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Overexpression of Runx2 Directed by the Matrix Metalloproteinase-13 Promoter Containing the AP-1 and Runx/RD/Cbfa Sites Alters Bone Remodeling In Vivo

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Abstract The activator protein-1 (AP-1) and *runt* domain binding (Runx/RD/Cbfa) sites and their respective binding proteins, c-Fos/c-Jun and Runx2 (Cbfa1), regulate the rat matrix metalloproteinase-13 (MMP-13) promoter in both parathyroid hormone (PTH)-treated and differentiating osteoblastic cells in culture. To determine the importance of these regulatory sites in the expression of MMP-13 in vivo, transgenic mice containing either wild-type (−456 or −148) or AP-1 and Runx/RD/Cbfa sites mutated ($-148A_3R_3$) MMP-13 promoters fused with the *E. coli lacZ* reporter were generated. The wild-type transgenic lines expressed higher levels of bacterial β-galactosidase in bone, teeth, and skin compared to the mutant and non-transgenic lines. Next, we investigated if overexpression of Runx2 directed by the MMP-13 promoter regulated expression of bone specific genes in vivo, and whether this causes morphological changes in these animals. Real time RT-PCR experiments identified increased mRNA expression of bone forming genes and decreased MMP-13 in the tibiae of transgenic mice (14 days and 6 weeks old). Histomorphometric analyses of the proximal tibiae showed increased bone mineralization surface, mineral apposition rate, and bone formation rate in the transgenic mice which appears to be due to decreased osteoclast number. Since MMP-13 is likely to play a role in recruiting osteoclasts to the bone surface, decreased expression of MMP-13 may cause reduced osteoclast-mediated bone resorption, resulting in greater bone formation in transgenic mice. In summary, we show here that the 148 bp upstream of the MMP-13 transcriptional start site is sufficient and necessary for gene expression in bone, teeth, and skin in vivo and the AP-1 and Runx/RD/Cbfa sites are likely to regulate this. Overexpression of Runx2 by these regulatory elements appears to alter the balance between the bone formation-bone resorption processes in vivo. J. Cell. Biochem. 99: 545–557, 2006.

Key words: bone formation; bone remodeling; bone resorption; Runx2; collagenase-3; matrix metalloproteinase-13

Bone is the body's main reservoir of calcium and phosphate ions, and is constantly regenerated through continuous formation and resorption in the process of bone remodeling. This physiological process occurs throughout adult life to maintain a constant bone mass. In several pathological conditions, the tight balance between bone formation and resorption is not preserved [Krane, 2002]. Bone formation in vivo is a complex phenomenon whereby recruitment

Abbreviations used: MMPs, matrix metalloproteinases; AP-1, activator protein-1; RD, runt domain binding site; Cbfa, core binding factor alpha; AML, acute myeloid leukemia; ECM, extracellular matrix; PTH, parathyroid hormone; RT-PCR, reverse transcriptase-polymerase chain reaction; BSP, bone sialoprotein; OC, osteocalcin; ALP, alkaline phosphatase; OPN, osteopontin; OPG, osteoprotegerin; RANKL, receptor activator of nuclear factor kappaB ligand.

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and replication of mesenchymal precursors of osteoblasts, differentiation into preosteoblasts, osteoblasts, and mature osteoblasts ultimately results in the accumulation and mineralization of the extracellular matrix [Aubin, 1998]. Osteoclasts responsible for bone resorption are derived from hematopoietic precursor cells belonging to the monocyte/macrophage lineage. Osteoclast differentiation is a multistep process that leads eventually to multinucleated bone-resorbing osteoclasts [Roodman, 1999].

Runx2, also called Cbfa1 or Pebp2αA, is a transcription factor that belongs to the runt-domain gene family [Komori and Kishimoto, 1998]. Runt proteins are a group of transcription factors conserved from *C. elegans* to humans. They share a typical 128-amino acid DNA-binding domain called the Runt domain. Runx2 acts as an inducer of osteoblast differentiation and it has all the characteristics of a differentiation regulator in the osteoblast lineage [Ducy et al., 1997; Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997; Karsenty, 1999]. Bone formation is not solely controlled at the level of osteoblast differentiation and Runx2 is also required for osteoblast function [Ducy et al., 1999]. Runx2 is able to induce both early and late markers for osteoblast differentiation, including alkaline phosphatase (ALP), type I collagen, osteopontin (OPN), bone sialoprotein, and osteocalcin (OC) in several cell lines [Harada et al., 1999]. Genes involved in the bone resorption process, such as RANKL, OPG, and MMP-13 are also regulated by Runx2 [Geoffroy et al., 2002].

MMP-13, a matrix metalloproteinase, is expressed as a late-differentiation gene in osteoblasts, and is primarily responsible for the degradation of extracellular bone matrix components (type I, II, and III fibrillar collagens). MMP-13 gene expression is regulated by bone-resorbing agents, such as PTH, cytokines such as interleukin-1 and -6, and growth factors that promote bone turnover [Scott et al., 1992; Varghese et al., 1995, 1996, 2000; Kusano et al., 1998]. In vivo, MMP-13 has been shown to be expressed in ossifying centers during bone development [Schorpp et al., 1995] and is detectable by immunohistochemistry in rat calvariae 14 days after birth [Davis et al., 1998]. The regulation of this gene is likely to have important consequences for both normal and pathological remodeling of bone where the balance between bone resorption and bone formation is disrupted. Using mutant mice homozygous for a targeted mutation in *Col1a1* that are resistant to collagenase cleavage of type I collagen, Zhao et al. [1999] showed that PTH-induced bone resorption and calcemic responses were markedly diminished. The number of osteoclasts was also reduced and the animals had thicker than normal bones [Zhao et al., 2000]. Studies with a null mutation of the MMP-13 gene in mice showed that in $Mmp13^{-/-}$ embryos, the growth plates were strikingly lengthened, a defect ascribable predominantly to a delay in terminal events in the growth plates, with failure to resorb collagens, as well as a delay in ossification at the primary centers [Inada et al., 2004].

