

Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo

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The $\alpha 1(X)$ collagen gene (*Col10a1*) is the only known hypertrophic chondrocyte-specific molecular marker. Until recently, few transcriptional factors specifying its tissue-specific expression have been identified. We show here that a 4-kb murine *Col10a1* promoter can drive β -galactosidase expression in lower hypertrophic chondrocytes in transgenic mice. Comparative genomic analysis revealed multiple *Runx2* (Runt domain transcription factor) binding sites within the proximal human, mouse, and chick *Col10a1* promoters. In vitro transfection studies and chromatin immunoprecipitation analysis using hyper-

trophic MCT cells showed that *Runx2* contributes to the transactivation of this promoter via its conserved *Runx2* binding sites. When the 4-kb *Col10a1* promoter transgene was bred onto a *Runx2*^{+/-} background, the reporter was expressed at lower levels. Moreover, decreased *Col10a1* expression and altered chondrocyte hypertrophy was also observed in *Runx2* heterozygote mice, whereas *Col10a1* was barely detectable in *Runx2*-null mice. Together, these data suggest that *Col10a1* is a direct transcriptional target of *Runx2* during chondrogenesis.

Introduction

Type X collagen gene (*Col10a1*) is the only known hypertrophic chondrocyte-specific molecular marker. Mutations in *COL10A1* in humans have been associated with Schmid metaphyseal chondrodysplasia (SMCD; Warman et al., 1993). It has also been reported that *Col10a1*-null mice have subtle growth plate compressions partially resembling SMCD (Kwan et al., 1997). However, the molecular mechanisms conferring its tissue-specific expression pattern are still poorly understood. Although in vitro transfection studies of the human and mouse promoters have identified putative cis regulatory elements (Beier et al., 1997; Riemer et al., 2002), transgenic mouse studies have not yielded tissue-specific expression using murine control elements (Eerola et al., 1996). Comparative sequence analysis demonstrates a high level of conservation between murine, human, and chick promoters in the immediate vicinity of the start site of transcription (-120 to +1 bp) identifying this as the likely basal promoter (Beier et al., 1996). However, whereas a

transgenic reporter mouse line containing 1.7 kb of the chick promoter resulted in tissue-specific expression in hypertrophic chondrocytes (Jacenko et al., 1993), the similar murine construct containing the corresponding mouse promoter resulted in expression within the soft connective tissues, but not in the hypertrophic chondrocytes, suggesting that sequence divergence from chick to humans and mice resulted in transposition of the tissue-specific cis regulatory element to another region of the promoter (Eerola et al., 1996). Several in vitro transfection/reporter studies using the human *COL10A1* promoter have suggested the existence of negative regulatory elements in the distal promoter (-2.8 to -2.4 kb), which are active in resting chondrocytes and nonchondrogenic cell lines, whereas a hypertrophic chondrocyte cell type-specific enhancer element resides in -2.4 to -0.9 kb region (Beier et al., 1997). Similar types of studies with chick *Col10a1* confirmed the importance of both negative and positive regulatory sequences in the chick proximal 4.4-kb promoter (Dourado and LuValle, 1998). Finally, a recent study of the human promoter identified a c-fos responsive element that might mediate parathyroid hormone/parathyroid hormone

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Abbreviations used in this paper: CCD, cleidocranial dysplasia; EMSA, electrophoretic mobility shift assays; P1, postnatal day 1; SMCD, Schmid metaphyseal chondrodysplasia.

related peptide regulation of *COL10A1* gene expression (Riemer et al., 2002). These data show that at least in cell culture, multiple cis elements together specify high level gene expression in hypertrophic chondrocytes.

Although several signaling molecules including Indian Hedgehog and parathyroid hormone related peptide have been shown to be required for chondrocyte hypertrophy by mouse genetic studies, few transcriptional determinants specifying hypertrophic chondrocyte-specific type X collagen gene expression have been identified to date (Karaplis et al., 1994; Schipani et al., 1995, 1997; Lanske et al., 1996; Vortkamp et al., 1996; St-Jacques et al., 1999; Chung et al., 2001). One candidate gene likely important for *Col10a1* gene regulation is the runt domain transcription factor *Runx2/Cbfa1*. *Runx2* has been shown in mouse genetic studies to be required for differentiation of the mesenchymal stem cell into the osteoblast lineage (Komori et al., 1997; Otto et al., 1997). *Runx2*-null mice do not have bone and accordingly *Runx2* has also been demonstrated to transactivate a host of genes highly expressed in osteoblasts including osteocalcin and type I collagen (Ducy et al., 1997). *Runx2* has also been shown to be important for chondrocyte differentiation, i.e., during endochondral ossification. Supporting this are recent histomorphologic studies showing alteration of chondrocyte maturation in some long bones of *Runx2*-null mice as well as cell culture studies indicating that *Runx2* is a positive regulatory factor for chondrocyte maturation (Inada et al., 1999; Kim et al., 1999; Enomoto et al., 2000). In fact, two studies mis-expressing *Runx2* in proliferating chondrocytes were able to induce chondrocyte hypertrophy and partially rescue *Runx2*-null mice (Takeda et al., 2001; Ueta et al., 2001). Although recent studies have demonstrated several transcriptional targets for *Runx2* in osteoblastic and chondrocytic cells (Jimenez et al., 1999; Zelzer et al., 2001), the direct link between *Runx2* and the tissue-specific expression of type X collagen, the only known hypertrophic chondrocyte-specific molecular marker, has not been identified yet.

Here, we present identification of the first murine *Col10a1* promoter that can direct reporter activity selectively in hypertrophic chondrocytes in transgenic mice. We further show that *Runx2* can directly transactivate this *Col10a1* promoter both in vitro and in vivo via putative *Runx2* binding sites found in this promoter region. Last, we discussed the altered endochondral ossification in the *Runx2* mutant mice. We surmise that this is probably due to down-regulation of *Runx2* targets in the hypertrophic chondrocytes including *Col10a1*.

Results

Comparative genomic analysis of type X collagen gene promoter

We isolated a murine *Col10a1* containing BAC clone. Sequence analysis on the 5' promoter region revealed several putative *Runx2* binding sites (PuACCPuCA or TGTGGT; Ducy et al., 1997; Jimenez et al., 1999). Then we performed comparative genome analysis of type X collagen gene promoter across species. Multiple potential *Runx2* binding sites were also observed within 5 kb of the human

