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Abstract

The purpose of this study was to clarify the mechanism responsible for the transcriptional regulation of the mouse Col5a3 gene in osteoblastic cells. Transient transfection into rat osteosarcoma ROS17/2.8 cells demonstrated that a region from nucleotides 337 to 1 was involved in the transcriptional activity of the Col5a3 gene. An electrophoretic mobility shift assay showed that Sp1/Sp3 and CBF/NF-Y bound to a GC-rich domain (194/186) and a CCAAT box (134/130) in the Col5a3 gene, respectively. Introduction of mutations or deletion into a GC-rich domain, the CCAAT box, or both elements decreased the transcription activity. Overexpression of Sp1 increases the transcription activity and interferes with Sp family binding to the GC-rich domain to decrease promoter activity. Therefore, the transcription of the mouse Col5a3 gene is cooperatively regulated by Sp1 and CBF/NF-Y in osteoblastic cells.

KEYWORDS: type V collagen, Sp1, CBF/NF-Y, gene regulation, osteoblastic cell

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Original Article

The Sp1 and CBF/NF-Y Transcription Factors Cooperatively Regulate the Mouse Pro-*α*3(V) Collagen Gene (*Col5a3*) in Osteoblastic Cells

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The purpose of this study was to clarify the mechanism responsible for the transcriptional regulation of the mouse *Col5a3* gene in osteoblastic cells. Transient transfection into rat osteosarcoma ROS17/2.8 cells demonstrated that a region from nucleotides −337 to +1 was involved in the transcriptional activity of the *Col5a3* gene. An electrophoretic mobility shift assay showed that Sp1/Sp3 and CBF/NF-Y bound to a GC-rich domain (−194/−186) and a CCAAT box (−134/−130) in the *Col5a3* gene, respectively. Introduction of mutations or deletion into a GC-rich domain, the CCAAT box, or both elements decreased the transcription activity. Overexpression of Sp1 increases the transcription activity and interferes with Sp family binding to the GC-rich domain to decrease promoter activity. Therefore, the transcription of the mouse *Col5a3* gene is cooperatively regulated by Sp1 and CBF/NF-Y in osteoblastic cells

Key words: type V collagen, Sp1, CBF/NF-Y, gene regulation, osteoblastic cell

Vertebrate collagens, a large family of extracellular proteins, are crucial to the formation and function of virtually every organ system [1]. Among them, fibrillar collagen, which includes five molecular types (I, II, III, V, and XI), participates in the formation of fibrils with molecules packed in quarter-staggered arrays [2]. The fibrillar collagens are divided into major types (I–III) and minor types (V and XI) based on their relative expression levels. Minor fibrillar collagen types V and XI are incorporated into the fibrils of the much more abundant collagen types I and II, respectively, and act as regulators of the sizes and shapes of the resultant heterotypic fibrils [3–7].

Type V collagen is broadly distributed in type I collagen-containing tissues as a heterotrimer $[\alpha1(V)]_2\alpha2(V)$ and is found in a limited number of cell types and tissues as a rare $[\alpha1(V)]_3$ homotrimer. The former regulates the physical properties of type I/V heterotypic collagen fibrils via partially processed NH_2-terminal globular sequences [5]. Defects in the human *COL5A1* and *COL5A2* genes underlie at least half of the cases of classic Ehlers-Danlos syndrome [8–10], and the complete dependence of fibril formation on type V collagen is indicative of the critical role in early fibril initiation [11]. In addition to the $[\alpha1(V)]_2\alpha2(V)$ and $[\alpha1(V)]_3$ isoforms, a relatively uncharacterized $\alpha1(V)\alpha2(V)\alpha3(V)$ heterotrimer has been isolated from human placenta and has also been reported in the uterus, skin, and synovial membranes [12–14]. The detection of $\alpha3(V)$ expression in nascent ligamentous attachments of developing joints, membranous linings of developing skeletal muscles, and in

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developing and regenerating peripheral nerves in mouse and rat suggests roles for the α1(V)α2(V)α3(V) heterotrimer in these tissues as well [15, 16]. The N-terminal globular domain of the pro-α3(V) chain influences bone formation by osteoblasts through proteoglycans on the cell surface during development or regeneration [17].

The promoter of the human *COL5A1* gene has been characterized. A minimal promoter region within 212 bp immediately upstream of the major transcriptional start site contains the region where Sp1 binds. Furthermore, GAGA boxes in the promoter and the first exon may affect the level of transcription [18]. In the mouse *Col5a1* gene, CBF/NF-Y binds to the CCAAT-like, CAAAT sequence, and activates the gene [19]. The *COL5A2* gene depends on a cis-acting element that contains 2 contiguous protein binding sites, FPA and FPB, located between nucleotides −149 and −95 bp [20]. Combinatorial interactions among PBX and PREP or HOX proteins are involved in regulating tissue-specific production of collagen V [21].

The sequences of human and mouse full-length pro-α3(V) chains were determined [15]. These were closely related to the pro-α1(V) chain. The core promoter of *COL5A3* was characterized, thus demonstrating that CBF/NF-Y acts as a transcriptional activator of *COL5A3* in a human schwannoma cell line [22]. The present study characterized the proximal promoter of the mouse *Col5a3* gene using ostoblastic cell lines. The expression of the mouse *Col5a3* gene was regulated through the GC-rich domain as well as through the CCAAT box in osteoblastic cells, and the Sp1 was responsible for the up-regulation in combination with CBF/NF-Y.

Materials and Methods

Cells and cell culture conditions. The cell lines used in this study were rat ROS17/2.8 osteosarcoma cells, rat RCS chondrosarcoma cells, and mouse preosteoblast MC3T3-E1 cells. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (Sanko Junyaku, Tokyo, Japan) at 37℃ in a 5% CO_2/air environment.

Isolation of RNA. Total RNA was isolated from cultured cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions. RNA was quantified by optical density (A260) and stored at −80℃ until used.

Reverse transcription-polymerase chain reaction (RT-PCR). The expression levels of *Col5a3*, Sp1, and Sp3 mRNA in various cell lines were investigated by RT-PCR. Three micrograms of total RNA was reverse transcribed by random hexamer priming using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The single-stranded cDNA was amplified by PCR using specific primer pairs (Table 1). PCR was carried out for 30 cycles using a step cycle of 94℃ for 30 sec, 55℃ for 30 sec, 72℃ for 30 sec, followed by 72℃ for 7 min. The PCR products were analyzed by electrophoresis on a 1.5% ethidium bromide-stained agarose gel. The amplified fragment was eluted from the gel and sequenced.