Previous work in our laboratory has determined that the activator protein-1 (AP-1) and *runt* domain binding (Runx/RD/Cbfa) sites and their respective binding proteins, c-Fos/c-Jun and Runx2 (Cbfa1), regulate the MMP-13 promoter in both PTH-treated and differentiating osteoblastic cells in culture [Selvamurugan et al., 1998; Winchester et al., 2000]. Furthermore, protein–protein interaction studies indicate that Runx2 and the runt domain of Runx2 alone interact with c-Fos and c-Jun [D'Alonzo et al., 2002]. Also, co-transfection of Runx2 with the MMP-13 promoter in UMR 106-01 cells has been shown to enhance transactivation of the MMP-13 promoter [D'Alonzo et al., 2002]. In the present study, we wished to investigate the importance of the AP-1 and Runx/RD/Cbfa sites and Runx2 in the expression of MMP-13 and bone specific genes in vivo. To determine if the regulatory elements responsible for MMP-13 expression in vitro operated in vivo, transgenic mice containing either wild-type (−456 or −148) or AP-1 and Runx/RD/Cbfa site mutant ($-148A_3R_3$) MMP-13 promoters fused with the *E. coli lacZ* (β-galactosidase) reporter were generated. Since Runx2 is involved in bone development and bone remodeling, we also investigated if overexpression of Runx2 under the control of the MMP-13 promoter in vivo regulates bone specific genes, and whether this causes changes in the bone phenotype of these animals.

MATERIALS AND METHODS

Transgenic Mice Generation

The rat MMP-13 promoter fragments were generated by PCR, with SalI linkers engineered

onto the 5′ and 3′ ends. The fragments were subcloned into pSV0CAT (Promega, Madison, WI) vectors and the AP-1 and RD mutations were generated using the Chameleon double-stranded site-directed mutagenesis kit (Stratagene). The fragments were released by SalI digestion, ligated to bacterial β-galactosidase and mouse protamine 1 which is a protein necessary for haploid DNA packaging and paternal procreation. These transgenes were then gel purified and used for injection into mouse blastocysts. The pCMV-c-myc mammalian expression vector expressing the N-terminal c-myc epitope tag was used to clone the rat MMP-13 promoter (−148 containing the AP-1 and RD sites) driving Runx2 (type II). The entire fragment containing the rat MMP-13 promoter, c-myc epitope tag, and Runx2 was released by a SphI and PvuII digestion and used for injection into mouse blastocysts. The Institutional Animal Care and Use Committee at Saint Louis University approved all procedures for the generation of the mice and collection of tissues from the mice bearing the MMP-13 promoter/reporter genes and the MMP-13 promoter/Runx2 genes. The Institutional Animal Care and Use Committee of UMDNJ-Robert Wood Johnson Medical School approved all treatments and procedures for collection of tissues from both sets of mice.

RNA Isolation and Real Time RT-PCR

Bone samples were ground with the grinding mill in a TRIZOL (Invitrogen) solution. RNA extraction was performed according to the instructions given by the company. Reverse transcriptase (RT) reaction was carried out using the TaqMan Reverse Transcription reagents (Roche). PCR reactions were performed according to the real-time thermocycler machine manufacturer's instructions (DNA Engine Opticon, MJ Research, MA), which allow real-time quantitative detection of the PCR product by measuring the increase in SYBR green fluorescence caused by binding of SYBR green to double-stranded DNA. The SYBR green kit for PCR reactions was purchased from Perkin Elmer Applied Biosystems. Primers used in this study were designed using the PrimerExpress software (Perkin Elmer Applied Biosystems). For PCR amplification, the following sets of primers were used: OC, 5′ AAGCAGGAGGGCAATAAGGT 3′ and 5′ AGCTGCTGTGACATCCCATAC 3′; MMP-13, 5′ GCCACCTTCTTCTTGTTGAGCTG 3′ and 5′ ATCAAGGGATAGGGCTGGGTCAC 3′; ALP, 5′ AGGCAGGATTGACCACGG 3′ and 5′ TGTAGTTCTGCTCATGGA 3′; OPN, 5′ CCAATGAAAGCCATGACCACA 3′ and 5′ CGTCAGATTCATCCGAGTCCAC 3′; Osteoprotegerin (OPG), 5′ CGAGGACCACAATGAACAAG 3′ and 5′ TCTCGGCATTCACTTTGGTC 3′; RANKL, 5′ CAGAAGACAGCACTCACTGC 3′ and 5′ ATGGGAACCCGATGGGATGC 3′.

Immunohistochemistry

Soft tissue samples were harvested and fixed in fresh 4% paraformaldehyde in PBS at 4°C for 24 h. Bone tissue was harvested and immediately placed into fresh 4% paraformaldehyde and incubated at 4°C for 1 h. The bones were demineralized in 0.1 M Tris-HCl, 0.3 M EDTA, pH 7.4 at 4°C for 4 days with daily changes. Fixed samples were embedded in cryopreservative (O.C.T. Compound), frozen in nitrogen-cooled isopentane and stored at −70°C until sectioning. Five-micrometer frozen sections were thawed at room temperature, and washed in PBS twice, 5 min each. The sections were postfixed in 4% paraformaldehyde for 15 min, and after rinsing with PBS/BSA (1 mg/ml), were kept for 30 min in 0.3% H_2O_2 in methanol to block endogenous peroxidase activity. After brief rinsing, non-specific antibody binding was suppressed by normal rabbit serum diluted 1:70 in PBS/BSA for 30 min. After a brief rinse, the sections were incubated with polyclonal rabbit anti-bacterial β-galactosidase antibody (Rockland, Inc., Gilbertsville, PA) at 2 μg/ml (1:500 dilution) in PBS/BSA at 4°C overnight. After rinsing, the sections were incubated with a biotinylated anti-rabbit antibody. The bound antibody complex was visualized by the avidin-biotin-peroxidase procedure using the Vectastain ABC Elite Kit (Vector Laboratories, Burlingame, CA) and 3-amino-9-ethylcarbazole (AEC) as chromogen. Control sections incubated with biotinylated rabbit IgG or sections from non-transgenic animals showed very low background and no specific cell staining. Photomicrographs were taken with the use of a Spot insight color digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) attached to a Nikon Microphot-FXL microscope (Nikon, Melville, NY), and Spot Advanced imaging software (Diagnostic Instruments, Inc., Sterling Heights).

Histology

Bones from the wild-type mice and transgenic mice were decalcified and fixed in 4% paraformaldehyde/0.1 M phosphate buffer. They were then paraffin-embedded, sectioned (5 μm thick), and stained with hematoxylin and eosin.

