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Identification of USF2 as a key regulator of Runx2 expression in mouse pluripotent mesenchymal D1 cells

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Abstract

Runx2 is one of the most important transcription factors directing the osteogenesis of mesenchymal stem cells and osteoblastic functions. It is likely that the factors controlling Runx2 expression would trigger the early steps of osteoblast differentiation. By using a reporter gene assay for 4.5 kb Runx2 promoter, it was found that the first 305 bp of Runx2 promoter are active in D1 cells. Within this region, electromobility shift assays (EMSAs) delineated a 6 bp of CACATG bound specifically by the proteins from D1 cell nuclear extract. Antibody super-shift and DNA-coupling magnetic bead pull-down assay indicated that the protein bound to this sequence is USF2. Site-specific mutagenesis revealed that this sequence contributed to the activity of 305 bp Runx2 promoter. Thus, we suggest that USF2 might be one of the regulators for the expression of the Runx2 gene in D1 cells. (Mol Cell Biochem **292:** 79–88, 2006)

Key words: Runx2, USF1, USF2, E-box, D1 cells, osteogenesis, mesenchymal stem cells, osteoblast, promoter, transcription

Introduction

The bone-related transcription factor, Runx2, is one of three mammalian members of the runt homology domain family of transcription factors [1]. Functionally, this transcription factor is essential for bone development and osteoblast differentiation. The deficiency of the Runx2 gene in a mouse model results in osteoblast differentiation arrest at the earliest stages of bone formation [2] and disturbance of chondrocyte maturation [3]. Heterozygous mice with Runx2 null allele display a delay in ossification, similar to the phenotype of cleidocranial dysplasia (CCD) [4–7]. In contrast to the Runx2 deficient model, overexpression of Runx2 genes induces osteoblastic differentiation for mesenchymal non-osteoblastic cells [8]. Moreover, expressions of a broad spectrum of bone and cartilage-related genes regulated by Runx2 have been reported [9–12]. With these characteristics, the factors controlling Runx2 expression would induce osteoblast lineage commitment and differentiation at early stages.

Two isoforms of mRNA transcripts and proteins of Runx2, designated TypeII/Runx2/MASNSL/p57 and TypeI/Runx2/MRIPVD/p56, are present in cell lines [13–15]. These transcripts are expressed from two separate promoters, P1 for Type II and P2 for Type I [15]. The upstream P1 promoter drives expression of the Type II isoform, which utilizes the most 5′ exon of the Runx2 gene and is the most dominant pattern in mouse embryos [16]. The P1 promoter of Runx2, among human, mouse and rat, is composed of two highly conserved regulatory domains, proximal (nt −113 to −1) and distal (nt −458 to −304), linked by a purine-rich

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sequence [15]. Within this region, both a FosB binding site (nt −415 to −375) and a non-consensus Runx2 binding site (5′TCCCAC3′) are responsible for enhancement of promoter activity [17, 18]. However, a region (nt −104 to −78) contains a Runx2 binding site and a vitamin D responsive element, conferring negative regulation of Runx2 promoter activity [15, 19].

To gain additional insight into the regulatory cues that mediate osteogenesis, we characterized the 4.5 kb flanking region of Runx2 gene in pluripotent mesenchymal D1 cells. This cell is originally cloned from a multipotent mouse bone marrow stromal precursor, possessing bone-producing characteristics *in vitro* including high levels of alkaline phosphatase, exhibiting a mineralized matrix and expression of osteocalcin mRNA [20]. *In vivo*, this cell repopulates bone marrow and retains osteogenic properties [21]. Significantly, systemically administrated D1 cells transduced with insulin-like growth factor-1 can localize to a fracture site and potentiate bone healing [22]. With all these properties, it might be ideal model for studying the regulation of Runx2 expression. By using D1 cells, we identified a cis-element, CACATG, which contributes to Runx2 promoter activity. The transcription factor that interacts with this sequence is USF2.

Materials and methods

Plasmid constructs

The pSEAP2/4.5Runx2P reporter plasmid containing a 4.5 kb Runx2 5′-flanking region was constructed as follows. The 4.5 kb Runx2 5′-flanking region was amplified by PCR using sequence-specific primers, 5′-TGC CCC ACA CTT ACC CCT TA-3′, and 5′-ATG AAG CGT TCA CAC AAT CC-3′ from commercial mouse genomic DNA (Clontech, Palo Alto, CA), followed by cloning into pGEM-easyT vector (Promega, Madison, WI). The 4.5 kb fragment was then excised and subcloned into a pSEAP2/basic (Clontech, Palo Alto, CA). For two deletion mutants, pSEAP2/3.5Runx2P and pSEAP2/0.305Runx2P, PCR was applied to generate two different lengths of the 5′-flanking region of Runx2 gene, 3.5 kb, and 0.32 kb, by using pSEAP2/4.5Runx2P as the template with respective primers. Then PCR products were cloned into pGEM-easyT. After amplification, the different fragments of the 5′-flanking region of Runx2 gene were excised and subcloned into a pSEAP2/basic. A DNA fragment bearing the β-globin basic promoter was placed into the *EcoR I* site of pSEAP2/basic to generate pSEAP2-β. The chemical synthesized oligonucleotides with 4 copies of OSE2 (5′-GCT CCC AAC CACA TAT CCT-3′) [10] was cloned into pSEAP2-β between *EcoR I* and *Bgl II* sites to generate pSEAP2-β-4XOSE2.