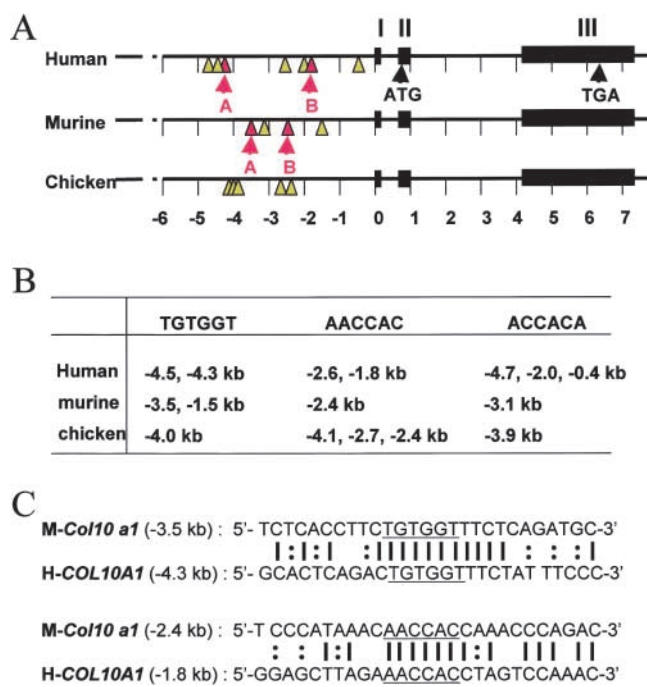


Figure 1. Comparative genomic analysis of type X collagen gene promoter. (A) Schematic illustration of human, mouse and chicken *Col10a1* promoter. Putative *Runx2* binding sites within the promoter region are indicated by yellow triangle. The two conserved sites A and B between human and mouse are highlighted as red triangle. (B) Sequence and position of the potential *Runx2* binding sites within 5-kb of the human, mouse, and chick *Col10a1* promoter. The putative *RUNX2* binding sequence and their position within the type X promoter are shown in the Table. There are 7, 4 and 5 potential *RUNX2* binding sites within 5-kb of the human, mouse, and chicken *Col10a1* promoters, respectively. (C) Sequence comparison between mouse and human A and B elements. The sequence including the mouse A and B elements and 10 bp of immediate flanking sequence show 60–70% homology to the corresponding human elements. No significant similarity was found among other putative *RUNX2* binding sites (not depicted). Core sequences of the putative *RUNX2* binding sites are underlined. Bars represent identical nucleotides and vertical dots represent conserved nucleotide base pairing.

and chicken *Col10a1* promoters (Fig. 1, A and B). Interestingly, no significant conservation within long stretches of sequences flanking the *Runx2* binding sites was observed between the human and mouse type X collagen genes. However, immediate flanking sequences, i.e., 10 bp on either side of sites -3485 and -2379 bp (A and B elements; Fig. 1 A and Fig. 2 A) in the mouse *Col10a1* gene, showed 60–70% homology between the human and murine *Col10a1* promoter region (Fig. 1 C).

4-kb *Col10a1* murine promoter can direct expression in hypertrophic chondrocytes both in vitro and in vivo

To study whether a 4-kb *Col10a1* promoter containing the conserved *Runx2* binding sites A and B is in fact up-regulated during chondrocyte hypertrophy in vitro, we measured the activity of a reporter construct containing this 4-kb promoter in hypertrophic MCT cells (Fig. 2 A). MCT cells are mouse chondrocytes that have been immortalized with a temperature-sensitive mutant of SV40 large T antigen. Although they proliferate at a permissive temperature of 32°C, MCT cells

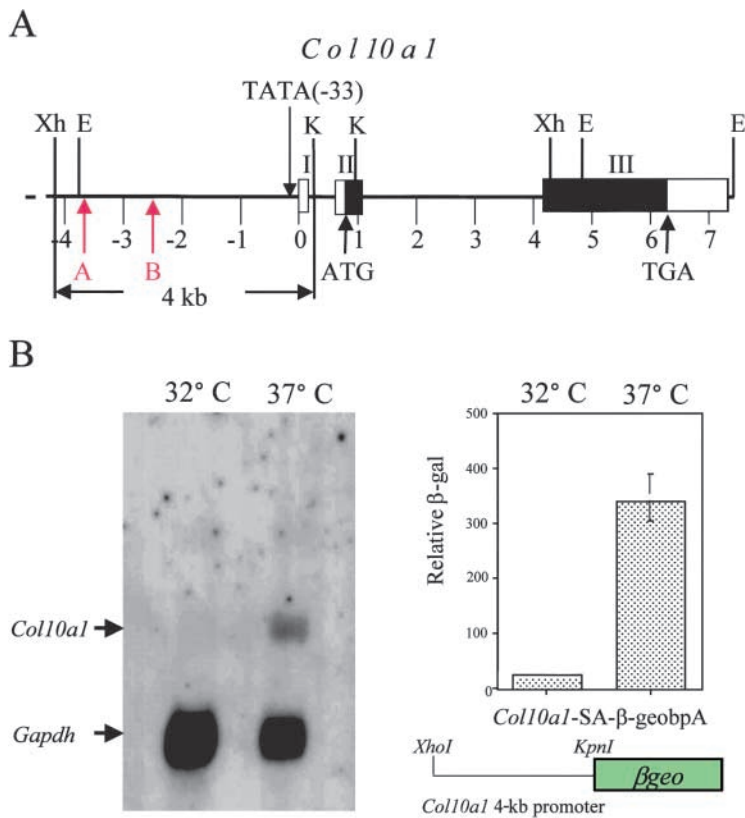


Figure 2. *Col10a1* promoter and its upregulation in MCT cells. (A) Restriction map of murine *Col10a1*. *Col10a1* is composed of three exons numbered I, II and III. Position of TATA box in the minimal promoter, ATG start codon, and TGA stop codon are marked. Positions of the two putative *Runx2* binding elements are highlighted as A and B. The 4-kb promoter used in transgenic mice studies is indicated below by arrows. E, EcoRI; Xh, XhoI; K, KpnI. (B) Endogenous *Col10a1* mRNA and *Col10a1* 4-kb promoter activity are up-regulated in hypertrophic MCT cells. (left) Northern analysis of *Col10a1* expression from MCT cells grown for 3 d at either 32°C or 37°C. Loading was normalized by hybridization with *Gapdh*. *Col10a1* was expressed abundantly in hypertrophic MCT cells at 37°C but not in proliferating MCT cells at 32°C. (right) *Col10a1*-SA-β-geobpA reporter plasmid was transfected into MCT cells grown at 32°C and then further cultured at either 32°C or 37°C. A RSV-luciferase expression plasmid was cotransfected as internal control for transfection efficiency. Bars represent the average ratios of β-galactosidase to luciferase activity. The standard deviations from three independent transfections of one representative experiment are indicated by the error bars.

terminally differentiate and become hypertrophic and specifically express type X collagen and other hypertrophic chondrocyte markers when grown at the nonpermissive temperature of 37°C (Lefebvre et al., 1995). Indeed, Northern analysis showed that MCT cells expressed type X collagen when cultured at 37°C but not at 32°C (Fig. 2 B, left; Lefebvre et al., 1995). We transfected the *Col10a1* 4-kb promoter driving the β-galactosidase reporter into MCT cells. Consistent with significant up-regulation of endogenous *Col10a1* gene expression in hypertrophic MCT cells, β-galactosidase activity was increased 42-fold compared to the activity in proliferating MCT chondrocytes (Fig. 2 B, right).