μCT (Microcomputed Tomography)

Measurements of trabecular architecture were done on the proximal tibiae cleared of all soft tissue using a μCT 20 (Scanco Medical AG, Bassersdorf, Switzerland). After an initial scout scan, a total of 100 slices with an increment of 22 μm were obtained on each bone sample, starting 1.5 mm below the growth plate in the area of the secondary spongiosa. The area for analysis was outlined within the trabecular compartment, excluding the cortical and subcortical bone. Every 10 sections were outlined, and the intermediate sections were interpolated with the contouring algorithm to create a volume of interest. Segmentation values used for analysis were sigma 1.2, support 2, and threshold 286. A three-dimensional (3-D) analysis was done to determine bone volume (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and connectivity density (Conn.D). Cross-sectional area was determined by outlining the periosteal surface and performing a two-dimensional analysis.

Cortical bone was measured on the tibia 2 mm below the tibia-fibula junction, where the diaphysis is most uniform in shape. Ten slices of the diaphysis were made, and the same segmentation parameters were used for analysis. The periosteal surface was outlined, and a two-dimensional analysis was done to determine cross-sectional area, bone volume, and periosteal perimeter (Ps.Pm). The endocortical surface was outlined, and the analysis repeated to determine endocortical perimeter (Ec.Pm). The mean cortical thickness (Ct.Th) was determined by distance measurements at eight different points on the cortical slice.

Histomorphometric Analyses

For the assessment of dynamic histomorphometric indices, mice were injected twice with calcein at a dose of 16 μg per g body weight and analyzed at 14 days or 6 weeks of age. The 14 days group received dual injections at 7 and 2 days before sacrifice, and the 6-week group received them at 12 and 2 days before sacrifice. Tibiae were fixed with ethanol, and the calcified bones were embedded in glycolmethacrylate. Three-micrometer longitudinal sections from the proximal parts of tibiae were stained with toluidine blue and analyzed using a semiautomated system (Osteoplan II; ZEISS). Nomenclature, symbols, and units used are those recommended by the Nomenclature Committee of the American Society for Bone and Mineral Research [Parfitt et al., 1987]. The histomorphometric service was provided by the Center for Metabolic Bone Disease, University of Alabama, Birmingham, AL.

Statistical Analysis

The results are expressed as means ± standard errors of the means (SEM). Statistical analyses were carried out using Student's *t* test (MicroSoft Excel 97). All statistical tests were two tailed and unpaired.

RESULTS

We have previously shown that 148 bp upstream of the transcriptional start site of the rat MMP-13 promoter retains all PTH-responsiveness and differentiating elements in osteoblastic cells [Selvamurugan et al., 1998; Winchester et al., 2000]. Three transgenic mouse lines were generated which carried the *E. coli* β-galactosidase reporter (marker) gene attached to 148 bp sequence upstream of the MMP-13 gene (Fig. 1). Transgenic founder mice were identified by PCR and Southern blot analyses of DNA isolated from tail snips. The bone and soft tissues

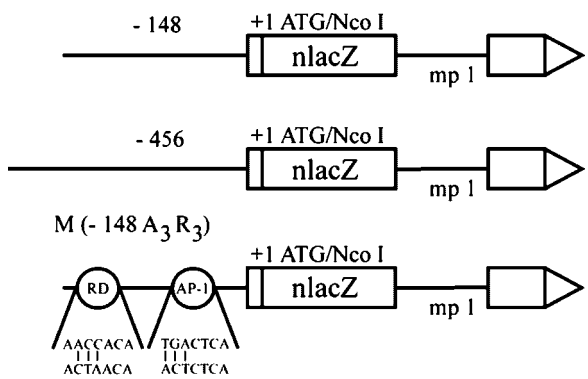

Fig. 1. Generation of transgenic mice overexpressing *E. coli lacZ* (β-*galactosidase*). The structure of the rat MMP-13 promoter fragments and nlacZ (nuclear lacZ) hybrid genes that were microinjected into mouse blastocysts.

from transgenic mice and non-transgenic siblings at various ages were analyzed for expression of β-galactosidase by immunohistochemical staining using anti-β-galactosidase antibody. Expression could be detected in all of the lines at 14–17 days postnatally in bone (Fig. 2) and teeth (data not shown). Some signal was also seen in skin (data not shown). No signal was seen in other tissues. Faint staining from expression of the transgene (−148 promoter/β-galactosidase) can be observed in developing cartilage in the 14.5-day embryo, which is more evident in the cartilage of the spine in 17.5-day embryos. There is also some expression of this transgene in the skin of postnatal animals (data not shown). Thus, it appears that expression of MMP-13 gene in bone, teeth, and skin is regulated by elements in the 148 bp upstream of the transcription start site.

To be sure that no other elements are involved in tissue-specific expression in vivo, transgenic mice harboring −456 bp upstream sequence of the rat MMP-13 gene attached to the *E. coli* β-galactosidase marker gene were also generated (Fig. 1). Two transgenic founders were identified by PCR of genomic DNA obtained from tail biopsies. Similar experiments were conducted as for the −148/β-galactosidase construct. We have found that the transgene is expressed in bone at 14–17 days postnatally. The level of expression of the reporter gene seems greater with the −456 bp promoter compared with the −148 bp promoter. This may be due to other elements such as Smad Binding Elements (SBEs) in the −456 to −148 region. Tissues such as heart, liver, and lung show no expression of either of these constructs (Fig. 2). We have also generated three lines of mice with the Runx/RD/Cbfa and AP-1 sites mutated (Fig. 1) similar to our in vitro experiments [Selvamurugan et al., 1998; Winchester et al., 2000; Inada et al., 2004]. The −148 construct with the RD and AP-1 site mutated ($-148A_3R_3$) showed expression but it is far less than the wild-type −148 or −456 constructs at 14 days postnatal in calvariae (Fig. 2). It appears that 148 bp upstream of the transcriptional start site of the rat MMP-13 promoter is sufficient to confer gene expression in bone, teeth, and skin.