Cell transfection

D1 cells (ATCC) were plated at 8×10^4 cells/well in 24-well plates for 1 day before transfection. Transfections were performed using FuGENE 6 (Roche Applied Science) according to the manufacturer's instructions. Briefly, 0.4 μg of the tested construct plasmids and 0.1 μg of the internal control pCAT3-Control vector (Promega) were mixed, and then added to a tube containing 1.5 μl of FuGENE 6 in 25 μl of DMEM medium with 10% fetal bovine serum (FBS) (Life Technologies, Inc.). DNA-FuGENE 6 complexes were allowed to form for 15–30 min at room temperature prior to their addition to each well containing 0.5 ml of DMEM medium with 10% FBS. Cells were collected for secreted alkaline phosphatase (SEAP) assay after 72 h additional incubation.

SEAP assay

Twenty-five microliters of supernatant were incubated at 65 °C for 30 min and cooled down at 4 °C for 5 min. This supernatant was mixed with an equal volume of reaction buffer and chemiluminescent substrate (Tropix) for 20 min and measured by a TopCount (Packard Instrument Company) in the chemiluminescent mode. Each chemiluminescent reading was normalized by chloramphenicol acetyltransferase assay, performed using the CAT-ELISA kit according to the manual of the supplier (Roche Applied Science).

Cell culture and preparation of nuclear extracts

D1 cells were grown in DMEM plus 0.1% ascorbic acid medium supplemented with 10% FBS. Nuclear extracts were prepared by high salt extraction according to standard procedures [23]. Briefly, cells grown at approximately 90% confluence were collected, washed with phosphate-buffered saline solution, and homogenized in a hypotonic buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and protease inhibitor cocktail (Roche Applied Science)). After centrifugation at 2000 g, the nuclear pellets were incubated for 30 min on ice with continuous stirring in a high salt buffer (20 mM HEPES, pH 7.9, 25% glycerol, 1.5 mM $MgCl_2$, 0.6 M KCl, 0.2 mM EDTA, 0.5 mM DTT and protease inhibitor cocktail). The extracts were centrifuged, and the supernatants were dialyzed against dialysis buffer (20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT and protease inhibitor cocktail). The protein concentration of nuclear extracts was quantified by Bradford solution (Bio-Rad).

Electrophoretic mobility shift assay (EMSA)

EMSA was performed as described previously [23]. Briefly, 8 μg of nuclear extracts were incubated for 20 min. at room temperature with 0.014 pmole ^{32}P end-labeled oligonucleotide-probes with or without unlabeled DNA competitors and 2 μg of poly (dI/dC) in binding buffer (20 mM HEPES, pH 7.9, 1 mM EDTA, 100 mM KCl, 1 mM dithiothreitol and 10% glycerol). For super-shift assay, the respective antibody, anti-C-myc (UPSTATE), -USF1 (Santa Cruz Biotechnology) and -USF2 (Santa Cruz Biotechnology) were included into the above reaction. The samples were electrophoresed on a pre-run 6% native acrylamide-bisacrylamide (28:2) gel in 1 X Tris-borate-EDTA at 4 °C. The gel was dried and autoradiographed.

Preparation of DNA-coupled magnetic beads

Biotin-5′- TCA CAA ACC ACA TGA TTC TG -3′ (RE1) or biotin-5′-GCT CCC AAC CACA TAT CCT-3′ (OSE2) was used as the oligonucleotide. The double-stranded oligonucleotide was created by annealing this oligomer with its antisense oligomer. Twenty microliters of the double-stranded oligomer (10 μM) were incubated with 0.6 mg of Strepavidin MagneSphere Paramagnetic particles (Promega) in 10 mM Tris-HCl buffer (pH 7.9) containing 1M NaCl for 20 min to prepare DNA-coupled magnetic beads. Then DNA-coupled magnetic beads were washed by 500 μl of the same buffer three times. Coupling efficiency was evaluated by comparing the DNA content before and after incubation using 1.5% agarose gel and EtBr staining.

DNA binding assay

Before use, DNA-coupled magnetic beads were washed three times with 500 μl of buffer (20 mM Hepes, pH7.9, 1 mM EDTA, 100 mM KCl, 1 mM dithiothreitol and 10% glycerol). After collecting the beads with a MagneSphere Magnetic Separation Stand (Promega), supernatant was carefully removed. Binding reactions were carried out with 3 mg of D1 nuclear extract, and incubated at room temperature for 20 minutes. Then beads were collected with a magnet stand, unbound protein in supernatant was removed and subjected to further analysis, and beads were washed three times in 500 μl of buffer (20 mM Hepes, pH7.9, 1 mM EDTA, 100 mM KCl, 1 mM dithiothreitol, 10% glycerol, and protease inhibitor cocktail). Afterward, beads were collected with a magnet stand, and resuspended in 50 mM Tris-Cl (pH 6.8), 100 mM dithiothreitol, 2% SDS and 10% glycerol to extract the proteins that bound to DNA.