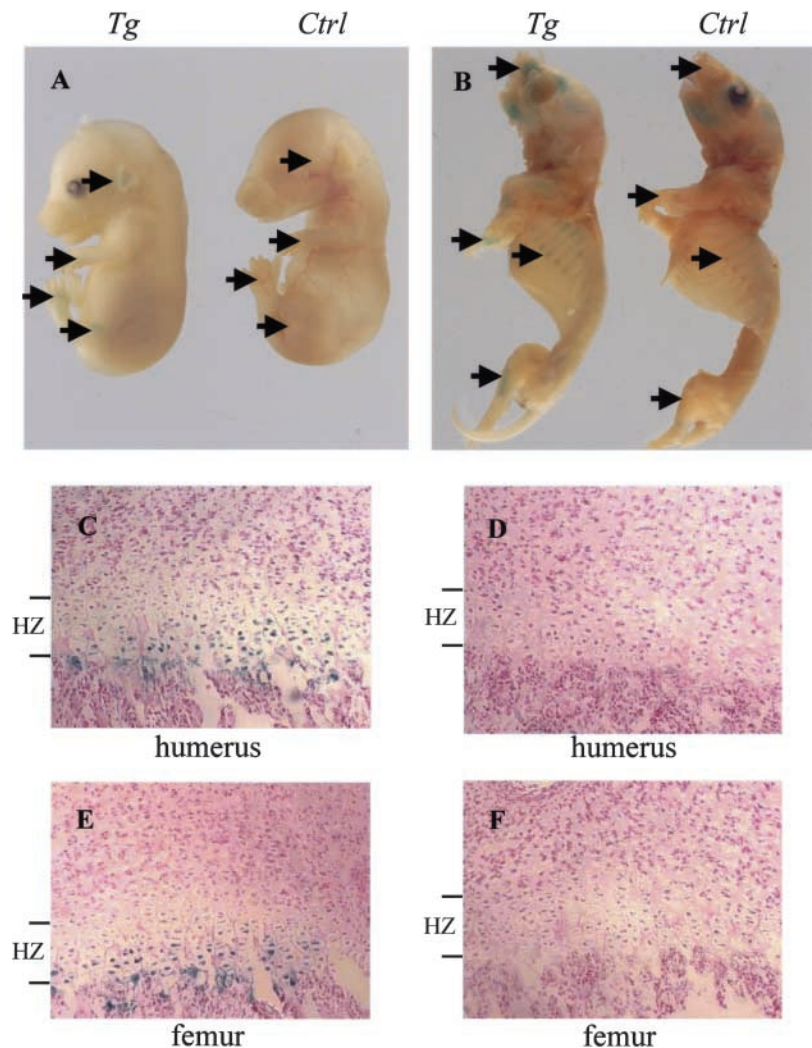
To determine the *in vivo* relevance of these data, we generated four independent transgenic mouse lines harboring the 4-kb proximal *Col10a1* promoter upstream of the β-galactosidase reporter gene. Three lines of mice exhibited similar X-gal staining, whereas the fourth one did not show any staining. We performed whole embryo staining of E15.5 mouse embryos and blue staining was noted only at the ends of long bones (Fig. 3 A). No blue staining was observed in any other tissues when analyzing sagittal sections of the whole embryos featuring a variety of tissues (unpublished data). Although some background staining in craniofacial region probably due to endogenous β-galactosidase, activity was observed both in transgenic and control postnatal day 1 (P1) mice, specific blue staining was observed only in the chondro-osseous junction of limbs, ribs and also in the nasal cartilage of transgenic mice (Fig. 3 B). Indeed, histological analysis confirmed that β-galactosidase expression was observed in the lower zone of hypertrophy of rib sections and in the long bone sections of the limbs including proximal

humerus and proximal femur at P1 stage (Fig. 3, C and E, and not depicted). Although some weak staining was also present in bone marrow along trabeculae presumably in osteoblasts, no blue staining was observed in other tissues including perichondrium, resting or proliferating chondrocytes, muscle fibers, or adhering connective tissues (Fig. 3 B and not depicted). These data show that the proximal 4-kb *Col10a1* promoter was able to direct hypertrophic chondrocyte expression of the β-galactosidase reporter *in vivo* and, therefore, contributes to *Col10a1* expression in these cells. Because a 1.7-kb mouse promoter was previously reported to be unable to direct expression of β-galactosidase to hypertrophic chondrocytes (Eerola et al., 1996), we hypothesized that the positive regulatory elements in this 4-kb construct most likely reside in the 5' portion where the two conserved *Runx2* binding sites A and B are found.

***Runx2* regulates transcription of *Col10a1* via *Runx2* binding elements within *Col10a1* promoter**

Electrophoretic mobility shift assays (EMSA) showed that His-tagged recombinant DNA-binding RUNT domain polypeptide bound to each of the putative RUNX2 binding sites A and B found in the *Col10a1* promoter (Fig. 4 A). As expected, mutations within the core binding sequences abolished binding, whereas mutations outside of the core sequence had no significant effect on formation of the DNA-protein complex (Fig. 4 A). EMSA, using nuclear extracts from hypertrophic MCT cells, showed DNA-protein complexes specific for each of these two cis elements (Fig. 4 B). These DNA-protein complexes were effectively competed for by unlabeled probe. Moreover, the addition of anti-

Figure 3. *Col10a1* promoter activity in transgenic reporter mice. (A and B) X-gal stained E15.5 (left) and postnatal day 1 (P1; right) transgenic mice line 41 showed blue staining in the chondro-osseous junction (arrows) of mouse limbs and ribs but not in the wild-type littermate controls. Background staining probably due to endogenous β -galactosidase activity was also observed in craniofacial region both in transgenic and control P1 mice. (C and E) Sagittal sections of proximal humerus and femur from P1 transgenic mouse line 41 shows blue staining in lower hypertrophic zone. Although strongest staining is observed in the lower hypertrophic zone, weak staining is also occasionally present in the bone trabeculae. (D and F) Comparative sagittal sections from wild-type littermates control show no staining. *Tg*, transgenic mice; *Ctrl*, wild-type littermate control; HZ, hypertrophic zone of growth plate.



Runx2 antibody resulted in the formation of a new low mobility complex, suggesting that Runx2 is able to bind the DNA elements and might be a component of the higher mobility complex(es) formed in the absence of antibody (Fig. 4 B). Interestingly, the EMSA with MCT nuclear extracts showed two complexes that were both effectively competed for by cold probe suggesting that different molecular complexes may form on these cis elements. The two additional putative *Runx2* binding sites in the mouse 4-kb promoter bound RUNX2 weakly and the addition of antibody did not generate bands of delayed mobility (unpublished data).

To test whether Runx2 interacts with *Col10a1* promoter via A and B elements in vivo, we also performed chromatin immunoprecipitation analysis using hypertrophic MCT cells and Runx2 antibody. Indeed, we were able to coimmunoprecipitate both A and B elements with Runx2 antibody but not with control antibody. Furthermore, a 300-bp *Col10a1* intron II fragment, which does not contain a Runx2 site, could not be immunoprecipitated with Runx2 antibody. These results indicated that *Runx2* binds to A and B elements within *Col10a1* promoter in vivo (Fig. 4 C).

In cotransfection studies in COS7 cells, RUNX2 was able to transactivate reporter constructs containing eight copies of either of these RUNX2-binding elements upstream of a

44-bp *Col10a1* minimal promoter and the luciferase reporter gene (Fig. 5 A). Promoter constructs containing the A element or B element were transactivated >20- and 40-fold above baseline, respectively (Fig. 5 A). These data show that RUNX2 can bind to the distal sequences of the 4-kb *Col10a1* promoter and transactivate a *Col10a1* minimal promoter via these sequences. We also transfected the *Col10a1* 4-kb promoter- β -galactosidase reporter plasmid with or without the *RUNX2* expression plasmid into hypertrophic MCT cells. The endogenous activity of the 4-kb promoter was 10-fold greater than that of the basal promoter (Fig. 5 B, left). In addition, when RUNX2 is over-expressed in these cells, reporter activity is further upregulated more than two-fold above the endogenous activity of this promoter (Fig. 5 B, left). In converse, when the two RUNX2 binding sites are mutated in the 4-kb promoter, reporter activity is decreased by 35% compared to the wild-type promoter (Fig. 5 B, right). Together, these data show that the 4-kb *Col10a1* promoter is up-regulated when MCT chondrocytes hypertrophy in culture and that RUNX2 binding contributes to this transactivation.

