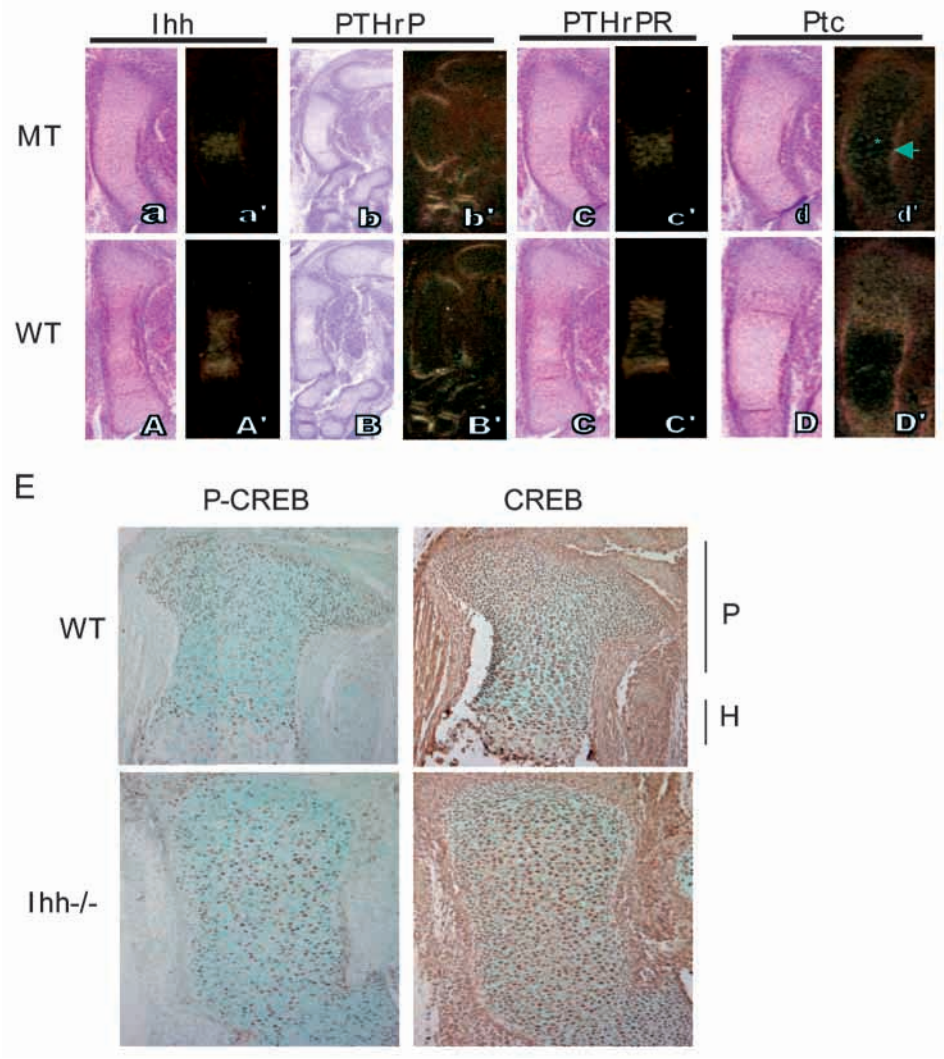

Fig. 7. CREB activity is critical for Ihh signaling during endochondral bone development. (A-D) In situ hybridization assays of tibial sections from 14.5dpc embryos. A-CREB homozygous mutant (top) and wild-type embryos (lower panel) shown. Bright (left) and dark (right) field photos shown. Analysis of patched (Ptch), Indian hedgehog (Ihh), parathyroid hormone related protein (PTHrP) and PTHrP receptor (PTHrPR) indicated above corresponding photos. *Ptch* expression in cartilage is reduced in the A-CREB mutant tibia (*); *Ptch* expression in the perichondrium appears normal (arrow). (E) Comparison of CREB Ser133 phosphorylation in tibial sections from 16.5dpc wild-type and $Ihh^{-/-}$ mice using phospho (Ser133) specific CREB antiserum. Sections incubated with non-discriminating CREB antiserum shown on right. Position of proliferating (P)and hypertrophic (H) zones indicated.

target for Ihh activity (Ingham, 1998; Tabin and McMahon, 1997), prompted us to examine the expression pattern of this gene in A-CREB transgenic mice. In tibial sections from wild-type 14.5dpc mouse embryos, *Ptch* was expressed in a gradient throughout the proliferative zone, with highest levels of *Ptch* in cells closest to *Ihh*-expressing chondrocytes as well as in perichondrial cells flanking the *Ihh*-expressing chondrocytes (Fig. 7D′). Compared with wild-type littermates, *Ptch* expression at 14.5dpc was markedly lower in chondrocytes of A-CREB transgenic mice (Fig. 7d′,D′). Consistent with the absence of A-CREB transgene expression in perichondrium, *Ptch* expression in perichondrial cells was comparable between transgenic and wild-type littermates (Fig. 7d′,D′). Taken together these results suggest that A-CREB interferes with endochondral bone formation, at least in part, by attenuating Ihh signaling.