SDS-polyacrylamide gel analysis and western blotting

The bound fraction from the above assay was heated at 100 °C for 3 min in 60 mM Tris-Cl (pH 6.8), 1% 2-mercaptoethanol, 2% SDS, and 10% glycerol, and then separated on 10% SDS-polyacrylamide gels (SDS-PAGE) followed by Western blotting. In the Western blotting assay, the proteins on the SDS-PAGE gel were transferred onto nitrocellulose membrane (Millipore). Then the membrane was treated by the respective antibody, followed by horseradish peroxidase-conjugated secondary antibody (PIERCE). The proteins that interacted with anti-Runx2 (ONCOGENE), -C-myc (UPSTATE), -Max (Santa Cruz Biotechnology), -USF1 (Santa Cruz Biotechnology) or -USF2 (Santa Cruz Biotechnology) antibody, were detected by using SuperSignal West Pico Chemiluminescent Substrate (PIERCE), and exposed to X-ray film.

Results

Transcription activity of Runx2 in D1 cells

D1 cell, a pluripotential mesenchymal cell, can differentiate along the osteoblastic lineage. Runx2 is an essential transcription factor involved in the differentiation and function of osteoblasts. Thus, we examined the transcription activity of Runx2 in D1 cells. The transcription activity was assessed by the transfection of Runx2-mediated and control reporter system, pSEAP2-β-4XOSE2 and pSEAP2-β into D1, respectively. Runx2-mediated transcription activity had been observed in D1 cells (Fig. 1A). Furthermore, we demonstrated that D1 cells contained Runx2 protein with immuno-blotting assay (Fig. 1B). Thus, we concluded that the D1 cell was a suitable model for studying regulation mechanism of Runx2 expression.

Localize regulatory motif of Runx2 promoter

To explore the regulatory pathways of Runx2 expression, we cloned 4.5 kb of the 5′ flanking region of Runx2 gene into secreted alkaline phosphatase (SEAP) gene to create a reporter system, pSEAP2/4.5Runx2P. To localize the regulatory motif of this Runx2 promoter, we also generated two deletion mutations of pSEAP2/4.5Runx2P, pSEAP2/3.5Runx2P and pSEAP2/0.305Runx2P (Fig. 2A). By transient transfection assay in D1 cells, we identified the major regulatory motif localized within 0.305 kb of Runx2 promoter (Fig. 2B). This region is highly conserved among humans, rats and mice [15]. The result indicated that this 305 bp region was responsible for expression of the Runx2 gene.

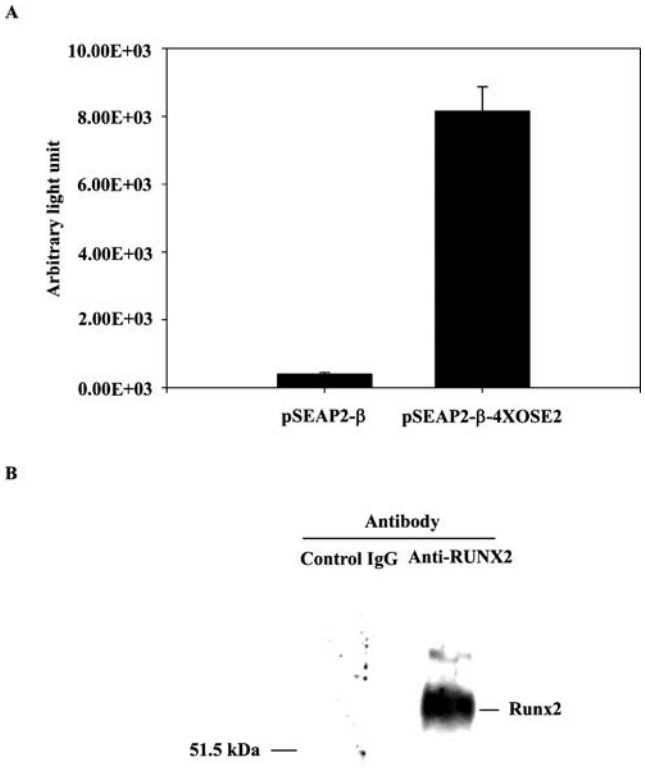

Fig. 1. A, Runx2-mediated transcription activity in D1 cells. D1 cells were transfected with pSEAP2-β-4XOSE2, or pSEAP2-β as negative control. The SEAP activities of both constructs were determined, normalized by the amount of CAT enzyme with ELISA, and presented as a bar graph. B, OSE2-coupled magnetic beads specifically pulled down Runx2 transcription factor. 600 μg of NE isolated from D1 cells were subjected to OSE2-coupled magnetic beads. The proteins bound to OSE2-coupled magnetic beads were extracted and applied to SDS-PAGE, followed by Western blotting analysis. Antibodies against Runx2 and control IgG were marked.