To quantify the correlation between *Runx2* and *Col10a1* expression, quantitative real time RT-PCR was used to determine their respective levels of expression in both the

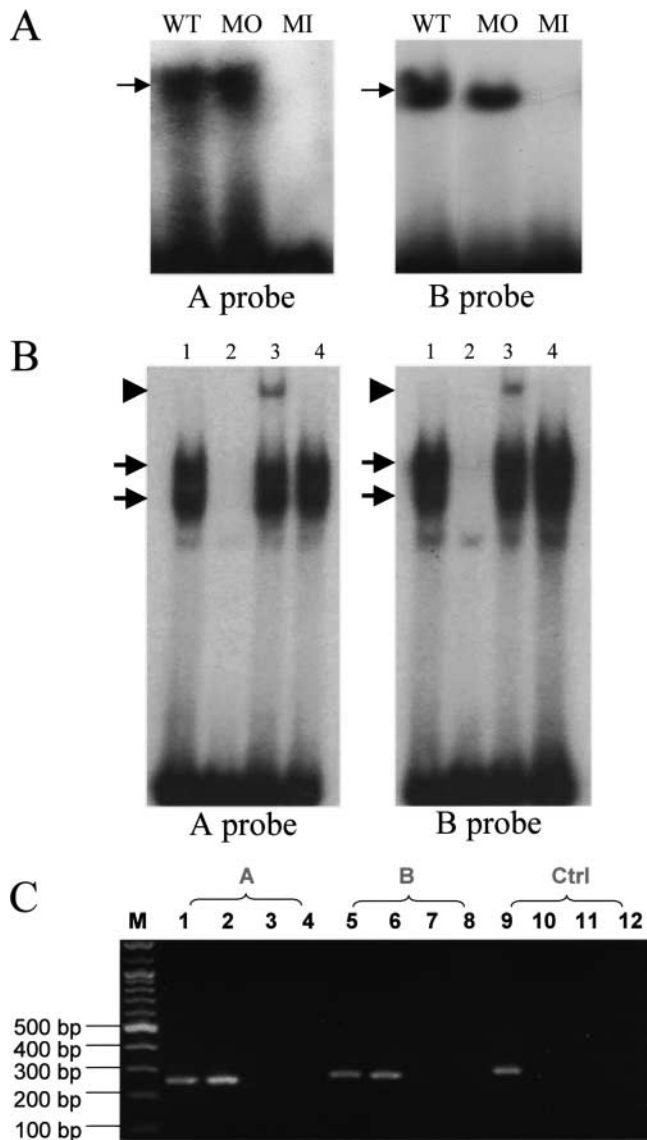


Figure 4. RUNX2 binds to cis elements A and B within *Col10a1* promoter region. (A) EMSA using His-tagged recombinant RUNX2 Runt polypeptide with cis element A or B as probe (Fig. 1 A). 32 P-End-labeled wild-type (WT) probe A or B (Fig. 1) and the probe mutated outside of the core sequence (MO) exhibited binding (arrow), whereas a mutation in the core sequence (MI) abolished binding. (B) EMSA using nuclear extracts from hypertrophic MCT cells grown at 37°C with A or B probe. Both 32 P-end-labeled wild-type probe A or B (lane 1) shows two specific DNA-protein complexes (arrows). These complexes diminish with competition with 100-fold cold probe (lane 2). Presence of Runx2 in the DNA-protein complex is demonstrated by the formation of a new low mobility complex (arrowhead) with the addition of anti-Runx2 antibody to the MCT cell nuclear extracts (lane 3) but not in the control with preimmune serum (lane 4). (C) Runx2 binds to the mouse A and B elements in hypertrophic MCT cells. A representative chromatin immunoprecipitation experiment with hypertrophic MCT cells is shown. Immunoprecipitation was performed with anti-Runx2 antibody (lanes 2, 6, and 10), preimmune antiserum (lanes 3, 7, and 11) or no antibody (lanes 4, 8, and 12). Following DNA purification, samples were subject to 35 cycles of PCR with primers designed for the A (lanes 1–4), B (lanes 5–8), and negative control elements (lane 9–12). A portion of input was used as positive control for PCR (lanes 1, 5, and 9). The results indicated that Runx2 binds to these putative Runx2 binding A and B elements within *Col10a1* promoter in vivo (lanes 2 and 6).

MCT cells and in *Runx2*^{+/+}, *Runx2*^{+/-}, and *Runx2*^{-/-} mice (Otto et al., 1997; Livak and Schmittgen, 2001; Pfaffl, 2001). *Runx2* was weakly expressed in MCT cells grown at 32°C by Northern and qualitative RT-PCR analysis (unpublished data). However, when cells were terminally differentiated by growth at nonpermissive temperatures (from 32°C to 37°C), *Col10a1* was upregulated (Fig. 2 B, left, and Fig. 6 A, right) in conjunction with a two fold up-regulation of *Runx2* (Fig. 6 A, left). In limbs from newborn mice, real time RT-PCR assay showed less *Col10a1* expression in *Runx2* heterozygotes (~50%) compared to that of the wild-type littermate controls (Fig. 6 B, right). Furthermore, *Col10a1* expression was at a level barely detectable in *Runx2*^{-/-} mice limbs (Fig. 6 B, right; Inada et al., 1999). As expected, *Runx2* expression was decreased by 45% in *Runx2* heterozygotes (Fig. 6 B, left). Thus, our data suggest that *Runx2* might directly regulate the activation of the type X collagen gene during chondrocyte maturation in vivo.