Real-time RT-PCR. For a quantitative analysis of the expression level of *Col5a3* mRNA, real-time PCR was performed using a LightCycler TaqMan Master (Roche, Indianapolis, IN, USA). The thermal cycling conditions included 1 cycle at 95℃ for 10 min, and 45 cycles at 95℃ for 10 sec and at 60℃ for 30 sec. The relative mRNA expression level of *Col5a1* was normalized against that of the GAPDH gene from the same RNA preparations using a comparative threshold cycle method. The primer sequences are listed in Table 1.

Oligo-nucleotide-capping rapid amplification of cDNA ends (RACE). To determine the transcriptional start sites, oligo-nucleotide-capping RACE was performed using the GeneRacer kit according to the manufacturer's protocol (Invitrogen) with the *Col5a3*-specific primers. Amplification products were subcloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) for sequencing.

Construction of chimeric plasmids. To generate the 5' stepwise deletion constructs, PCR procedures were applied. PCR was performed using sets of oligonucleotide primers that are SacI site-linked 5' and XhoI site-linked 3' primers specific to the *Col5a3* sequence (Table 1) and the BAC genomic plasmid RP23-349D13 as a template. These PCR products were subcloned into the pGEM-T Easy vector, followed by digestion with SacI and XhoI, and subcloned into the SacI/XhoI site of the pGL4.10-Basic vector (Promega). Internal deletion and substitution mutation constructs were generated by site-

Table 1 Primers used for PCR procedures

1. For RT-PCR		
Col5a3 (BC138419):	(sense)	5'-gatgaaccagaaaccccctgc-3'
	(antisense)	5'-agcaccaggaaagatctgga-3'
Sp1 (AB077988):	(sense)	5'-agtgcagcaggatggttctg-3'
	(antisense)	5'-taaagttcataattcccatg-3'
Sp3 (AY902330):	(sense)	5'-ttcagatcattcctggctct-3'
	(antisense)	5'-aggcaactgtgatgaagagg-3'
β-actin (X03672):	(sense)	5'-aagagaggtatcctgaccct-3'
	(antisense)	5'-tacattggctggggtgttgaa-3
2. For real time RT-PCR		
Col5a3 (BC138419):	(sense)	5'-cggggaggagtcttttgag-3'
	(antisense)	5'-gcctgagggtctggaattaac-3'
GAPDH (BC145812):	(sense)	5'-caatgaatacggctacagcaac-3'
	(antisense)	5'-ttactccttggaggccatgt-3'
3. For generation of luciferase constructs of 5' stepwise deletion (AC170598)		
−1824/+92: (sense; −1824)		5'-at**gagctc**gggctccacccccttctc-3'
−1509/+92: (sense; −1509)		5'-at**gagctc**tggagatgaagagcttgg-3'
−1209/+92: (sense; −1209)		5'-at**gagctc**aggtccaggttcaacgag-3'
−909/+92: (sense; −909)		5'-at**gagctc**cggcttctcaaagggcag-3'
−609/+92: (sense; −609)		5'-at**gagctc**gggctatacagagaaacc-3'
−337/+92: (sense; −337)		5'-at**gagctc**ggtctgagacagaactca-3'
−141/+92: (sense; −141)		5'-at**gagctc**cagccagccaatagcgc-3'
−59/+92: (sense; −59)		5'-at**gagctc**cgggccccgcccgcaag-3'
−9/+92: (sense; −9)		5'-at**gagctc**gtctcgggcagtctccag-3'
+1/+92: (sense; +1)		5'-at**gagctc**agtctccagcagatcctc-3'
Common: (antisense; +92)		5'-at**ctcgag**cctggtgcaccaaggcca-3'
(The bold letters indicate the tagged *SacI* and *XhoI* sites in the sense and antisense primers for cloning.)		
4. For CHIP assay (AC170598)		
P1: (sense)		5'-ttaagtgcctcttcttgctgagc-3'
P2: (antisense)		5'-gcgggggcgggaggggggaccca-3'
P3: (sense)		5'-tagatatgtgccaccacaactgt-3'
P4: (antisense)		5'-acgtcagatcccctcggagctggg-3'
P5: (sense)		5'-agcaggacctggctcttccaaga-3'
P6: (antisense)		5'-gactggttttatcaccttctaac-3'

The accession number is shown in the parentheses.

directed mutagenesis using the pGL4.10 −337/+92 plasmid as a template. The PCR products were digested with endonuclease, followed by self-ligation. All mutagenesis plasmids were digested with SacI and XhoI and then re-cloned into the SacI/XhoI I site of the pGL4.10-Basic vector.

The pCMV-Sp1, PRC/CMV-Sp3 expression plasmid and the dominant-negative CBF-B/NF-YA were previously described [23-25].

Transient transfection and luciferase assays. Transient transfection experiments were carried out using ROS17/2.8, MC3T3-E1, and RCS cells. The cells were plated at a density of 2×10^5 per 35-mm dish 18h before transfection. For transient transfection, 0.5μg of plasmid DNA was transfected into these cells by using calcium phosphate precipitation methods [26]. Plasmid pRL-TK vector (Promega) was always cotransfected as an internal control for transfection efficiency. After additional cultivation for 48h, the transfected cells were harvested, lysed, centrifuged to pellet the debris, and subjected to luciferase assay. The luciferase activities were measured as chemiluminescence in a luminometer (Lumat LB 9507, Perkin-Elmer Life Sciences, Waltham, MA, USA) using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The cotransfection experiments were performed using 1.5μg of Sp1or Sp3 expression plasmids or 1.5μg of

the dominant-negative CBF/NF-Y plasmid. Mithramycin A (Sigma, St Louis, MO, USA) was incubated with a concentration of 100 nM for 24h before the luciferase assays. All transfections were repeated in triplicate and the results were expressed as the mean ± S.D. of four independent experiments.

Preparation of nuclear extracts. Nuclear extracts from ROS17/2.8 cells were prepared according to the method of Dignam *et al.* [27]. Briefly, ROS17/2.8 cells, 1×10^8, were scraped in ice-cold phosphate-buffered saline (PBS), centrifuged for 5 min at 1,500 × g, and washed with PBS before being recentrifuged. The pellets were resuspended in buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, 0.1% Nonidet P-40), incubated on ice for 10 min, and homogenized. Nuclei were pelleted by centrifugation at 3,000 × g for 10 min at 4℃, followed by resuspension in buffer (50 mM HEPES, pH 7.8, 420 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, 20% glycerol) and mixed by rotation at 4℃ for 1h. After centrifugation at 24,000 × g for 30 min at 4℃, the supernatants were collected and stored at −80℃ until used. The protein concentration of the nuclear extracts was determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) using bovine serum albumin as a standard.