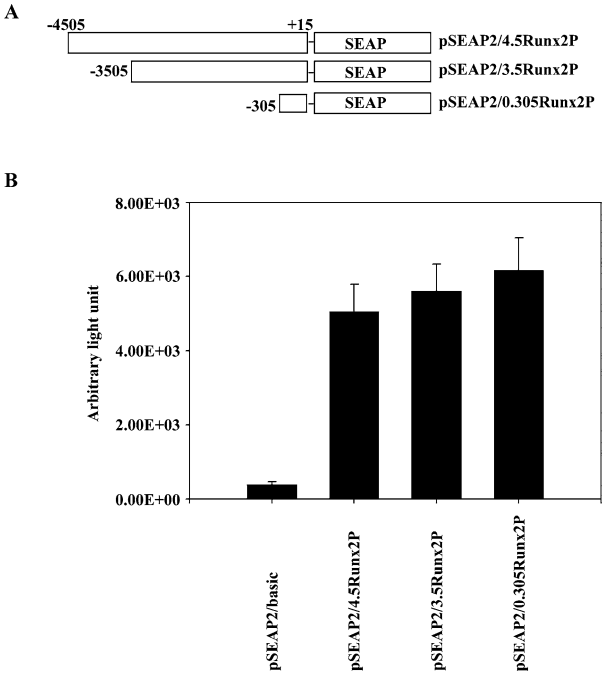

Fig. 2. A, different deletion constructions of Runx2 promoter-fused SEAP reporter gene. B, reporter gene activity of different lengths of Runx2 promoter plasmids. D1 cells were transfected with the Runx2 promoter as indicated in the figure. The SEAP activities of various constructs were determined, normalized by the amount of CAT enzyme with ELISA, and presented as a bar graph.

Identification of DNA-protein interaction domain of 305 bp Runx2 promoter

To locate the interaction region of the 305 bp Runx2 promoter with proteins, 15 oligonucleotides were synthesized with 1 representing 1–40, 2 representing 20–60, 3 representing 40–80, and so on (Fig. 3A). Among 15 oligonucleotides, 8 oligonucleotides cover the whole 320 bp and 7 oligonucleotides cover the junction area. In EMSA, only 3 oligonucleotides exhibited specific interaction using D1 cell nuclear extracts, and oligo #12 demonstrated the most significant and highest binding activity among the 3 oligonucleotides (Fig. 3B). The sequence of this region is identical between rat and mouse, and only one nucleotide is different from humans (Fig. 4A). We further characterized this oligonucleotide by EMSA. The results indicated that oligo #12 could be specifically competed by its own sequences, but not by other DNA sequences (Fig. 4B). This suggests that this oligonucleotide interacts with certain DNA binding proteins in D1 cells (Fig. 4B). Unfortunately, oligo #11 failed to label and did not show any protein binding. We did not explore this oligonucleotide further.

The specific DNA motif essential for protein binding

From the last experiment we found that oligo #12 can specifically interact with certain proteins in D1 cells. Oligo #12 is located at junction between oligo #11 and #13 (Fig. 3A). The first 20 nucleotides of oligo #12 overlap with the last 20 nucleotides of oligo #11, and the last 20 nucleotides of oligo #12 overlap with the first 20 nucleotide of oligo #13 (Fig. 3A). Logically, we could predict the protein binding region of oligo #12 just based on the binding pattern of oligo #13. Oligo #13 did not demonstrate any specific binding pattern, indicated that the last 20 nucleotides of oligo #12 do not contain the region for this protein to bind (Fig. 3B). Thus, we speculated that the interaction region of oligo #12 might be located in the first 20 nucleotides, TCACAAACCACATGATTCTG, denoted as RE1. From this sequence, we further synthesized 5 20-mer oligonucleotides, including RE1, m1, m2, m3, and m4, to perform EMSA for identification of the putative cis-element of the Runx2 promoter (Fig. 5A). In cold