Runx2 transactivates *Col10a1* promoter in transgenic study

To further delineate the contribution of *Runx2* to transactivation of *Col10a1* in vivo, we bred the 4-kb promoter- β -galactosidase transgene (*Tg*) onto a *Runx2* heterozygote background to generate four different genotypes for histological analysis: *Tg*^{+/-}/*Runx2*^{+/-}, *Tg*^{+/-}/*Runx2*^{+/+}, *Runx2*^{+/-}, and *Runx2*^{+/+} (wild type; Otto et al., 1997). Sections of long bones including the distal ulna and proximal tibia from P1 mice showed X-gal staining in hypertrophic chondrocytes in *Tg*^{+/-}/*Runx2*^{+/+} (Fig. 7 A and not depicted). X-gal staining was decreased in *Tg*^{+/-}/*Runx2*^{+/-} mice (Fig. 7 A). Although the *Runx2* targeted allele does contain a knock-in *LacZ* reporter, *Runx2* expression in hypertrophic chondrocytes is lower compared to its expression in osteoblasts and X-gal blue staining in the *Runx2*^{+/-} mice was undetectable in hypertrophic chondrocytes with our protocol (Fig. 7 A, right). As expected, X-gal staining was stronger in the bone marrow of *Tg*^{+/-}/*Runx2*^{+/-} mice than in *Tg*^{+/-}/*Runx2*^{+/+} mice because of the strong *LacZ* expression of the *Runx2* targeted allele in osteoblast (unpublished data). To quantify and compare the transgene expression in *Tg*/*Runx2*^{+/+} and *Tg*/*Runx2*^{+/-} mice, we performed semiquantitative densitometric analysis of transgene expression in mouse limb sections (Ma et al., 2001). 200 blue-stained cells in the hypertrophic zone from twenty limb sections from each of the genotype were chosen for analysis. The gray value, which is inversely related to the intensity of the blue staining, is significantly lower in cells from *Tg*/*Runx2*^{+/-} mice limb sections than that from *Tg*/*Runx2*^{+/+} mice ($P < 0.01$). This result suggested that the transgene is expressed at a lower level in chondrocytes from *Tg*/*Runx2*^{+/-} mice compared to that of *Tg*/*Runx2*^{+/+} mice (Fig. 7 B, left). We also performed real time RT-PCR quantification of transgene expression using RNAs from transgenic mice limbs with *Runx2* wild-type (*Tg*/*Runx2*^{+/+}) or heterozygote background (*Tg*/*Runx2*^{+/-}). Transgene expression decreased by approximately 40% in *Tg*/*Runx2*^{+/-} compared to that of *Tg*/*Runx2*^{+/+} (Fig. 7 B, right). These data support genetic interaction between the *Col10a1* *Tg* reporter allele and *Runx2* expression in hyper-

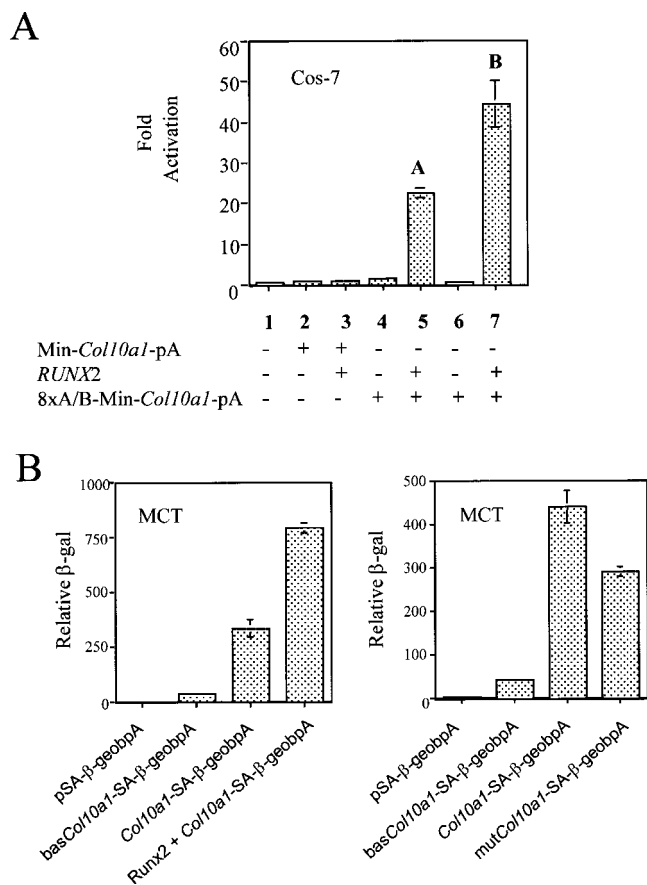


Figure 5. *Col10a1* promoter activity is up-regulated via RUNX2 binding elements in vitro. (A) Transactivation of *Col10a1* via RUNX2-binding A and B elements. The RUNX2 expression plasmid (RUNX2) was cotransfected in COS7 cells with reporter plasmids Min-*Col10a1*-pA or 8xA/B-Min-*Col10a1*-pA. Transfection of Min-*Col10a1*-pA with or without RUNX2 expression plasmid produced no transactivation (lanes 2 and 3). Transfections of 8xA/B-Min-*Col10a1*-pA alone also produced no transactivation (lanes 4 and 6). However, the addition of the RUNX2 expression plasmid resulted in strong transactivation of 8xA-Min-*Col10a1*-pA and 8xB-Min-*Col10a1*-pA reporter plasmids, respectively (lanes 5 and 7). A SV2 β gal plasmid was cotransfected as an internal control for transfection efficiency. Representative data are presented as fold activation relative to the activity obtained with pcDNA3.1 empty vector plasmid (lane 1). Each transfection experiment was performed in triplicate and the standard deviations are shown by the error bars. (B) Contribution of RUNX2 binding sites to 4-kb *Col10a1* promoter activity. (left) Overexpression of RUNX2 in MCT cells further upregulates the 4-kb *Col10a1* promoter. MCT cells were transfected only at the nonpermissive temperature (37°C) with the reporter plasmids bas*Col10a1*-pSA β -geobpA, 4-kb *Col10a1*-SA β geobpA alone, and 4-kb *Col10a1*-SA β -geobpA along with the *Runx2* expression plasmid. A RSV-luc plasmid was cotransfected as internal control for transfection efficiency. The endogenous activity of the 4-kb promoter was 10-fold higher than that of the basal promoter, whereas over-expression of RUNX2 further increased the promoter activity. (right) RUNX2 binding sites contribute to 4-kb *Col10a1* promoter activity in MCT cells. MCT cells were transfected only at nonpermissive temperature (37°C) with reporter plasmids bas*Col10a1*-pSA β -geobpA, 4-kb *Col10a1*-SA β -geobpA, and mut 4-kb *Col10a1*-SA β -geobpA. When the two Runx2 binding sites were mutated, reporter activity was decreased by 35% compared to that of the wild-type promoter.