Runx2 is a bone specific transcription factor and is able to induce expression of genes that are involved in bone formation and bone resorption [Ducy et al., 1997; Harada et al., 1999; Liu et al., 2001; Geoffroy et al., 2002]. We wanted to investigate whether overexpression of Runx2 by the MMP-13 promoter regulates expression of bone specific and bone related genes (MMP-13) in vivo, and whether this causes morphological changes in these animals. The pCMV-myc mammalian expression vector (Clontech) expressing the N-terminal c-myc epitope tag was used to clone both the rat MMP-13 promoter (−148 containing the AP-1 and RD sites) and Runx2 (type II). The entire fragment containing the rat MMP-13 promoter, c-myc epitope tag, and Runx2 was released by SphI and PvuII digestion (Fig. 3A) and used for injection into mice blastocysts for the transgenic work. To determine that the transgene is expressed and is regulated by PTH, the transgenic DNA construct (−148/c-myc/Runx2), the negative control constructs (pSV0/-148, pCMV/c-myc), and the positive control (pCMV/c-myc/Runx2) were transiently transfected into COS-7 cells for 48 h and then treated with or without 8-bromo-cAMP (8BrcAMP) for 24 h. The cells were then lysed and the c-myc-tagged Runx2 (Cbfa1) was identified by Western blot using the c-myc antibody (Fig. 3B). The result indicated that the transgene (c-myc-tagged Runx2) is expressed and its level was increased in response to 8BrcAMP treatment in COS-7 cells. Five lines of mice were generated that carry the rat MMP-13 promoter (−148) driving expression of the c-myc tagged Runx2 gene. None of the transgenic mice lines had significant visual phenotypic changes compared to the wild-type mice.

In order to determine tissue specific expression of c-myc tagged Runx2, total RNA was isolated from the bones and soft tissue of the 14 days and 6 weeks old wild-type and transgenic mice. Semi quantitative RT-PCR was carried out to determine the level of expression of the transgene (Fig. 4). In 14 days and 6 weeks old transgenic mice, MMP-13 promoter-directed expression of c-myc-Runx2 was seen in highly mineralized tissues such as tibiae, calvariae and teeth, whereas in soft tissue (liver) there appears to be no expression of c-myc-Runx2. There was also expression of the transgene in skin of the 6 weeks old mice. Expression of the transgene was not detected in either the bone or soft tissues of wild-type mice. Thus, these results indicate that c-myc-Runx2 is expressed only in transgenic mice and its expression under the control of the MMP-13 promoter seems to be restricted to bone, teeth, and skin.