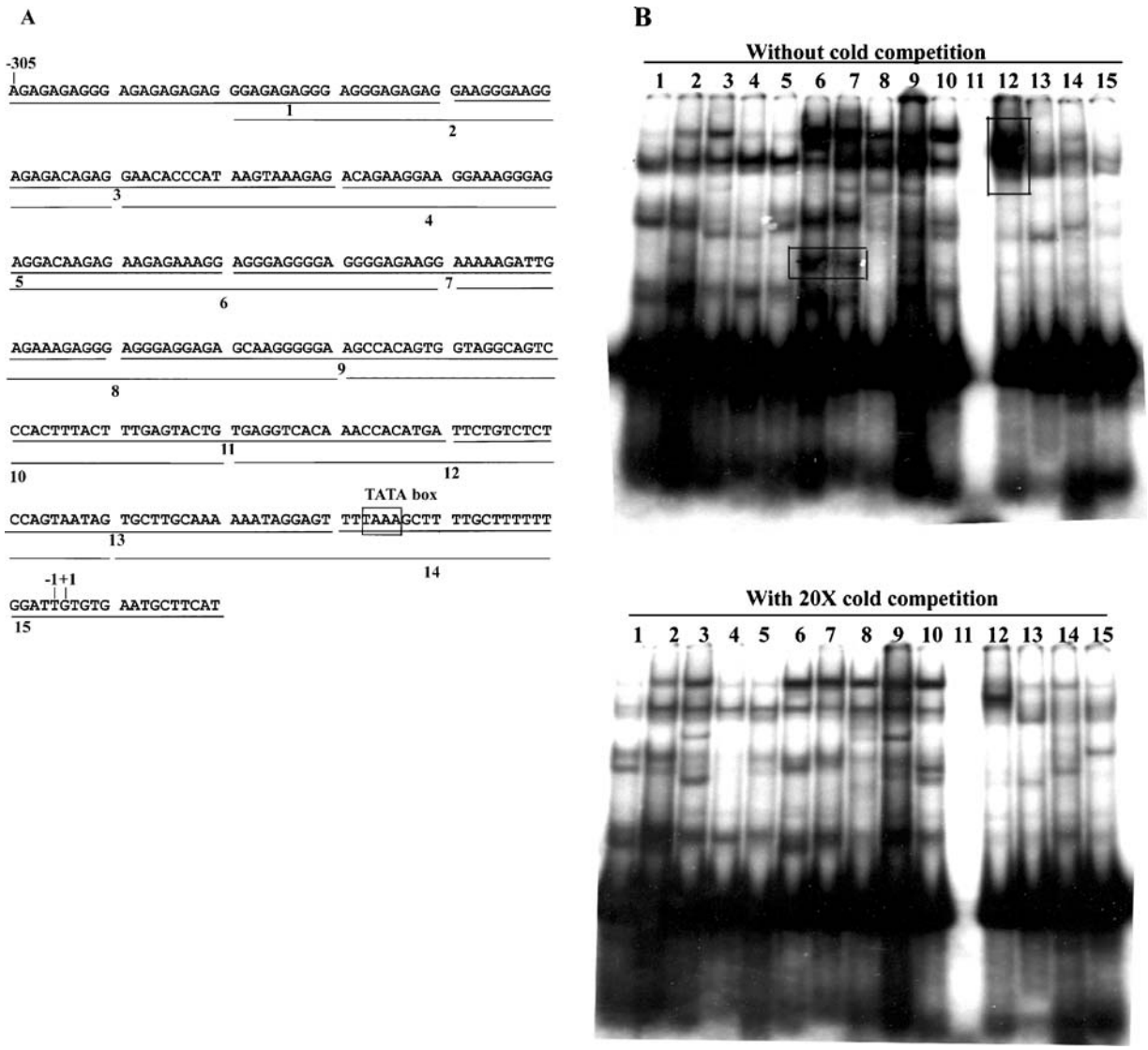

Fig. 3. A, the sequence of 305 bp of Runx2 promoter. The probes used in EMSA are underlined and numbered. B, EMSA of DNA-protein complexes, demonstrating specific and non-specific interaction between oligonucleotide and proteins. The oligonucleotide corresponding to the position of 305-bp Runx2 promoter was denoted as number in A. 0.014 pmol of about 1×10^5 cpm of the respective oligonucleotide was incubated with D1 nuclear extracts, and protein-DNA interaction was analyzed by EMSA as described under "Materials and Methods". EMSA condition in the presence or absence of 20X cold oligonucleotide is indicated. The possible specific DNA-protein complex is indicated as a box.

competition assay, m1 has slightly decreased its competition capacity with hot DNA, whereas m2, m3 and m4 have almost lost all competition activity with hot DNA probe (Fig. 5B). This result suggested that this DNA-protein interaction motif is located in the CCACATGAT proximity.

CACATG is the sequence responsible for binding the transcription factors

The sequence of CCACATGAT contains one putative transcription factor binding site, CACATG. This sequence is only one nucleotide different from the consensus sequence of the c-myc transcription factor family, CACGTG, known as E-box [24]. This family belongs to the transcription factors designated by the presence of a basic helix-loop-helix/leucine zipper domain accountable for DNA binding and dimerization [25]. To determine whether CACATG might be the putative c-myc family binding sequence, we performed EMSA to examine whether the CACGTG consensus sequence could compete with RE1, and also if the binding pattern of the CACGTG consensus sequence was identical with that of RE1. Our results revealed that the E-box consensus sequence could compete with RE1 for the binding of transcription