Electrophoretic mobility shift assay (EMSA). Wild-type and mutant probes used for EMSA were generated by PCR using each set of HindIII site-linked primers, and all PCR products were subcloned into the pGEM-T Easy vector. All plasmids were digested with HindIII, and the digested fragments were radiolabeled with [α-^{32}P] dCTP using the Klenow fragment to fill in the HindIII overhanging sites. The binding reaction was carried out for 30 min at 25℃ in 25 μl of binding buffer (50 mM HEPES, pH 7.8, 250 mM KCl, 25 mM $MgCl_2$, 5 mM EDTA, 50% glycerol) containing 20,000–30,000 cpm of labeled probe, 3 μg of poly(dI-dC), 2 μg of ssDNA, and 5–15 μg of nuclear extracts. For the competition assays, double-stranded oligonucleotides containing a consensus CBF/NF-Y, CBF/NF-Y mutant, Sp1, and Sp1 mutant binding sites were generated by annealing equimolar complementary oligonucleotides. For the supershift assay, anti-CBF-A/NF-YB, anti-CBF-B/NF-YA, anti-CBF-C/NF-YC, anti-Sp1, and anti-Sp3 polyclonal antibodies were purchased (Santa Cruz Biotechnology, Santa Cruz, CA, USA). For these interference assays, a 5 to 100-fold molar excess of unlabeled competitor or 2 μg of antibody was added to the reaction mixture for 1h at 4℃ before the addition of the radiolabeled probe. The DNA-protein complexes were separated on a 4.5% non-denaturing polyacrylamide gel in a 0.25× Tris borate electrophoresis buffer at 200 V. After the running was completed, the gel was transferred onto 3 MM paper and dried under a vacuum. The dried gel was then visualized by autoradiography using a BioImaging Analyzer FLA-5000 (Fuji Film, Tokyo, Japan).

Chromatin immunoprecipitation (CHIP) assay. CHIP assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's protocol [25, 28]. Briefly, ROS17/2.8 cells were inoculated at a density of 5×10^6 per 100-mm dish. The cells were fixed in a final concentration of 1% formaldehyde for 10 min at room temperature. After washing twice with PBS, the cells were removed from dishes in PBS containing 1 mM EDTA and harvested by centrifugation at 1,000 g for 1 min at 4℃. The cell pellets were resuspended in SDS lysis buffer, incubated for 10 min at 4℃, and sonicated four times for 10 sec, yielding DNA fragments 200–1,000 bp in length. After centrifugation, the supernatant was diluted in CHIP dilution buffer, precleared with ssDNA/protein A-agarose slurry, and immunoprecipitated with the anti-Sp1 or anti-Sp3 (Santa Cruz Biotechnology) antibodies overnight at 4℃. Immunocomplexes were captured on the ssDNA/protein A-agarose slurry and washed with a low-salt wash buffer, a high-salt wash buffer, and LiCl wash buffer. Then they were washed twice with TE buffer. The immunocomplexes were eluted by incubation for 15 min at 25℃ with 200 μl of elution buffer (1% SDS, 100 mM $NaHCO_3$, 1 mM dithiothreitol). To reverse the cross-linking of DNA, the eluates were treated with 8 μl of 5 M NaCl and incubated for 4h at 65℃, followed by treatment with proteinase K for 1h at 45℃. The DNA fragments were extracted with phenol/chloroform and precipitated with ethanol. PCR was carried out for 40 cycles using a step cycle of 94℃ for 30 sec, 55℃ for 20 sec, 72℃ for 30 sec, and 72℃ for 8 min. The sets of primers are listed in Table 1. The PCR products were analyzed by electrophoresis on 1.5% ethidium bromide-stained agarose gel.

DNA sequencing. Nucleotide sequences were determined by automated DNA sequencing (ABI PRISM 310 Genetic Analyzer, Applied Biosystems, Foster City, CA, USA) using the BigDye Terminator Cycle Sequencing Kit (Applied Biosystems).

Results

RNA expression of Col5a3 in cultured cells. Initially, the expression of the *Col5a3* was examined in various cultured cells using the RT-PCR technique. As shown in Fig. 1A, the predicted band was observed in ROS17/2.8 and MC3T3-E1 cells, but not in RCS cells. The PCR products were verified by a nucleotide sequence to confirm the specificity of the amplified *Col5a3* sequence. A real-time RT-PCR experiment showed the expression to be higher in ROS17/2.8, thus corresponding to mature osteoblasts, than in MC3T3-E1, which correspond to preosteoblastic cells (Fig. 1B).