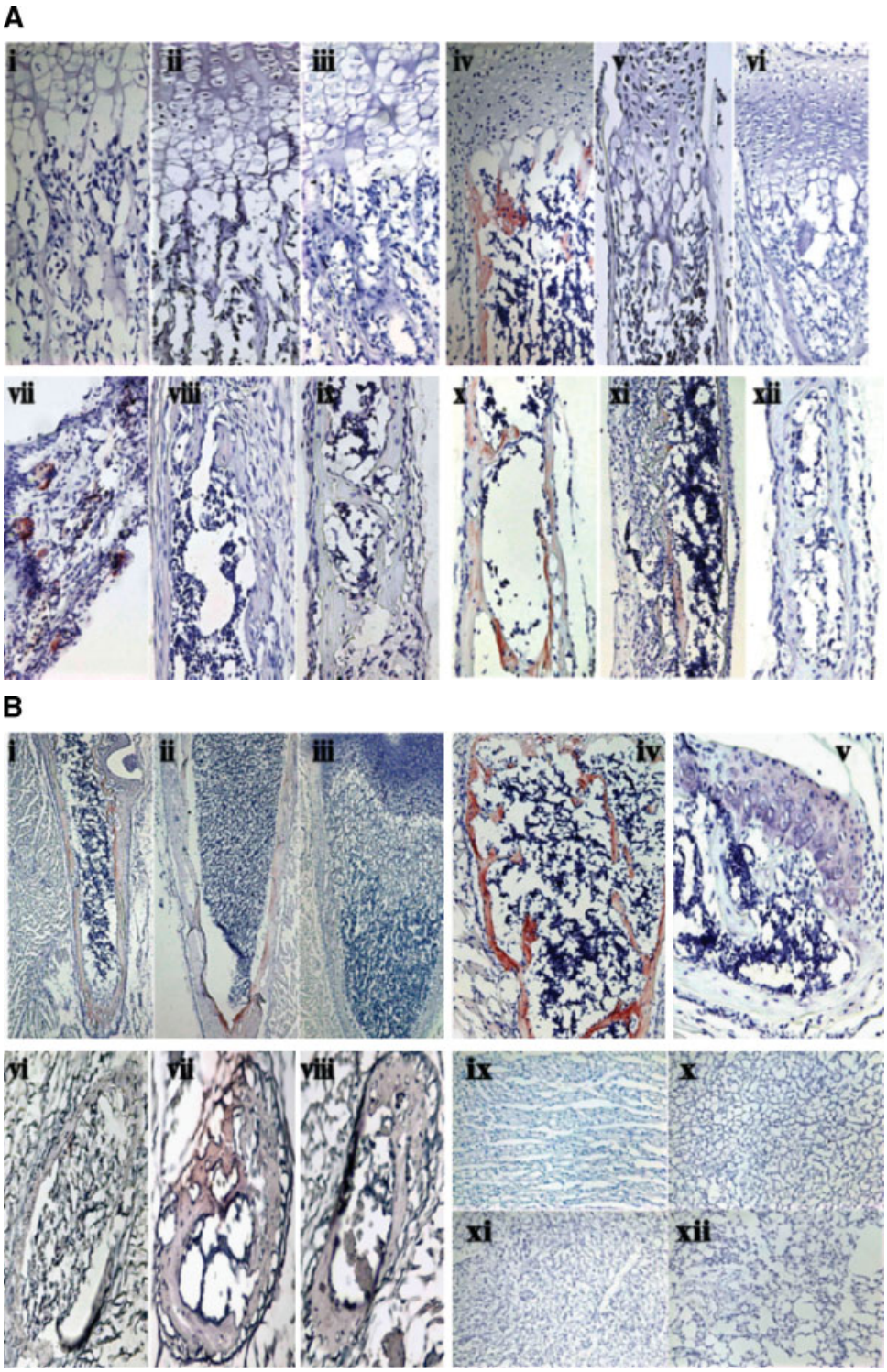

Fig. 2. Photomicrographs of −456*lacZ*, −148*lacZ*, −148(A_3R_3)*lacZ* transgenic mice, and wild-type mice. Bones and tissues from 14, 16, and 17 day postnatal mice, and 17.5 day prenatal mouse embryos were analyzed immunohistochemically for *E. coli* β-galactosidase. **A**: 14 day postnatal −148*lacZ* mouse femur (i), 14 day postnatal −148(A_3R_3)*lacZ* mouse femur (ii), and 14 day postnatal wild-type mouse femur (iii), 14 day postnatal −456*lacZ* mouse tail (iv), 14 day postnatal −148(A_3R_3)*lacZ* mouse tail (v), and 14 day postnatal wild-type mouse tail (vi), 16 day postnatal −148lacZ mouse calvaria (vii), 16 day postnatal −148(A_3R_3)*lacZ* mouse tail (viii), and 16 day postnatal wild-type mouse tail (ix), 17 day postnatal −456*lacZ* mouse calvaria (x), 17 day postnatal −148*lacZ* mouse calvaria (xi), and 17 day postnatal −148(A_3R_3)*lacZ* mouse calvaria (xii). **B**: 17 day postnatal −148*lacZ* mouse femur (i), 17 day postnatal −456*lacZ* mouse femur (ii), and 17 day postnatal −148(A_3R_3)*lacZ* mouse femur (iii), 17 day postnatal −456*lacZ* mouse rib (iv), and 17 day postnatal −148(A_3R_3)*lacZ* mouse rib (v), E17.5 day wild-type mouse vertebra (vi), E17.5 day −148*lacZ* mouse vertebra (vii), and E17.5 day −148(A_3R_3)*lacZ* mouse vertebra (viii), 14 day postnatal −148*lacZ* mouse heart (ix), kidney (x), liver (xi), and lung (xii).

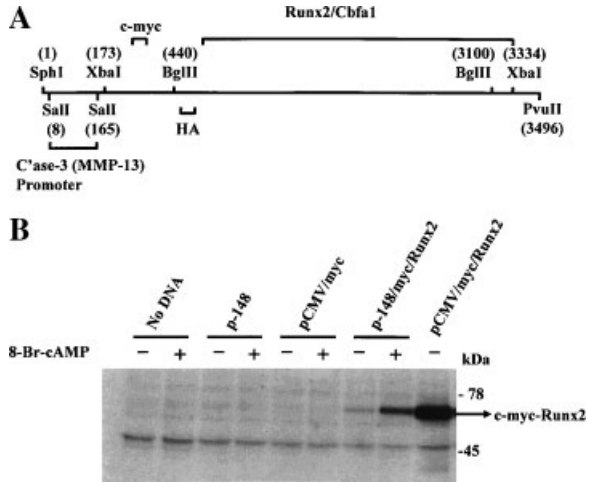

Fig. 3. Generation of transgenic mice overexpressing Runx2. **A**: Structure and restriction map analysis of the −148/c-myc/Runx2. The pCMV-myc mammalian expression vector expressing the N-terminal c-myc epitope tag was used to clone the rat MMP-13 promoter (−148 containing the AP-1 and RD sites) and Runx2 (type II). The entire fragment containing the rat MMP-13 promoter, c-myc epitope tag, and Runx2 was released by a Sph1 and PvuII digestion and used for injection into mouse blastocysts. **B**: Expression of the transgene in vitro. The transgenic DNA construct (p-148/c-myc/Runx2) as well as control constructs (p-148 and pCMV/c-myc) were transiently transfected into COS-7 cells using Lipofectamine reagent for 48 h and then treated with or without 8BrcAMP (10^{-3} M) for 24 h. The cells were then lysed and the c-myc-tagged Runx2 was identified by Western blot using the monoclonal antibody to the c-myc epitope tag.

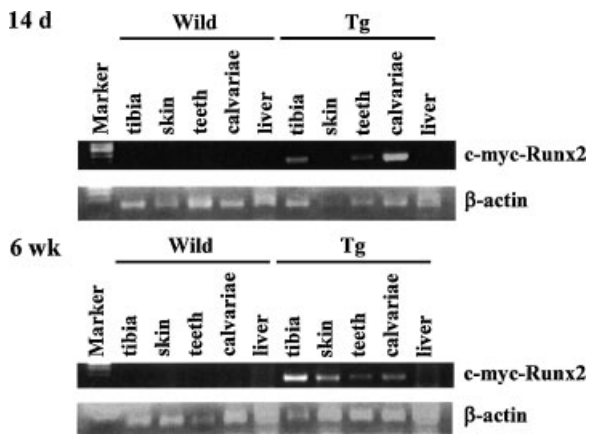

Fig. 4. Semiquantitative RT-PCR analysis of the temporal expression of the transgene in transgenic mice. Total RNA was extracted from tibiae, skin, teeth, calvariae, and liver of wild-type and transgenic mice at the indicated ages. One step RT-PCR was carried out using an RT-PCR kit (Invitrogen) with the forward (c-myc) and the reverse (Runx2) primers. To normalize the amounts of RNA used in the experiment, β-actin was included as a control. The products were identified on a 2% agarose gel.

The wild-type and transgenic mice overexpressing Runx2 transcription factor under the control of the MMP-13 promoter were examined for changes in their bone phenotype. The proximal tibiae of wild-type and transgenic mice (14 days and 6 weeks) were analyzed by microcomputed tomography (microCT) measurements. There were no statistically significant differences between the wild-type and transgenic animals. However, the μCT analysis of the proximal tibiae of the 6 weeks old animals suggested a tendency for the transgenic animals to have slightly greater trabecular bone than the wild-type (Table IA,B). Histological sections of the midtibial metaphysis from wild-type and transgenic mice (6 weeks old) showed that there is increased size and number of trabeculae in transgenic mice, compared to wild-type mice (Fig. 5).

To further understand if the slightly increased bone in 6 weeks old transgenic mice is due to increased bone formation or reduced bone resorption, kinetic analyses of bone formation were performed. Calcein was injected twice at 12 and 2 days before sacrifice in 6 weeks old mice. Bone histomorphometric studies were carried out with proximal tibial metaphyses, a standard site for this study. As shown by histomorphometric data (Fig. 6), the percentage of mineralizing surface, the amount of secreted and mineralized matrix per osteoblast (i.e., the mineral apposition rate), and the rate of bone formation were significantly increased in transgenic mice. Moreover, there was no change in the number of osteoblasts but the number of osteoclasts was significantly decreased in the transgenic mice.