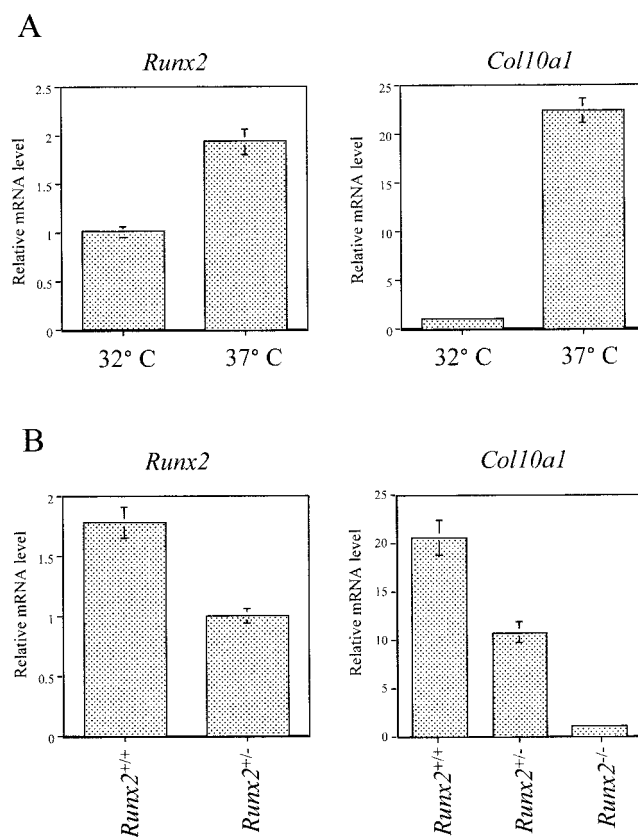


Figure 6. Runx2 directly regulates the activation of the type X collagen gene. (A) *Runx2* is upregulated in hypertrophic MCT cells. When MCT cells were shifted from 32°C to 37°C, *Runx2* showed two-fold upregulation as assayed by real time RT-PCR (left). Meanwhile, *Col10a1* showed more than 20-fold up-regulation in hypertrophic MCT cells (right). Each real time PCR experiment was performed in triplicate and the standard deviations are shown by the error bars. Similar results were obtained from three independent experiments. (B) Endogenous downregulation of *Col10a1* in *Runx2*^{+/-} and *Runx2*^{-/-} mouse limbs. *Runx2* expression was also decreased by 45% in *Runx2* heterozygotes (left). Endogenous *Col10a1* expression was also decreased by 50% in *Runx2* heterozygotes and barely detectable in *Runx2*^{-/-} mice as compared to that of the wild-type littermate control by real time RT-PCR assay (right). Similar results were obtained from five wild-type, eight *Runx2*^{+/-} and two *Runx2*-null mice. One representative set of results is presented here with the standard deviations shown by the error bars.

trophic chondrocytes. Interpreted in the context of the in vitro and in vivo data, this is most likely explained by direct transactivation of the *Col10a1* promoter by *Runx2*. Interestingly, as shown in the ulna sections in Fig. 7 A, both *Runx2*^{+/-} and *Tg*^{+/-}/*Runx2*^{+/-} mice had a slightly shortened zone of hypertrophy as compared to *Runx2*^{+/+} mice. Similar differences were also observed in sections of the growth plates of the humerus and radius (unpublished data). Furthermore, RNA in situ hybridization on distal femur sections of *Runx2*^{+/+} or *Runx2*^{+/-} mice using a *Col10a1* riboprobe showed that *Col10a1* expression was detected throughout the hypertrophic zone. Moreover, the *Runx2*^{+/-} mice had a shortened hypertrophic zone in the growth plate region similar to that observed in *Tg*^{+/-}/*Runx2*^{+/-} mice (Fig. 7 C).

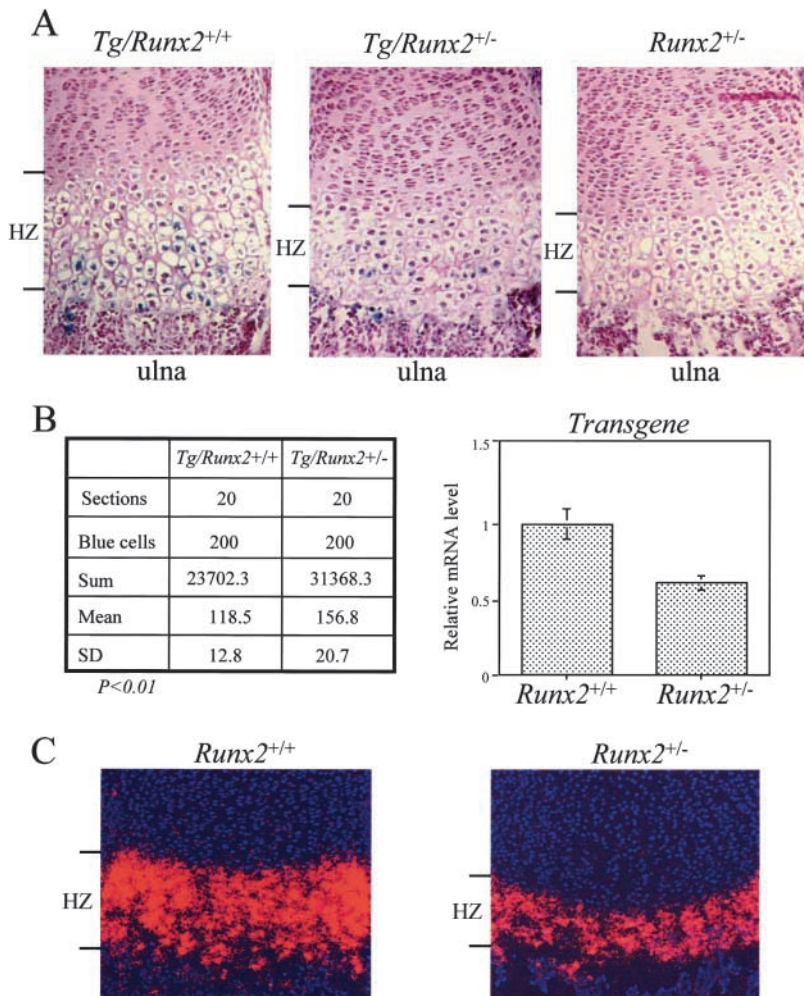


Figure 7. Genetic interaction of *Runx2* and *Col10a1*. (A) *Runx2* and *Col10a1* promoter/reporter transgene. The 4-kb *Col10a1* promoter- β geo transgene (*Tg*) was bred onto *Runx2*^{+/+} and *Runx2*^{+/-} backgrounds and compared to *Runx2*^{+/-} mice without *Tg*. X-gal blue staining is much weaker in the lower hypertrophic zones of ulna of *Tg/Runx2*^{+/-} mice (middle) than in that of *Tg/Runx2*^{+/+} mice (left), whereas no staining is observed in the hypertrophic zone of *Runx2*^{+/-} mice (right). The hypertrophic zone (HZ) appeared shorter in sections from mice with *Runx2*^{+/-} than that of *Runx2*^{+/+} background. (B) Transgene expression in *Tg/Runx2*^{+/+} versus *Tg/Runx2*^{+/-} mice. (left) Semi-quantitative densitometric analysis of transgene expression in mouse limb sections by comparing the gray values generated for blue-staining cells as described in Materials and methods. Sum, total gray value of blue cells; SD, standard deviation. (right) Transgene expression was decreased by 40% in *Runx2* heterozygotes as compared to that of the wild-type littermate control by real time RT-PCR assay. Similar results were obtained from two *Tg/Runx2*^{+/+} and three *Tg/Runx2*^{+/-} mice. One representative set of results is presented here with the standard deviations shown by the error bars. (C) *Col10a1* expression pattern in *Runx2*^{+/-} mice. *Runx2*^{+/+} mice showed signal of *Col10a1* transcripts throughout the hypertrophic zone of femur by in situ hybridization (left), whereas *Runx2*^{+/-} mice also have shortened hypertrophic zone as indicated by *Col10a1* expression pattern (right).