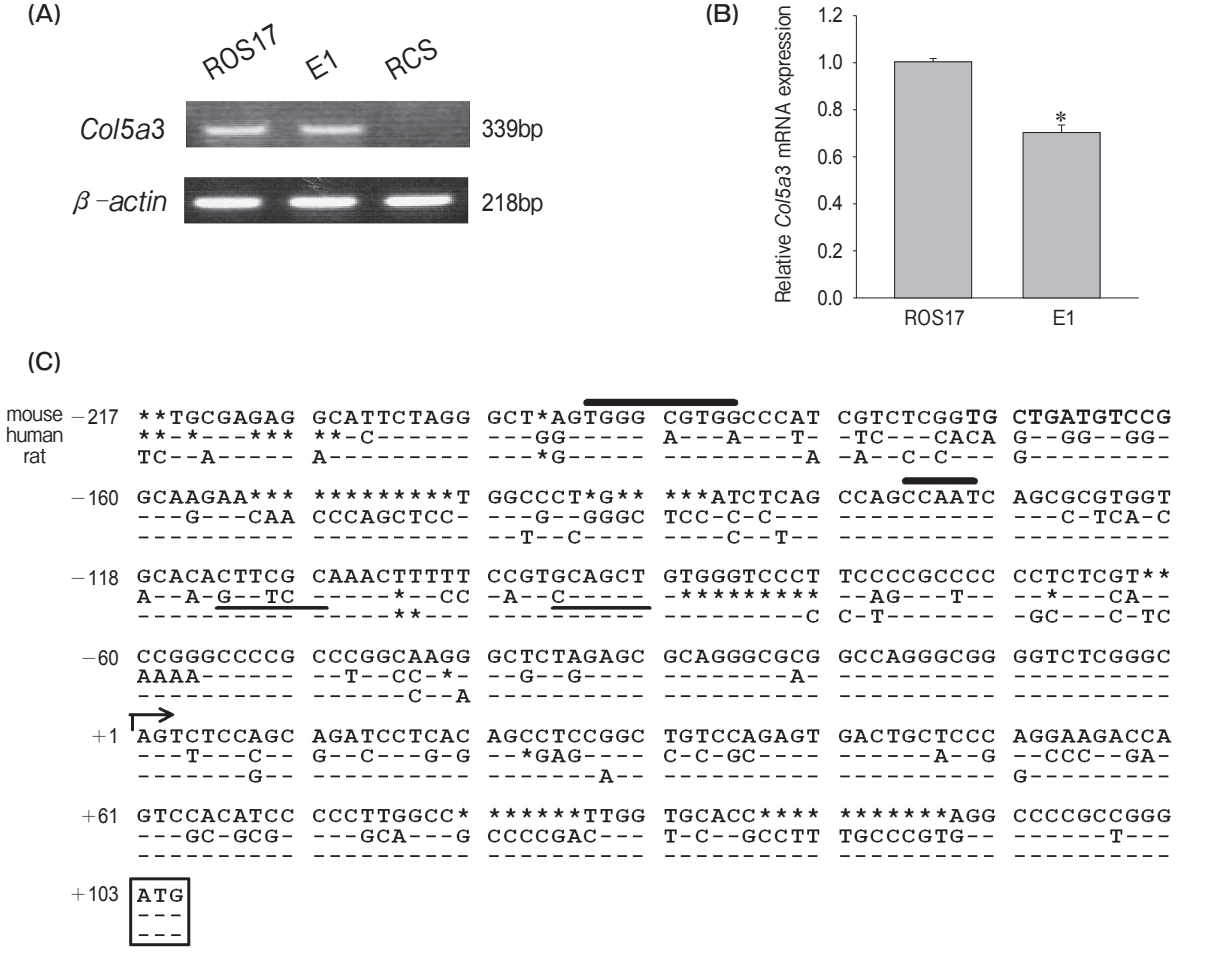

Fig. 1 **A**, RNA expression of *Col5a3* in various cell lines. RT-PCR analysis was performed on total RNA from ROS17/2.8, MC3T3-E1, and RCS cell lines. β-actin was included as a positive control for PCR; **B**, A real-time RT-PCR was performed using ROS17/2.8 and MC3T3-E1 cells. The relative expression of *Col3a5* was normalized against that of the GAPDH gene. The data are the mean ± S.D. of three independent experiments. The asterisks indicate statistically significant results ($p<0.05$); **C**, A comparative sequence analysis of the *Col5a3* promoter regions. The dashes and asterisks indicate the same nucleotides as those of the mouse and missing nucleotides in comparison to those of other species. The transcription start site positions of mouse *Col5a3* and human *COL5A3* are shown with arrows above. The bold lines indicate *cis*-elements in the mouse promoter described in this paper. The ATG codon of the translation start site is indicated with a box. The putative repressor binding sites described previously in the human gene [22] are underlined. (The GenBank accession numbers for the nucleotide sequence of pro-α3(V) collagen genes: mouse NT-039472, human NT-011295, rat NW-047798).

The transcription initiation sites of Col5a3. Oligo-nucleotide-capping RACE experiments were performed to determine the transcription initiation sites of *Col5a3*. The oligo-nucleotide-capping RACE was carried out using total RNA derived from MC3T3-E1 cells. Twenty independent clones were selected and sequenced. The 5'-ends of the *Col5a3* cDNA were located at 102 bp upstream from the initiating ATG codon, and 15 of the 20 RACE products were generated from the same position, thus suggesting that the major transcription initiation site is located 102 bp upstream from the initiating ATG codon (Fig. 1C). The nucleotide sequence upstream from the major transcription initiation site is similar to those of the human and rat counterparts. The *Col5a3* promoter lacks a canonical TATA box and has a high GC content.

Functional analysis of the Col5a3 promoter region. To define the proximal regulatory regions in the *Col5a3* promoter, a series of chimeric constructs containing progressive 5' ends linked to the luciferase gene were transfected into ROS17/2.8 and MC3T3-E1 cells, which express the *Col5a3* gene, and into RCS cells, which do not express that gene, and luciferase assays were carried out (Fig. 2). A higher activity level was observed in ROS17/2.8 cells, while MC3T3-E1 cells showed moderate activity, but there was almost no activity in RCS cells. These results were consistent with those of the RT-PCR analysis (Fig. 1A and 1B). The longest construct, pGL4.10 −1824/+92, had a strong transcriptional activity, and deletion to the −337 bp still showed strong transcriptional activity in ROS17/2.8 cells and MC3T3-E1 cells. The deletion to −141 bp began to decrease the activity, and deletion to the +1 showed almost a complete loss of luciferase activity in both cell lines. These results indicate that the region between the −337 and +1 bp is important for basal transcriptional activity of the *Col5a3* promoter in osteoblastic cells.

Identification of nuclear factor binding sites in the core promoter of the Col5a3. EMSA experiments were conducted to investigate the binding of nuclear proteins to the proximal *Col5a3* promoter. Five overlapping oligonucleotide probes covering the region between −370 and −1 bp were prepared based on the luciferase experiment data (Fig. 2). The nuclear protein was prepared from ROS17/2.8 cells because they had the highest activity.