To determine whether CREB is a direct target for Ihh signaling, we performed immunocytochemical studies on tibial sections from 16.5dpc $Ihh^{-/-}$ mice. Compared with wild-type cartilage, the proportion of phospho Ser133-positive chondrocytes was comparable in $Ihh^{-/-}$ sections (Fig. 7E). These results suggest that CREB potentiates *Ptch* expression in developing cartilage via an Ihh-independent pathway.

DISCUSSION

A number of signaling molecules that regulate endochondral bone formation have been identified (Ingham, 1998; Tabin and McMahon, 1997). But the mechanisms by which these signals induce expression of the cartilage and bone genetic programs remain largely uncharacterized. Our results suggest that the CREB family of activators function as critical intermediates in this process. CREB and its paralogs are phosphorylated and hence activated in a subpopulation of chondrocytes within the proliferative zone. Correspondingly, expression of the dominant negative A-CREB inhibitor in developing chondrocytes reduced the height of the proliferative zone in developing limbs and lowered the proportion of BrdU-positive cells in the growth plate. Although the shortened proliferative zone in the A-CREB mutant mice may simply reflect the lowered proliferation rate, this inhibitor may reduce both the rate of proliferation and the number of cell cycles that chondrocytes undergo prior to exiting the cell cycle.

Other CRE-binding proteins in addition to CREB, most notably the transcription factor ATF2, also appear to have important functions in bone development (Reimold et al., 1996). The phenotypes of $Atf2^{-/-}$ and A-CREB mice are

readily distinguishable; most *Atf2*$^{-/-}$ mice survive into adulthood whereas all of the A-CREB mice die minutes after birth. Histologically, capillary invasion of the developing growth plate is absent in *Atf2*$^{-/-}$ mice, resulting in loss of cartilage trabeculae that normally extend into the marrow cavity from the metaphyseal side of the growth plate (Reimold et al., 1996). By contrast, capillary invasion and trabecular bone formation are normal in A-CREB mice, indicating that ATF2 and CREB may either function at different times during development or may regulate distinct sets of genes. Cyclin D1 expression is severely attenuated in *Atf2*$^{-/-}$ mice, for example, whereas A-CREB transgenic mice show no such reduction compared to wild-type littermates. The underlying basis for target gene selectivity between ATF2 and CREB remains unclear. But differences between ATF2 and CREB DNA-binding specificity have been noted (Benbrook and Jones, 1994). Additionally, binding of ATF2 to DNA is strongly regulated by heterodimerization with Jun, and the relative occupancy of CRE-containing genes by ATF2 may therefore depend on levels of *Jun* expression in developing cells (MacGregor et al., 1990).

The decrease in chondrocyte proliferation in the A-CREB-expressing mice is much more dramatic than the modest decrease in chondrocyte proliferation seen in PTHrP-null mice (Amizuka et al., 1996; Karp et al., 2000). Indeed, CREB Ser133 phosphorylation was unaffected in PTHrP-null mice, suggesting that other pathways are regulating CREB family activity in the normal growth plate.

The phenotype of the A-CREB transgenic mice is consistent with the ability of CREB to potentiate Ihh signaling; *Ptch* expression was reduced in developing chondrocytes from A-CREB mice. CREB is unlikely to be a direct target for *Ihh* signaling, however; Ser133 phosphorylation in developing cartilage was unaffected in *Ihh*$^{-/-}$ mice compared with control littermates. Rather, our results are consistent with a model in which a parallel pathway potentiates *Ihh* signaling via a phospho (Ser133) CREB-dependent mechanism.

In this regard, a number of growth factor pathways, including IGF, TGFβ, FGF, EGF and PDGF, have been found to promote target gene expression via CREB (Cesare and Sassone-Corsi, 2000; Montminy, 1997; Shaywitz and Greenberg, 1999). The IGFs appear to be critical for skeletal growth in vertebrates, for example; *Igf1r*$^{-/-}$ mice exhibit dwarfism (Baker et al., 1993; Liu et al., 1993). Signaling by IGFs has been found to promote Ser133 phosphorylation and target gene expression via CREB (Pugazhenthi et al., 1999). It will be of interest to examine whether CREB promotes chondrocyte proliferation in response to IGF signaling.

This report constitutes the first example documenting a critical role for the CREB family in cellular proliferation. The mechanism by which CREB family members might regulate cell cycle progression is unclear; but a number of cell cycle-regulated genes with consensus CRE sites have been identified. The genes for cyclin A and PCNA (proliferating cell nuclear antigen), for example, contain functional CREs that appear to be important for high level expression of both genes (Desdouets et al., 1995; Lee and Mathews, 1997). The mouse model we describe here should be useful in identifying target genes in chondrocytes that are induced by CREB in response to mitogenic signals during development.

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