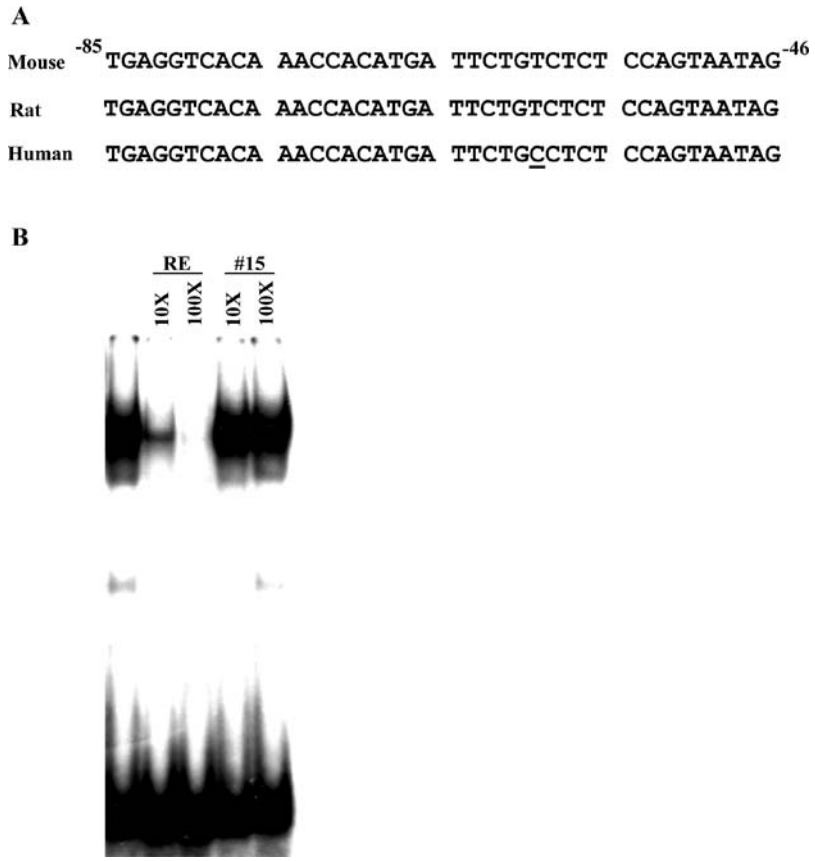

Fig. 4. A, Comparison of 40-bp sequence (RE) highly conserved in human, mouse and rat. Numbering is related to positions from respective transcription start sites; differences from the mouse sequence are underlined. B, EMSA of RE-protein complex demonstrated its highly specific binding pattern. 0.014 pmol of about 1×10^5 cpm of RE oligonucleotide was incubated with D1 nuclear extracts. The analysis method was the same as described in Fig. 3B. The non-specific cold competition oligonucleotide was #15 oligonucleotide, as indicated in Fig. 3A.

factor, and vice versa (Fig. 5C). Moreover, the binding patterns of RE1 and the binding patterns of the consensus E-box were identical (Fig. 5C).

In short, we concluded that CACATG is the sequence responsible for binding the transcription factors and might be a putative E-box.

CACATG sequence is a cis-element for Runx2 promoter activity

To examine whether CACATG binding motif was responsible for the activity of Runx2 promoter, we used site-directed mutagenesis by replacing the CAT sequence in CACATG with GTA on the pSEAP2/0.305Runx2P plasmid. We found that by substituting CAT with GTA, the 305 bp-mediated promoter activities were inhibited (Fig. 6). This result indicated that CACATG binding factor not only interacts physically with this 305 bp promoter but also plays an essential role in its function.

CACATG might be the binding sequences for USF2 homodimer

The sequence of CACATG is not only essential for the binding of transcription factors, but also responsible for the activity of the 305 bp promoter. USF1, USF2, and the Myc family members have all been demonstrated to recognize E-box [26]. To determine which transcription factors interact with CACATG, we utilized RE1-coupled magnetic beads to pull down the specific proteins, followed by Western blotting against anti-max, -myc, -USF1 and –USF2 antibodies. The finding suggested that USF2 is the only protein being pulled down by RE1-coupled magnetic beads (Fig. 7A). Furthermore, only anti-USF2 antibodies could supershift the CACATG-protein complex in EMSA (Fig. 7B). Both results indicated that USF2 is the transcription factor interacting with CACATG, and contributing to the expression of Runx2 gene.

During this study, we successfully identified one cis-element element (CACATG) and the trans-acting factor, USF2, involved in the regulation of Runx2 expression. Our study suggests that the USF2 regulatory pathway might