The −230/−141 and −160/−71 probes could

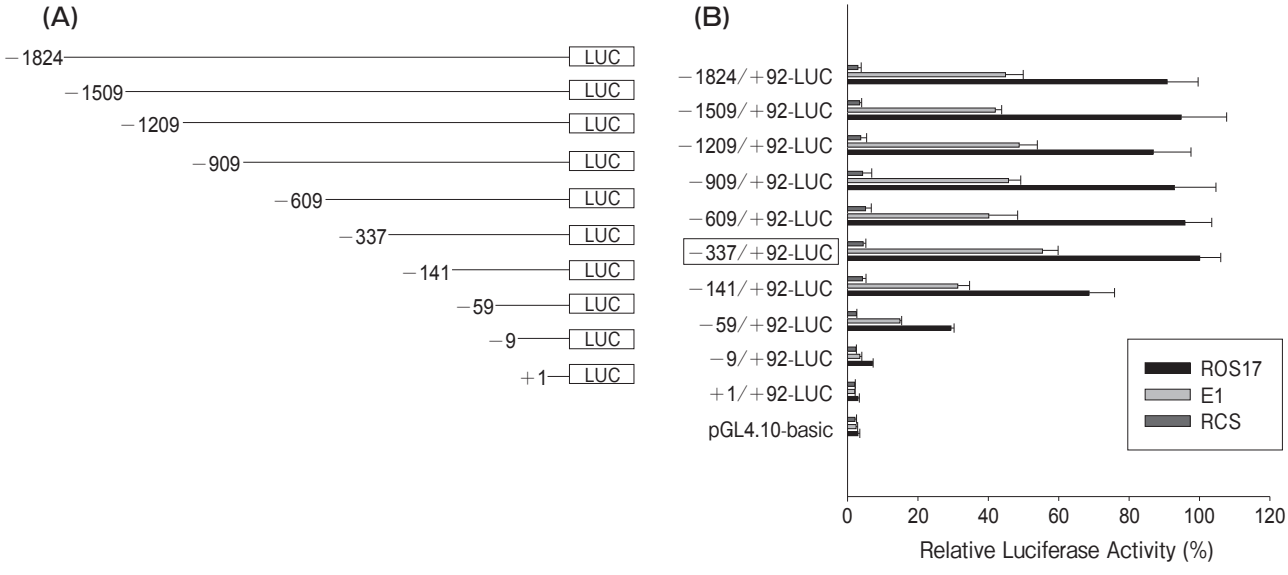

Fig. 2 Luciferase assay of the *Col5a3* promoter. A, A schematic illustration of the 5'-deletion constructs of the *Col5a3* promoter; B, Luciferase activity in ROS17/2.8 (closed histogram), MC3T3-E1 (light shaded histogram), and RCS cells (dark shaded histogram). Relative luciferase activities (%) were normalized against the activity of the pGL4.10 −337/+92-Luc construct, which is indicated with a box, in ROS17/2.8. All of the constructs were cotransfected with the pRL-TK vector as an internal control for transfection efficiency. Data are the mean ± S.D. of 4 independent experiments.

bind nuclear protein extracted from ROS17/2.8 cells in a dose-dependent manner, while the −370/−281, −300/−211, and −90/−1 probes could not bind nuclear protein (Fig. 3A). In the cold competition experiment, the DNA-protein complex of the region −230 to −141 bp disappeared completely in the presence of a 100-fold excess of unlabeled −230/−141 probe, but not in the presence of excess unlabeled −300/−211 and −160/−71 probes (Fig. 3B). Similarly, the DNA-protein complex of the region −160 to −71 disappeared completely in the presence of a 100-fold excess of unlabeled −160/−71 probe, but not in the presence of excess unlabeled −230/−141 and −90/−1 probes (Fig. 3B). Therefore, these complexes were specific, and the sites of the complex located were −210 to −160 and −140 to −90.

Six short oligonucleotide probes, which covered −220 to −181, −200 to −161, −180 to −141, −160 to −121, −140 to −101, and −120 to −81,