Discussion

Chondrocyte hypertrophy is the terminal step of chondrocyte differentiation. The identification of transcription factors controlling this process is essential for understanding the molecular mechanisms that specify endochondral ossification and the molecular pathogenesis of skeletal dysplasia such as SMCD. Here, we use the hypertrophic chondrocyte-specific molecular marker *Col10a1* to delineate cell-type-specific cis elements and the DNA-binding proteins that activate them. Our in vitro and in vivo studies show that *Runx2* directly transactivates *Col10a1*. Although it was previously reported that treatment with antisense oligonucleotides for *Runx2* reduced type X collagen expression in the chondrogenic cell line ATDC5 and that SMAD-RUNX2 interactions are important for chondrocyte hypertrophy, it was unclear whether *Runx2* directly regulates the activation of the type X collagen gene (Enomoto et al., 2000; Leboy et al., 2001). Our data provide several lines of evidence supporting *Runx2*'s role in the direct regulation of type X collagen. First, multiple *Runx2* binding sites are found in the type X collagen promoter of mouse as well as human and chick (Fig. 1, A and B), and some are conserved between human and mouse (Fig. 1 C). These sites bind to RUNX2 and can mediate transactivation of the *Col10a1* minimal promoter. Moreover, their location in the distal

promoter is in agreement with previous reports of positive regulatory elements in the corresponding 5' positions in the human promoter (Beier et al., 1997). Second, the 4-kb promoter containing these sites is activated in MCT cells that are undergoing hypertrophy and expressing endogenous type X collagen. *Runx2* is also concurrently upregulated. Importantly, mutations of these two sites decrease expression of the same promoter in hypertrophic cells, whereas overexpression of *Runx2* in the same cells further upregulates the promoter. Third, the 4-kb promoter, but not the 1.7-kb mouse promoter, can direct expression selectively though weakly in hypertrophic chondrocytes. Moreover, it does not direct expression in resting and proliferating chondrocytes or in any other tissues including perichondrium that contains high levels of *Runx2* in transgenic mice (Eerola et al., 1996). These in vivo data are correlated with the demonstration of a genetic interaction between the *Runx2* locus and the activity of the *Col10a1* 4-kb promoter/reporter transgene. Notably, we also detected decreased *Col10a1* expression in *Runx2* heterozygote mice, whereas *Col10a1* was barely detectable in *Runx2*-null mice.

This paper utilizes a unique type X collagen-expressing MCT cell line to delineate the cis-acting elements for chondrocyte hypertrophy. Compared to the bovine or chicken primary hypertrophic chondrocytes previously used in the

cell culture studies, the MCT cell line has the advantage of homogeneity and phenotypic stability (Lefebvre et al., 1995). Most importantly our results from MCT cells correlate with that of transgenic mice. Therefore, MCT cells could be used to further delineate the cis elements controlling chondrocyte hypertrophy and eventually to characterize the transcription factor(s) specifying this process.

The 4-kb *Col10a1* promoter described can direct reporter expression selectively to lower hypertrophic chondrocytes in transgenic mice. However, it is likely that additional positive and negative regulatory elements outside of this 4-kb *Col10a1* promoter are also required for tissue-specific expression during embryogenesis. This is supported by the detection of weak β -galactosidase expression in the bone trabeculae of our transgenic mice, as well as by the localization of reporter gene expression primarily in the lower hypertrophic zone, and less so in the upper hypertrophic zone. Together, these observations suggest the requirement of additional positive and negative regulatory elements outside this 4-kb promoter to achieve high level *Col10a1* expression in hypertrophic chondrocytes in vivo. *Runx2* is likely only one of several factors that are required for this coordinated process. *Runx2* is essential for osteoblast differentiation and it is also important for chondrocyte maturation. It has been shown that *Runx2* transcriptional regulation of collagenase-3, osteopontin, and VEGF is critical for the transition from chondrogenesis to ossification (Sato et al., 1998; Jimenez et al., 1999; Zelzer et al., 2001). *Runx2* may interact with different factors within osteoblast and hypertrophic chondrocytes to regulate different downstream genes. It alone is not sufficient to specify chondrocyte hypertrophy in all bones, because loss of hypertrophic chondrocytes is observed only in some of the long bones of *Runx2*-null mice (Inada et al., 1999; Kim et al., 1999). Identification of other transcription factors important for chondrocyte hypertrophy and their relation with *Runx2* will be crucial for our understanding of chondrogenesis.

Whether decreased *Col10a1* expression might directly affect chondrocyte hypertrophy is still controversial. There is some evidence to support that haploinsufficiency of *COL10A1* accounts for cases of SMCD (Chan and Jacenko, 1998; Chan et al., 2001; Wilson et al., 2002), whereas others have pointed to a possible dominant negative effect (Marks et al., 1999; Gregory et al., 2000). A very recent study showed that nonsense mutations leads to the complete degradation of mutant collagen X mRNA in cartilage in SMCD (Bateman et al., 2003). Interestingly, SMCD patients also have an altered zone of hypertrophy (Wasylenko et al., 1980; Lachman et al., 1988; Nielsen et al., 2000). However, it appears that chondrocyte hypertrophy by itself, at least in mice, does not require type X collagen (Rosati et al., 1994). Instead, defective mineralization, altered hematopoiesis, and growth plate compression were reported in *Col10a1*-null mice (Kwan et al., 1997; Gress and Jacenko, 2000). It may be that the structural integrity of the hypertrophic zone requires normal type X collagen expression in humans and a metaphyseal dysplasia develops in the presence of decreased expression. However, the same effect may not be seen in mice because of different biomechanical forces impinging upon metaphyseal development in a tetrapod.

From the point of view of skeleton pathogenesis, haploinsufficiency of *RUNX2* causes cleidocranial dysplasia (CCD), a dominantly inherited skeletal dysplasia in humans (Mundlos et al., 1995, 1996, 1997; Lee et al., 1997). The pathognomonic features of CCD include both defective intramembranous ossification and defective endochondral ossification (Cooper et al., 2001). Interestingly, it has been reported that *Runx2*^{+/-} mice exhibit some of the features of CCD including delayed closure of the fontanel and hypoplastic clavicles (Otto et al., 1997). Our findings of decreased *Col10a1* expression and altered chondrocyte hypertrophy in *Runx2* heterozygote mice suggest that they could also serve as a model to study the pathogenesis of long bone defects of CCD.

Materials and methods

Electrophoretic mobility shift assay

The mouse *Col10a1* BAC was mapped by oligonucleotide-specific Southern hybridization analysis of multiple restriction enzyme digested DNA. The 4-kb XhoI/KpnI promoter fragment was subcloned and sequenced (GenBank/EMBL/DBJ accession no. AF326473). EMSA were carried out essentially as described previously (Zhou et al., 1999) using His-tagged recombinant RUNX2 Runt polypeptide and a ³²P-end-labeled double-stranded probe corresponding to DNA elements A (5'-GATCCTCACCTTCTGTGGTTTCTCAGAA-3'), B (5'-GATCCCCATAAACCAACCACCAACCCAA-3'), and mutant versions of A or B with mutations outside of the core sequence (MO: A, 5'-GATCCTCATATTCTGTGGTTTCTCAGAA-3'; B, 5'-GATCCCCAGCAACAACCACCAACCCAA-3' [mutation in italics]) and mutations inside the core sequence (MI: A, 5'-GATCCTCACCTTCTGTGGTTTCTCAGAA-3'; B, 5'-GATCCCCATAAACACGTCACCAACCCAA-3' [mutation in italics]). Putative Runx2 binding sites are underlined.

For nuclear extracts, MCT cells were grown at 32°C until subconfluency. They were further incubated at 37°C for 2 d before nuclear extract preparation. Nuclear extracts were then prepared as described previously with 10 μ g/ml of leupeptin and pepstatin in all buffers (Dyer and Herzog, 1995). 3 μ g of hypertrophic MCT cell nuclear extracts were used for each binding assay. 50- to 100-fold cold competitor probe, or Runx2 antibody (Ducy et al., 1997), or preimmune serum were incubated with nuclear extracts 10 min on ice before addition of probe in parallel experiments. The MCT cells were provided by B. de Crombrugge (University of Texas, Houston, TX). The anti-Runx2 antibody was provided by G. Karsenty (Baylor College of Medicine, Houston, TX).