In order to evaluate the molecular events that underlie the slightly modified phenotype in transgenic mice, we analyzed the mRNA expression patterns of genes that are involved in bone formation (ALP, OC, OPN) and bone resorption (MMP-13, OPG, RANKL). Total RNA was obtained from tibiae, calvariae, teeth, skin, and liver and was subjected to real time (quantitative) RT-PCR. In the immature skeleton (14 days old animals), mRNA expression of bone formation genes such as OC and OPN were significantly increased in the tibiae of transgenic mice (Fig. 7A) and had returned to normal in 6 weeks old transgenic mice (Fig. 7B). In calvariae, there was increased mRNA expression of OC in both 14 days and 6 weeks old transgenic mice (Figs. 7A,B) and there was

TABLE IA. Structural Parameters of Trabecular Bone in the Proximal Tibia of 6 Week Old Mice Measured by Micro-Computed Tomography

Parameter	Wild-type	Transgenic
Percent bone volume, BV/TV (%)	22.33 ± 0.036	23.14 ± 0.016
Trabecular number, Tb.N (1/mm)	6.22 ± 0.361	6.48 ± 0.374
Trabecular thickness, Tb.Th (μm)	48.9 ± 0.003	48.6 ± 0.001
Trabecular separation, Tb.Sp (mm)	155.18 ± 0.005	148.74 ± 0.011
Connectivity density, Conn.D (1/mm)	221.99 ± 29.98	266.88 ± 25.97

Data was tabulated as the mean ± SEM. The number of animals/group was five.

TABLE IB. Structural Parameters of Cortical Bone in the Mid-Shaft Femur of 6 Week Old Mice Measured by Micro-Computed Tomography

Parameter	Wild-type	Transgenic
Percent bone volume, BV/TV (%)	52.77 ± 0.87	53.10 ± 0.44
Periosteal perimeter, Ps.Pm (mm)	8.41 ± 0.19	8.37 ± 0.10
Endocortical perimeter, Ec.Pm (mm)	5.33 ± 0.23	5.29 ± 0.33
Cortical thickness, Ct.Th (mm)	0.194 ± 0.006	0.191 ± 0.001

Data was tabulated as the mean ± SEM. The number of animals/group was five.

increased expression of OPG and RANKL mRNAs in 6 weeks old transgenic mice (Fig. 7B). In teeth, OPG and RANKL mRNAs were increased in 14 days old transgenic mice (Fig. 7A); whereas expression of OC and MMP-13 mRNAs were increased in this tissue in 6 weeks old transgenic mice (Fig. 7B). In skin, mRNA expression of the bone forming genes and bone resorbing genes was not significantly altered in either 14 days or 6 weeks old transgenic mice (Figs. 7A,B). Interestingly, MMP-13 mRNA expression was significantly reduced in the tibiae and calvariae of both 14 days and 6 weeks old transgenic mice (Figs. 7A,B).

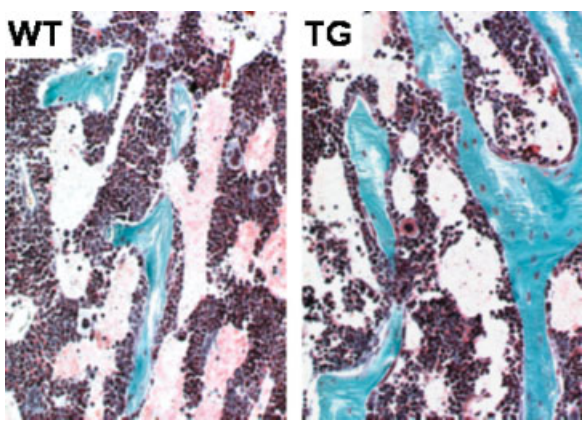

Fig. 5. Histological appearance of Runx2 transgenic bone. Longitudinal sections through the proximal tibiae of wild-type (WT) and transgenic (TG) mice at 6 weeks of age. The trabecular structure of both WT and TG tibiae has been magnified.

When we examined the endogenous Runx2 mRNA levels, there was no significant change between the wild-type and the transgenic mice.

DISCUSSION

Our previous work has determined the elements and proteins regulating the MMP-13 promoter in PTH-treated and differentiating osteoblastic cells in vitro [Selvamurugan et al., 1998; Winchester et al., 2000]. In both cases, the AP-1 and Runx/RD/Cbfa sites are involved. However, this does not mean that these elements are the functional elements directing osteoblast-preferential expression of this gene in vivo. While transient and stable transfection studies can provide insight into the biochemical and molecular interaction, it is only within the intact tissue that the true biological importance of the promoter and transcriptional environment can be appreciated. This is the rationale for the extensive and expensive effort required to carry out a promoter analysis in intact mice. Transgenic mice provide an excellent setting for studying complex regulatory systems that cannot be modeled in isolated experimental systems in vitro or in cellulo.

In this study, we show that wild-type transgenic lines (−456 and −148) express β-galactosidase expression in bone, teeth, and skin and none in heart, liver, or lung, compared to the mutant and non-transgenic lines (Fig. 2). Since