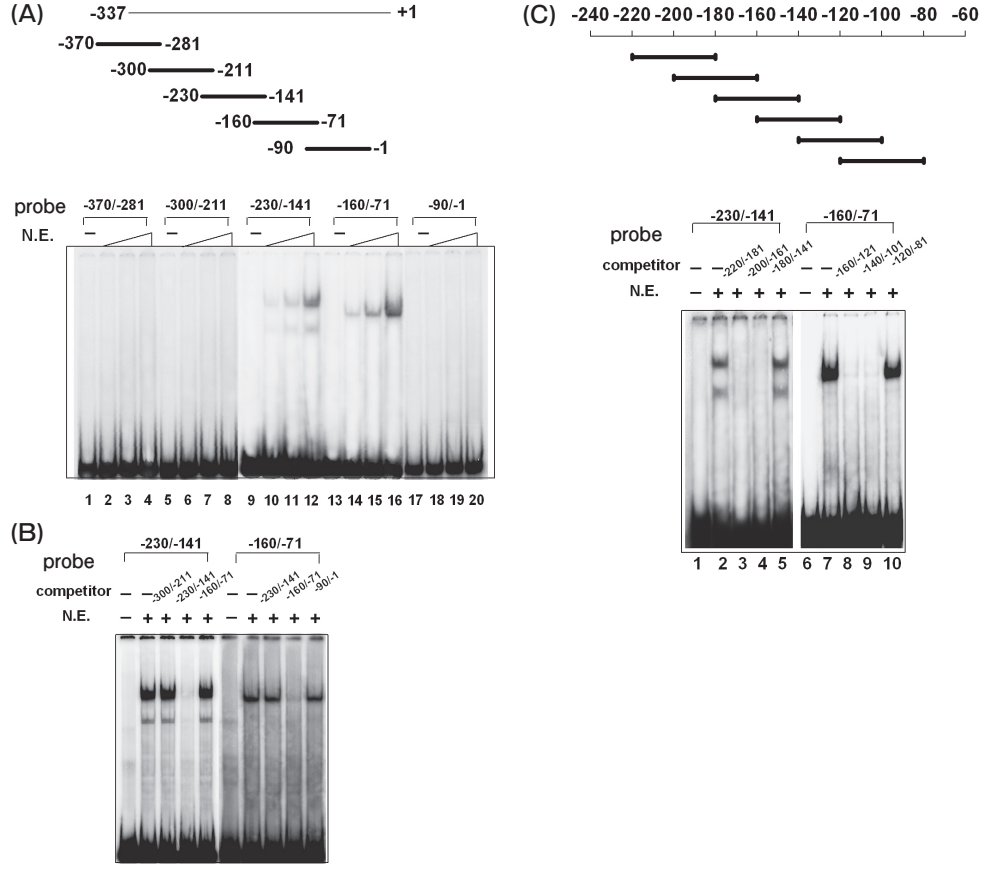

Fig. 3 EMSA analysis of the *Col5a3* promoter; **A**, Binding assay using overlapping probes in the *Col5a3* promoter. A schematic illustration of the probes used for EMSA is shown in the upper panel. The ^{32}P-labeled −370/−281 (lanes 1–4), −300/−211 (lanes 5–8), −230/−141 (lanes 9–12), −160/−71 (lanes 13–16), and −90/−1 (lanes 17–20) probes were incubated with or without (−) nuclear extracts (N.E.) from ROS17/2.8 cells; **B**, Competition analysis using an excess of unlabeled oligonucleotides. The ^{32}P-labeled −230/−141 and −160/−71 were incubated with nuclear extracts from ROS17/2.8 cells in the presence of a 100-fold excess unlabeled −300/−211 (lane 3), −230/−141 (lanes 4 and 8), −160/−71 (lanes 5 and 9), and −90/−1 (lane 10) probes, respectively. Control assays were performed without nuclear extracts (lanes 1 and 6), and with nuclear extracts but without a competitor (lanes 2 and 7); **C**, A competition analysis using an excess of unlabeled oligonucleotides that are shorter than those used in (B). A schematic illustration of the probes used for EMSA is shown in the upper panel. The ^{32}P-labeled −230/−141 (lanes 1–5) and −160/−71 (lanes 6–10) were incubated with or without (−) nuclear extracts (N.E.) from ROS17/2.8 cells in the presence of 100-fold excess unlabeled −220/−181 (lane 3), −200/−161 (lane 4), −180/−141 (lane 5), −160/−121 (lane 8), −140/−101 (lane 9), and −120/−81 (lane 10) probes. Control assays were performed without nuclear extracts (lanes 1 and 6) and with nuclear extracts but without a competitor (lanes 2 and 7).

were prepared to further investigate the binding sites (Fig. 3C). The complex of the region −230 to −141 was competitively inhibited with corresponding excess unlabeled oligonucleotides of −220/−181 and −200/−161, but was not inhibited with −180/−141 (Fig. 3C). It was therefore understood that the binding site of the complex was −200 to −181. The complex of the region −160 to −71 was competitively inhibited with the corresponding excess unlabeled oligonucleotides of regions −160/−121 and −140/−101, but it was not inhibited with −120/−81 (Fig. 3C). These results indicated that another site of the complex was located at −140 to −121.

Identification of nuclear factors in the core promoter of Col5a3. A data-base analysis was conducted to identify the nuclear binding proteins in the core promoter of the *Col5a3* (transfac. gbf. de/TRANSFAC/). The Sp1 binding site was recognized in the −200/−181 region, and the CCAAT motif, which is recognized by transcription factors of CBF/NF-Y and was proven to be in the core promoter of human *COL5A3* [22], was found in the −140/−121 region.

A competition assay was conducted with 2 shorter probes, −220/−161 and −160/−101 (Fig. 3D), to confirm these 2 complexes. The binding band of the −220/−161 probe disappeared completely in the presence of a 100-fold excess of unlabeled −220/−161 and the consensus Sp1-binding oligonucleotides, but not in the presence of excess unlabeled −220/−161 mt and Spmt oligonucleotides (Fig. 3D). The binding band of the −160/−101 probe disappeared completely in the presence of a 100-fold excess of unlabeled −160/−101 wt and the CBFwt oligonucleotide, but not in the presence of excess unlabeled −160/−101 mt and CBFmt (Fig. 3D). An interfer-

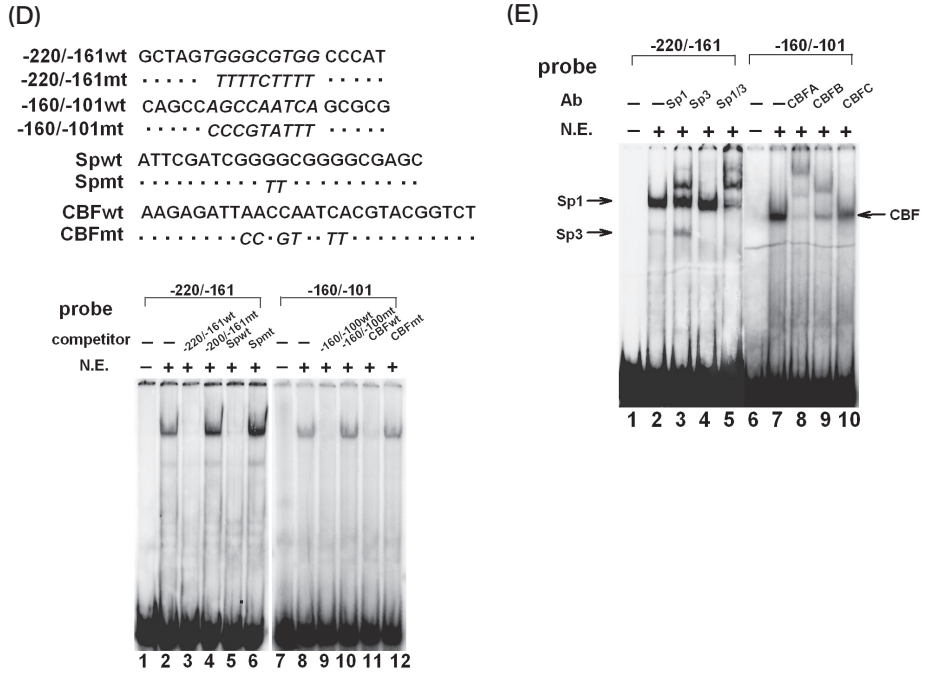

Fig. 3 (continued); D, A competition analysis using an excess of unlabeled mutated oligonucleotides. The identical and mutated nucleotides are indicated by dots and *italics*, respectively. The ^{32}P-labeled −220/−161 wt (lanes 1–6) and −160/−101 wt (lanes 7–12) were incubated with nuclear extracts (N.E.) from ROS17/2.8 cells in the presence of 100-fold excess unlabeled −220/−161 wt (lane 3), −220/−161 mt (lane 4), Spwt (lane 5), Spmt (lane 6), −160/−100 wt (lane 9), −160/−101 mt (lane 10), CBFwt (lane 11), and CBFmt (lane 12). Control assays were performed without nuclear extract or competitors (lanes 1 and 7), and with nuclear extract but without competitors (lanes 2 and 8); E, Supershift analysis using specific antibodies. The ^{32}P-labeled −220/−161 (lanes 1–5) and −160/−101 (lanes 6–10) were incubated with nuclear extracts from ROS17/2.8 cells anti-Sp1 (lane 3), anti-Sp3 (lane 4), and both of these (lane 5), anti-CBF-A/NF-YB (lane 8), anti-CBF-B/NF-YA (lane 9) and anti-CBF-C/NF-YC (lane 10). Control assays were performed without nuclear extract (lanes 1 and 6), and with nuclear extract but without the antibody (lanes 2 and 7). The bands of Sp1, Sp3, and CBF/NF-Y binding are indicated by arrows.

ence assay was performed using specific antibodies. As shown in Fig. 3E, the DNA-protein complex in the −220 to −160 region was supershifted by anti-Sp1 and anti-Sp3 antibodies. The complex in the −160 to −100 was also supershifted by anti-CBF/NF-Y antibodies, namely anti-CBF-A/NF-YB, anti-CBF-B/NF-YA, and anti-CBF-C/NF-YC. Therefore Sp1, Sp3, and CBF/NF-Y appeared to specifically interact with the *Col5a3* promoter in ROS17/2.8 cells.