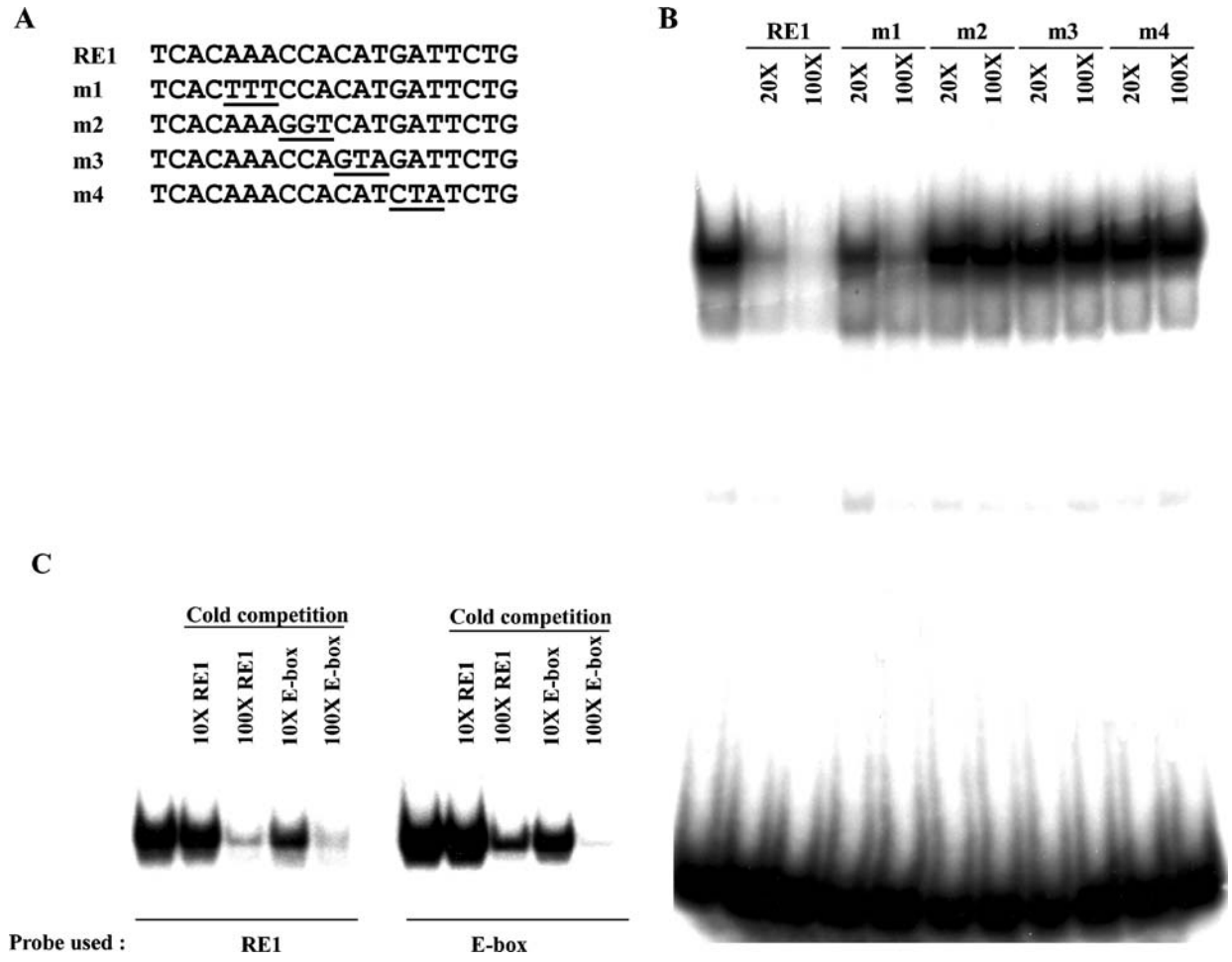

Fig. 5. A, sequences of RE1 and 4 different mutants of RE1. The mutated sequence was underlined. B, Cold competition in EMSA demonstrated that m2, m3 and m4 were unable to compete with RE1 to interact with its binding protein. 0.014 pmol of about 1×10^5 cpm of RE1 or four RE1 mutated oligonucleotide was incubated with D1 nuclear extracts. The analysis method was the same as described in Fig. 3B. Incubations were in the absence or presence of 20X and 100X cold RE1 and various mutated cold oligonucleotides as indicated. **C**, competition of RE1 by RE1 and E-box, and competition of E-box by E-box and RE1. The sequence containing E-box in this experiment is 5′- TCACAAACCACGTGTATCTG-3′. 0.014 pmol of about 1 X 10^5 cpm of the respective probe was incubated with D1 nuclear extracts. The analysis method was the same as described in Fig. 3B. Probes used, and incubations in the absence or presence of 20X and 100X cold RE1 and various mutated cold oligonucleotides were indicated.

control the early steps of osteoblast lineage commitment and differentiation.

Discussion

The expression of the bone-related Runx2 transcription factor (MASNS/p57 isoform) may be a principal element affecting skeletal development, since Runx2 plays an important role in osteoblast differentiation and function [9, 27, 28]. Elucidation of the regulatory mechanism controlling Runx2 expression in osteoblasts may reveal the upstream factors that mediate lineage commitment, growth, and differentiation. We identified CACATG as an essential cis-element for the expression of Runx2, and only USF2, not USF1 or c-myc/max, could interact with it.

USF1 and USF2 belong to the myc family of transcription factors distinguished by the presence of a basic helix-loop-helix/leucine zipper domain accountable for DNA binding and dimerization [29, 30]. The consensus binding sequence of this family of transcription factors is CACGTG, termed the E box. By binding E box target genes, USFs have been demonstrated to activate gene transcription in response to various stimulations, including glucose-dependent liver gene expressions [31–33], and glucose-induced osteopontin transcription in aortic vascular smooth muscles [34]. Usually, USFs form homo- or heterodimers to interact with their target DNA sequence [32, 33]. In D1 cells, USF2 might form

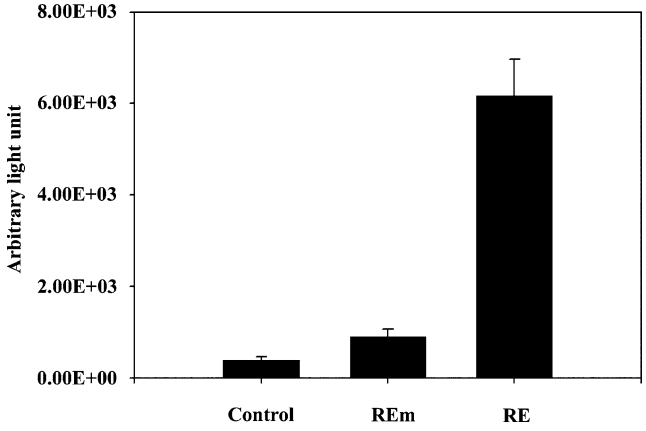

Fig. 6. Replacement of CAT with GTA of RE1 in pSEAP2/0.302Runx2P abolished 305-bp promoter activity. RE: wild type E-box (CACATG). REm: mutated form of E-box (CAGTAG). Control: pSEAP2-basic vector. D1 cells were transfected with the respective wild type and mutated form of pSEAP2/0.305Runx2P as indicated in the figure. The SEAP activities of various constructs were determined, normalized by the amount of CAT enzyme with ELISA, and presented as a bar graph.

a homodimer to activate Runx2 genes. The homogeneous USF2 knock-out mice are significantly smaller in size than their littermates, and only 20% of these mice survive a few hours after birth [29]. Although USF2 activates the genes involved in glucose-dependent pathways, the postnatal lethality is not obviously related to decreased transcription activation by glucose in liver [29]. Interestingly, Runx2 null mice die of respiratory failure shortly after birth [2], and our results suggest that Runx2 is down-stream of USF2 transcription factor. Whether deletion of the USF2 gene resulting in reduced transcription of Runx2 causes the postnatal lethality phenotype remains to be explored.