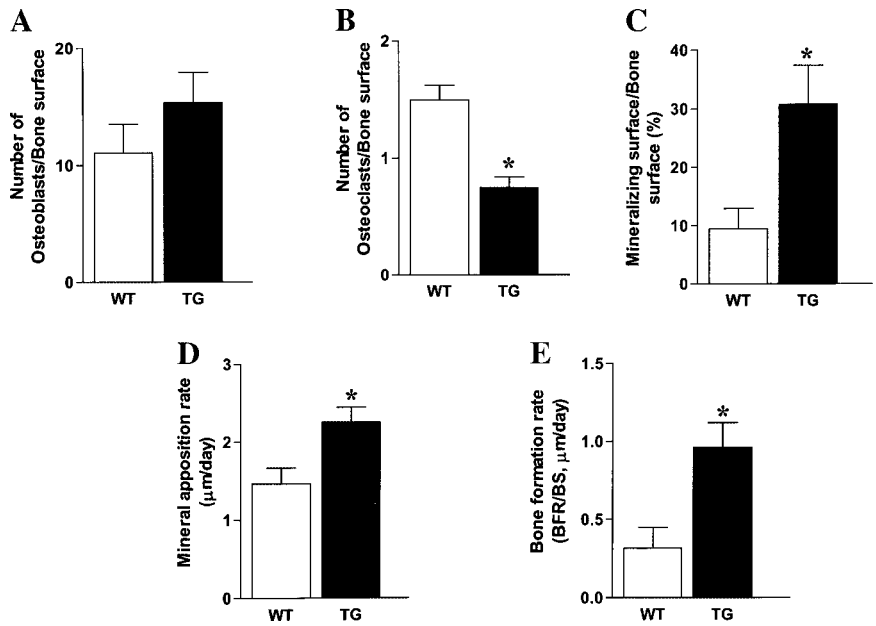

Fig. 6. Histomorphometric bone formation and resorption parameters in Runx2-overexpressing mice. Wild-type and transgenic female mice were labeled with calcein and sacrificed at 6 weeks of age. **A**: Number of osteoblasts and (**B**) number of osteoclasts/bone surface are compared between wild-type (white bars) and transgenic mice (black bars) at 6 weeks of age. The analyses were done using proximal parts of tibiae. **C**: mineralizing surface, (**D**) mineral apposition rate, and, (**E**) bone forming rate in trabecular bone of wild-type (white bars) and transgenic (black bars) mice. Bars show means ± SEM (n = 4). *Significant difference compared to the wild-type mice; $P < 0.05$.

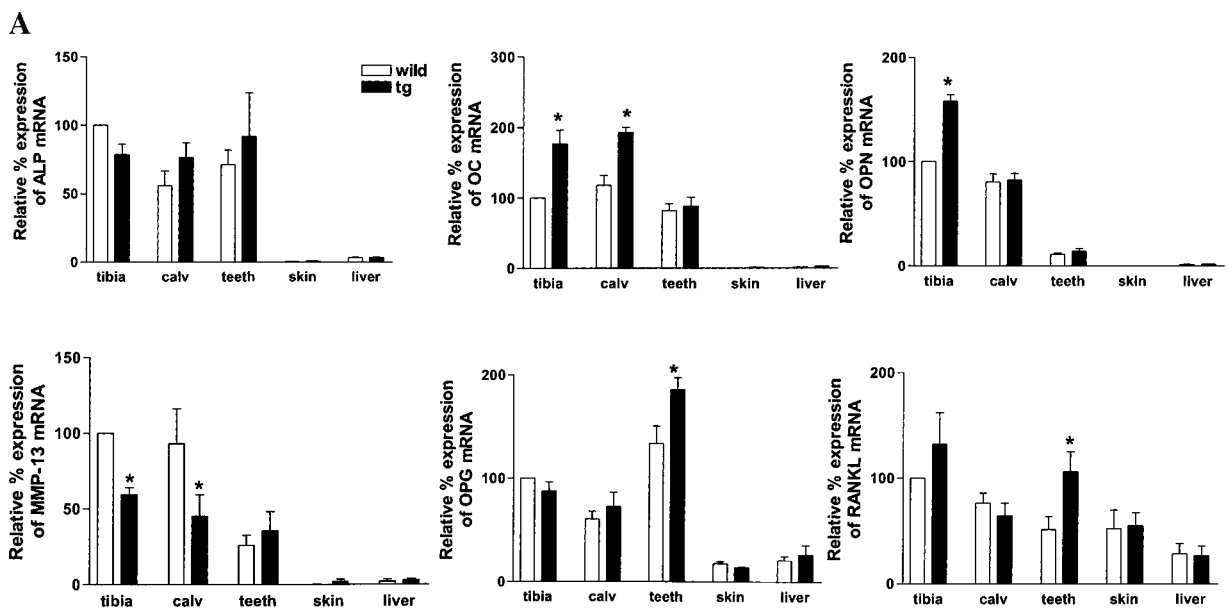

Fig. 7. Quantitative analysis of expression of genes involved in bone formation and bone resorption. Total RNA was isolated from tibiae, calvariae, teeth, skin, and liver of 14 d (**A**), and 6 weeks (**B**) old wild-type and Runx2 transgenic mice and subjected to real time quantitative RT-PCR using specific primers as outlined in the figure and methods section. The mRNAs were normalized to β-actin. The mRNA level for each gene in the tibiae of wild-type mice has been converted to 100% and from this the relative mRNA expression was compared in other tissues of both wild and transgenic mice. The data are represented as mean ± SEM (n = 3). The experiment was carried out at least three times. *Significant change compared to the tissues of the wild-type mice; $P < 0.05$.