In vivo occupancy of the Col5a3 promoter by transcription factors. Next, the binding of these transcription factors to *Col5a3* in ROS17/2.8 cells was tested by performing chromatin immunoprecipitation (CHIP) experiments. DNA-protein complexes were immunoprecipitated with antibodies, the cross-links were reversed, and the recovered DNA fragments were monitored by PCR using the primer set p1 and p2 for the −293 to −68 region in the proximal promoter region of the *Col5a3* gene. As shown in Fig. 4, DNA fragments immunoprecipitated with polyclonal antibodies against Sp1/Sp3 and CBF/NF-Y were amplified by PCR, whereas those immunoprecipitated with normal IgG were not. Other sets of primers, p3/p4 and p5/p6, which amplied segments that were located approximately 4 kb upstream and downstream of *Col5a3* promoter, respectively, were used as controls. Those primer sets did not amplify the DNA fragments immunoprecipitated with polyclonal antibodies against Sp1/Sp3 and CBF/NF-Y. These results indicate that Sp1/Sp3 and CBF/NF-Y specifically bind to the *Col5a3* core promoter *in vivo*.

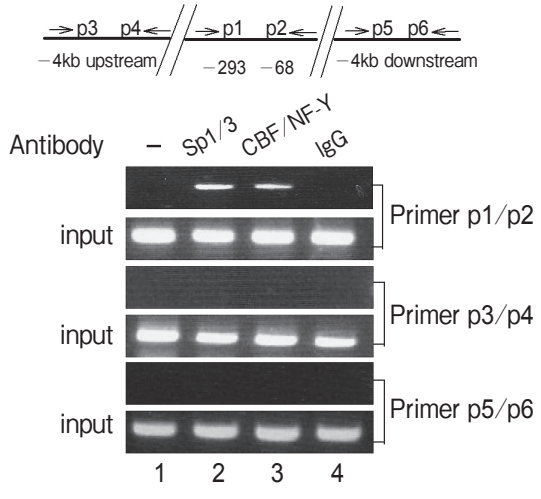

Fig. 4 Chromatin immunoprecipitation analysis. A CHIP assay was performed to confirm the binding of Sp1/Sp3 and CBF/NF-Y to the *Col5a3* promoter *in vivo*. Lane 1, immunoprecipitation without the antibody; Lane 2, immunoprecipitation with the mixture of polyclonal antibodies against Sp1 and Sp3; Lane 3, immunoprecipitation with the mixture of polyclonal antibodies against CBF-A/NF-YB, CBF-B/NF-YA, and CBF-C/NF-YC; Lane 4, immunoprecipitation with normal IgG. All immunoprecipitated DNA fragments were analyzed by PCR with the indicated primers.

Functional assay of transcription factor-binding sites in transient transfection experiments. Identification of potential binding sites for the transcription factors led to the question of whether or not these sites are involved in the activation of the *Col5a3* promoter in ROS17/2.8 cells. To test this hypothesis, deletion reporter constructs were generated. These constructs included two 5′-step-wise deletions (−141/+92-Luc and −59/+92-Luc), 3 internal deletions (del1-Luc, del2-Luc, and del3-Luc) and 3 site-directed mutations (mut1-Luc, mut2-Luc, and mut3-Luc) using the −337/+92-Luc wild construct. The mutation constructs were changed in the Sp1, CBF/NF-1, and both binding sites (Fig. 5A). The promoter activity decreased by approximately 30% in the constructs solely deleted or mutated in the Sp1 binding site (−141/+92, del1-Luc, and mut1-Luc), whereas it decreased by nearly 50% in the constructs changed in the CBF/NF-Y site (del2-Luc and mut2-Luc; Fig. 5A). The activity further decreased in the constructs that were changed at both sites (−59/+92-Luc, del3-Luc, and mut3-Luc), but still maintained more than 40% of their activity (Fig. 5A).

Functional analysis of Sp1/Sp3 and CBF/NF-Y in ROS17/2.8 cells. The roles of Sp1/Sp3 and CBF/NF-Y in *Col5a3* promoter activity were further examined by inhibiting their binding to the promoter. The effect of Sp1/Sp3 was examined using mithramycin A (100 μM), which recognizes GC-rich promoter regions and interferes with the binding of the Sp family [29]. The effect of CBF/NF-Y was examined by the overexpression of the dominant-negative CBF-B/NF-YA subunits, which interact with the CBF-A/NF-YB and CBF-C/NF-YC subunits to form CBF/NF-Y. Mithramycin A decreased activity in the −337/+92-Luc, del2-Luc, and mut2-Luc constructs, which contain the Sp1 binding site, but had no effect on −141/+92-Luc, −59/+92-Luc, del1-Luc, del3-Luc, mut1-Luc, and mut3-Luc, which do not contain that site (each lane 2 in Fig. 5B). The overexpression

of the dominant-negative CBF-B/NF-YA subunits also decreased the activity of −337/+92-Luc, −141/+92-Luc, del1-Luc, and mut1-Luc, which contain the CBF/NF-Y binding site, but had no effect on −59/+92-Luc, del2-Luc, del3-Luc, mut2-Luc and mut3-Luc, which do not contain that site (each lane 3 in Fig. 5B).

In addition, Sp1 and Sp3 were overexpressed with the expression plasmids pCMV-Sp1 and pCMV-Sp3. The results, in Fig. 6A, confirmed the increased expression of Sp1 and Sp3 mRNA in RT-PCR. Furthermore, Fig. 6B shows that overexpression of