D1 cells are pluripotent mesenchymal cells, and can develop into osteoblasts and adipocytes in the presence of vitamin C and dexamethasone, respectively [35, 36]. In comparison with bone-related cell lines, such as UMR106 or C2C12, D1 cells represent the very early differentiation stage of osteoblastic cells. AP1 is the major transcription factor responsible for the expression of Runx2 in UMR 106 cells [17]. In D1 cells, the deletion of the AP1 binding site does not affect the expression of Runx2. Why the transcription factor contributing to the expression of Runx2 is different between UMR 106 cells, osteosarcoma cells, and D1 (the early differentiation of osteoblastic cells) remains unclear. However, some transcription factors, such as AP1 and myc, are usually overexpressed in tumor cells, whereas they are tightly controlled in normal cells. The overexpression of transcription factors in cancer cells may upset normal physiological conditions and therefore the phenomenon that have appeared in UMR 106 cells may not reflect on D1 cells. One recent study indicates that Dlx5 up-regulates Runx2 expression in BMP-2

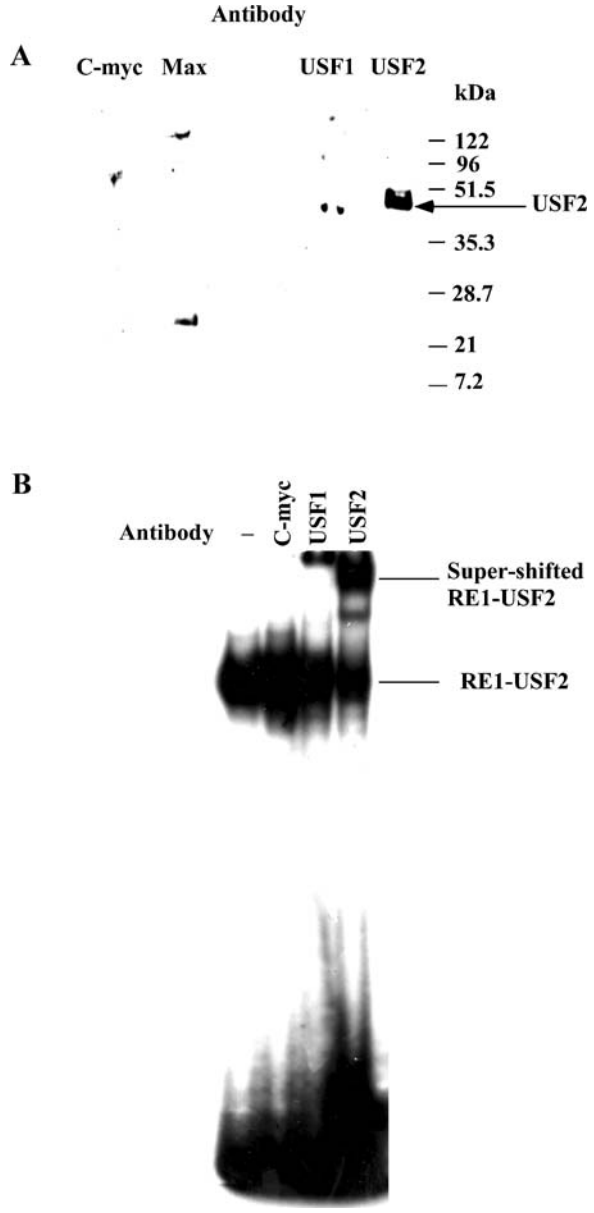

Fig. 7. A, RE1-coupled magnetic beads specifically pulled down USF2 transcription factor. 600 μg of NE isolated from D1 cells were subjected to RE1-coupled magnetic beads. The proteins bound to RE1-coupled magnetic beads were extracted and applied to SDS-PAGE, followed by Western blotting analysis. Antibodies used in this experiment were marked and USF2 protein was indicated. B, anti-USF2 antibodies super-shifted the RE1-protein complex in EMSA. Antibodies used in this experiment were marked and the RE1-USF2 complex is indicated by arrow.

signaling through interacting with three Dlx5-response elements located between −756 and −342 of P1 promoter in C2C12 cells [37]. We did not add BMP-2 into our reporter gene assay, and therefore, that might be the reason why the deletion of this region did not reduce P1 promoter activity in D1 cells (Fig. 2). Moreover, we have observed the slight

increase of the reporter gene activity by deleting the region between −4505 and −305 (Fig. 2). In this region, GAGATA sequence located at −1176 is a binding site of a transcription repressor, TRPS1 [38]. The system without this sequence might result in the slight increase of the reporter gene activity in D1 cells (Fig. 2).

In summary, our experiments demonstrated that USF2 is an important regulator of Runx2 expression. Mutation of the USF2 binding site almost completely abolishes Runx2 promoter activity in D1 cells.

Acknowledgments

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