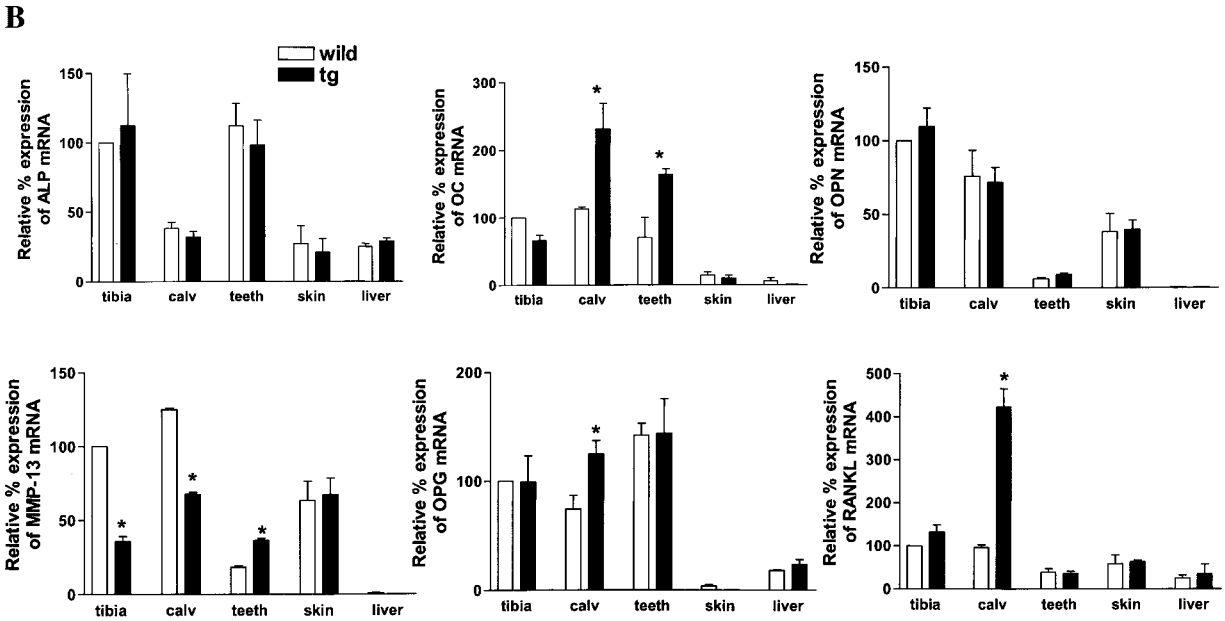

Fig. 7. (*Continued*)

both −456 and −148 reporter constructs exhibited a similar regulatory expression in bone, teeth, and skin, we suggest that the regulatory elements (AP-1 and Runx/RD/Cbfa) within the 148 bp upstream of the MMP-13 promoter are enough to confer this effect. In addition, it is evident that transgenic mice containing mutated AP-1 and Runx/RD/Cbfa sites ($-148A_3R_3$) in the MMP-13 promoter expressed very low β-galactosidase expression, compared to the non-transgenic lines (Fig. 2). Hence, it is reasonable to assume that activation of the MMP-13 promoter requires only the AP-1 and Runx/RD/Cbfa sites in both in vitro and in vivo conditions.

Runx2 is a major regulator of bone development [Karsenty, 2000; Komori, 2000]. Mouse models have enhanced our understanding of the basic functions of Runx2. Mice heterozygously mutated in the *Runx2* locus show a phenotype similar to that of cleidocranial dysplasia (CCD) in humans, in whom mutations of *Runx2* have been found [Komori et al., 1997; Mundlos et al., 1997; Otto et al., 1997]. A homozygous mutation of this gene in mice induced a complete lack of bone formation with arrest of osteoblast differentiation [Komori et al., 1997; Otto et al., 1997]. The dominant negative form of Runx2 developed an osteopenic phenotype in mice and was used to indicate the indispensability of the gene for postnatal bone formation by regulating the functions of mature osteoblasts [Ducy et al., 1999]. Through deletion of the C-terminal intranuclear targeting signal by homologous recombination, it has been shown that subnuclear targeting and the associated regulatory functions are essential for control of Runx-dependent genes [Choi et al., 2001]. A large number of in vitro studies have also implied that Runx2 is a positive regulator that can stimulate the expression of bone matrix genes, including *type I collagen*, *osteopontin*, *bone sialoprotein*, *osteocalcin*, and *fibronectin* [Banerjee et al., 1997; Ducy et al., 1997; Sato et al., 1998; Harada et al., 1999; Xiao et al., 1999; Lee et al., 2000; Kern et al., 2001; Prince et al., 2001].

A fundamental tool that is used in the transgenic experimental approach is a promoter that has tissue-restricted activity. Within the lineage of bone and cartilage cells, the type I and type II collagen promoters can be designed to have preferential expression at specific stages of differentiation. OC and BSP expression is specific to bones and thrombocytes and the OC promoter has been widely used in the transgenic mouse model system [Kalajzic et al., 2002]. When the MMP-13 promoter was used to overexpress the β-galactosidase reporter gene, we found its expression not only in bone but also in teeth, and skin (Fig. 2).

Liu et al. [2001] reported that transgenic mice expressing Runx2 directed by the pro-α-type I collagen promoter had osteopenia and fragility of bone that were caused by the inhibition of osteoblast maturation, and immature osteoblasts

accumulated in the bone of adult mice [Liu et al., 2001]. Their transgenic mice showed decreases in bone formation rate, matrix apposition rate, and mineralized surface area in trabecular bone as well as in cortical bone compared to those of the wild-type mice. Geoffroy et al. [2002] have reported that Runx2 controls not only genes that are important for osteoblast differentiation [Ducy et al., 1997] and function [Ducy et al., 1999] but also genes that are involved in osteoclast differentiation and bone formation-resorption coupling [Geoffroy et al., 2002]. Even though MMP-13 has been shown to be expressed in skin, uterus, and ovary, it is mostly expressed in bone [Balbin et al., 1996; Davis et al., 1998; Tuckermann et al., 2000; Shum et al., 2002]. We report here that overexpression of Runx2 directed by the MMP-13 promoter increases the bone mineralization surface, bone formation rate, and matrix apposition rate (Fig. 6). Since there was no change in the number of osteoblasts, this effect could be due to uncoupling and unbalancing of bone formation and bone resorption processes. Even though OPG and RANKL mRNA expression were not altered in the tibiae of transgenic mice at the ages examined, there was significantly reduced MMP-13 mRNA expression during development of transgenic mice (Fig. 7A,B) and it has been shown that MMP-13 is necessary for osteoclast-mediated bone resorption [Zhao et al., 2000]. The downregulation of MMP-13 at the ages examined by overexpression of Runx2 in transgenic mice could be due to negative feed back regulation of Runx2. This also could be due to the fact that MMP-13 is expressed at greater levels in long bones, in the fetus, and maximally at 14 days in the calvariae, while OC is mostly expressed postnatally [Davis et al., 1998; Tuckermann et al., 2000]. It is possible that the maturation of the bones is advanced and the usual peak in MMP-13 expression is at an earlier age. Perhaps the reduced expression level of MMP-13 in the transgenic mice could have led to decreased recruitment of osteoclasts (Fig. 6) to the bone surface, resulting in reduced bone-resorptive activity, reflected by increased bone formation in transgenic mice.

Overall, we provide evidence that the 148 base pairs of MMP-13 promoter is sufficient and necessary for tissue-restricted (bone, teeth, and skin) gene expression in vivo, and the AP-1 and Runx/RD/Cbfa sites are likely to regulate this. Using these regulatory elements, we further document that overexpression of Runx2 appears to alter the balance between the bone formation-bone resorption processes in vivo and does regulate the expression of MMP-13 and other bone marker genes.

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