Chromatin immunoprecipitation assay

Hypertrophic MCT cells were incubated at 37°C for 3 d before formaldehyde fixation. Cold PBS (with Protein Inhibitor Cocktail Tablets; Roche) washing, cell harvesting, and sonication to shear DNA to 500–1,000 bp was performed according to the manufacturer's protocol (Upstate Biotechnology). Precleared chromatin was incubated with 2 μ g of the anti-Runx2 antibody (Santa Cruz Biotechnology, Inc.), preimmune antiserum or no antibody and rotated at 4°C for 12 h. Immunoprecipitation, washing, and elution of immune complex were carried out as described previously (Boyd and Farnham, 1999). The specific primers for A element, B element, and the control primers within *Col10a1* intron II region were used for PCR amplification as described previously (Thomas et al., 2001; Weinmann and Farnham, 2002).

Transfection studies

The *Col10a1* minimal promoter (+7 bp to -37 bp) was inserted into the pLuc4 luciferase reporter plasmid (Min-*Col10a1*-pA; Zhou et al., 1999). Each of the *Runx2* binding elements A or B were concatamerized to form eight copy fragments, which were inserted upstream of the *Col10a1* minimal promoter (8xA/B-Min-*Col10a1*-pA). Transfections using an expression plasmid (control pcDNA3.1 or pcDNA3.1/RUNX2), a reporter plasmid (Min-*Col10a1*-pA or 8xA/B-Min-*Col10a1*-pA), and a normalizing plasmid (pSV2 β gal) were performed with the Lipofectamine-plus (GIBCO BRL) reagent in COS7 cells, and luciferase and β -galactosidase activities were assayed 24 h after transfection as described previously (Zhou et al., 1999). Transfections were performed in triplicate at three different doses (0.2, 0.5, and 1.0 μ g/well, respectively) to ensure a linear-dose response.

MCT cells were grown at 32°C in standard DME media with 8% FBS (GIBCO BRL) and 8% CO₂ as per published protocol (Lefebvre et al.,

1995). Transfection of MCT cells were conducted at both 32°C and 37°C using reporter plasmids containing a 221-bp basal *Col10a1* promoter upstream of the β geo reporter (bas*Col10a1*-SA β geobpA), the wild-type *Col10a1* 4-kb proximal promoter (*Col10a1*-SA β geobpA), or the same promoter with mutated RUNX2 binding sites A and B (mut*Col10a1*-SA β geobpA). The mut*Col10a1*-SA β geobpA reporter plasmid was generated by replacing a Xho/BlpI wild-type fragment with a mutant one within the 4-kb *Col10a1* promoter and it contained the same mutations within the RUNX2 binding A and B sites as described in the previous paragraph on the EMSA experiments.

MCT cells were transfected for 6 h using Lipofectamine-plus (GIBCO BRL), incubated for an additional 48 h at either 32°C or 37°C, and harvested for β -galactosidase activity assay. A luciferase expression plasmid pRSVluc was added to all transfections and used as internal control for normalizing the cell transfection efficiency. Transfections were also performed in triplicate at three doses to ensure a linear-dose response.

Northern analysis, real time RT-PCR, and in situ hybridization

For Northern hybridization analysis of *Col10a1* mRNA derived from MCT cells grown at either 32°C or 37°C, total RNA was prepared from MCT cells using TRIzol reagent according to the manufacturer's protocol (GIBCO BRL). 10 μ g RNA was fractionated, transferred to Hybond NH₂ nylon membrane (Amersham Biosciences), and hybridized with murine *Col10a1* cDNA (a 1.2-kb BamHI fragment) and *Gapdh* cDNA (a 300-bp fragment from the 3' untranslated region) probes as described previously (Lefebvre et al., 1995). The filter was then autoradiographed for 3 d.

For MCT cells, the same total RNAs for Northern analysis were also used for cDNA synthesis. Total mouse limb RNAs from littermates with wild-type, *Runx2*^{+/-}, or *Runx2*^{-/-} background at P1 stage was extracted by liquid nitrogen frozen, grinded on ice, and followed by TRIzol reagents extraction (GIBCO BRL). cDNA synthesis was performed by using the Superscript first-strand synthesis system RT-PCR kit (Invitrogen). The primer sequences used for PCR amplification for *Runx2*, *Col10a1*, transgene (the bovine growth hormone poly A region in the transgene construct) and *Gapdh* was performed using specific primers. The *Gapdh* gene was used as an internal control of the quantity and quality of the cDNAs. Real time PCR amplification was performed on LightCycler (Roche) according to the manufacturer's protocol and published procedures with modifications (Pfaffl, 2001). Analysis of the real time PCR results, i.e., the relative gene expression level, was achieved by using the 2^{- $\Delta\Delta$ C_T} method for fold induction, and C_T (the threshold cycle) for the fractional cycle number at which the amount of amplified target reaches a fixed threshold (Livak and Schmittgen, 2001; Pfaffl, 2001).

For *Col10a1* in situ hybridization, newborn mouse hind limbs were fixed in 4% PFA in PBS, dehydrated, and embedded in paraffin. Sectioning and in situ hybridization were carried out as described previously (Albrecht, 1998) with the same *Col10a1* probe used in the Northern analysis. Nine distal serial femur sections from each of two *Runx2*^{+/-} and one *Runx2*^{+/+} littermates were analyzed. Representative data for comparable sections are shown.

Generation and histochemical analysis of transgenic mice

The DNA fragment containing the 4-kb *Col10a1* promoter followed by SA β geobpA reporter was released from the above-described *Col10a1*-SA β geobpA construct by Apal digestion. Purified DNA's were redissolved and microinjected into the pronuclei of one-cell ICR X B6C3F1 mouse embryos and implanted into ICR pseudopregnant foster mothers (Hogan et al., 1986). Transgenic founder mice were analyzed by genomic southern analysis of tail DNA's with a 3-kb *lacZ*-specific probe (Zhou et al., 1995). *Runx2* heterozygote mice were obtained from M. Owen (Lincoln's Inn Fields, London, UK) and genotyped by PCR amplification as reported previously (Otto et al., 1997).

In brief, P1 mice were collected, skinned, and eviscerated. They were fixed and stained with X-gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) as described previously (Zhou et al., 1995). After staining, mice were paraffin embedded, sectioned, and counterstained with nuclear fast red (Poly Scientific R&D Corp.). Sections of all long bones were analyzed and comparisons were made only among littermates at the same magnifications. At least 30 sections of each growth plate were analyzed.

For densitometric analysis of transgene expression in *TgRunx2*^{+/+} and *TgRunx2*^{+/-} mouse limb sections, we calculated the gray value of blue staining cells corresponding to β -galactosidase activity using a microscope (Axioplan 2; Carl Zeiss Microimaging, Inc.) and AxioVision 3.1 software (Carl Zeiss Vision GmbH). Analyses were made by two independent observers blinded to genotype of the sections. 200 blue staining cells in the hypertrophic zone were randomly chosen from 20 serial limb sections. A

gray value with standard deviation was generated for each cell. This value inversely related to the intensity of the blue staining. Average gray values were calculated for the 200 cells of each genotype. Statistical analysis was performed with *t* test.

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