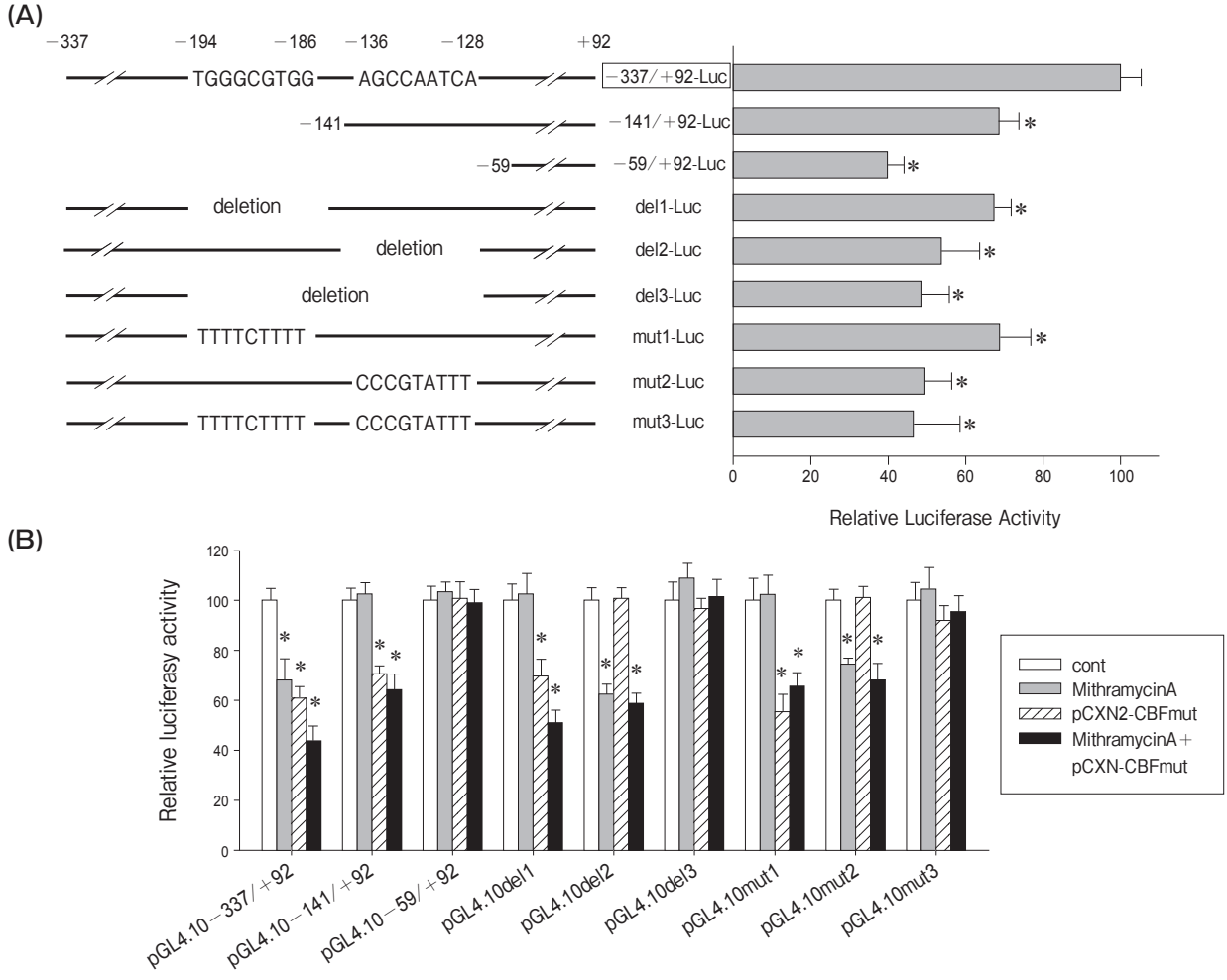

Fig. 5 A functional analysis using deletion and mutation constructs of the *Col5a3* promoter activity; **A**, Left panel: A schematic illustration of constructs that are mutated in putative nuclear protein binding sites in the *Col5a3* proximal promoter. Right panel: Luciferase activities in ROS17/2.8 cells transfected with the −337/+92-Luc, −141/+92-Luc, −59/+92-Luc, del1-Luc, del2-Luc, del3-Luc, mut1-Luc, mut2-Luc, and mut3-Luc constructs. All of the constructs were cotransfected with pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities (%) were normalized against the activity of the −337/+92-Luc construct indicated with a box. Data are the mean ± S.D. of 4 independent experiments. The asterisks indicate statistically significant results ($p<0.05$); **B**, A functional analysis by inhibition of the binding of Sp1/3 and CBF/NF-Y. ROS17/2.8 cells were transfected with 0.5 μg of −337/+92-Luc, −141/+92-Luc, −59/+92-Luc, del1-Luc, del2-Luc, del3-Luc, mut1-Luc, mut2-Luc and mut3-Luc. The cells were treated with mithramycin A (100 nM; shaded histogram), cotransfected with 1.5 μg mutant CBF/NF-Y expression vector (striped histogram), and treated with mithramycin A after cotransfection of 1.5 μg mutant CBF/NF-Y expression vector (closed histogram). The empty vector was cotransfected as a control in each group (open histogram). All of the constructs were cotransfected with pRL-TK vector as an internal control for transfection efficiency. The relative luciferase activities (%) were normalized against the activity of the control. Data are the mean ± S.D. of 3 independent experiments. Asterisks indicate statistically significant results ($p<0.05$).

Sp1 increased the activity of −337/+92-Luc, del2-Luc, and mut2-Luc (lane 2 in each construct), while the overexpression of Sp3 had almost no effect on activity (lane 3 in each construct). The overexpression of Sp1 or Sp3 had no effect on −141/+92-Luc, −59/+92-Luc, del1-Luc, del3-Luc, mut1-Luc, and mut3-Luc, which do not contain the Sp1 binding site.

Discussion

Fibrillar collagen plays a key role in regulating bone mineralization and bone integrity [30]. A previous study showed the pro-α3(V) collagen chain to be expressed in bones such as the calvaria, vertebra, maxilla, mandibula, and clavicula [17]. In the vertebral region, pro-α3(V) was detected in the periosteum and bone matrix, where ossification initially occurs. In the current study, the promoter region was characterized by a transfection assay using a luciferase reporter gene and EMSA to understand the transcriptional regulation of the *Col5a3* gene in osteoblastic cells. Basal transcriptional activity was seen from −337 to +1 in premature and mature osteoblastic cells, but not in chondrocytic cells. A GC box and a CCAAT box in the region were critical binding sites for nuclear protein. The Sp1/Sp3 bound to the GC box and CBF/NF-Y bound to the CCAAT box both *in vitro* and *in vivo*. Furthermore, a functional analysis by transfection with luciferase constructs showed that Sp1 and CBF/NF-Y were necessary for the basal transcriptional activity of the mouse *Col5a3* gene in osteoblastic cells (Fig. 7).

The gene transcription is controlled by a series of complex interactions of many transcription factors. Among the collagen genes, the transcriptional regulation of type I collagen has been the most extensively studied, and shows common features of the proximal promoter and tissue-specific enhancer. Previous stud-

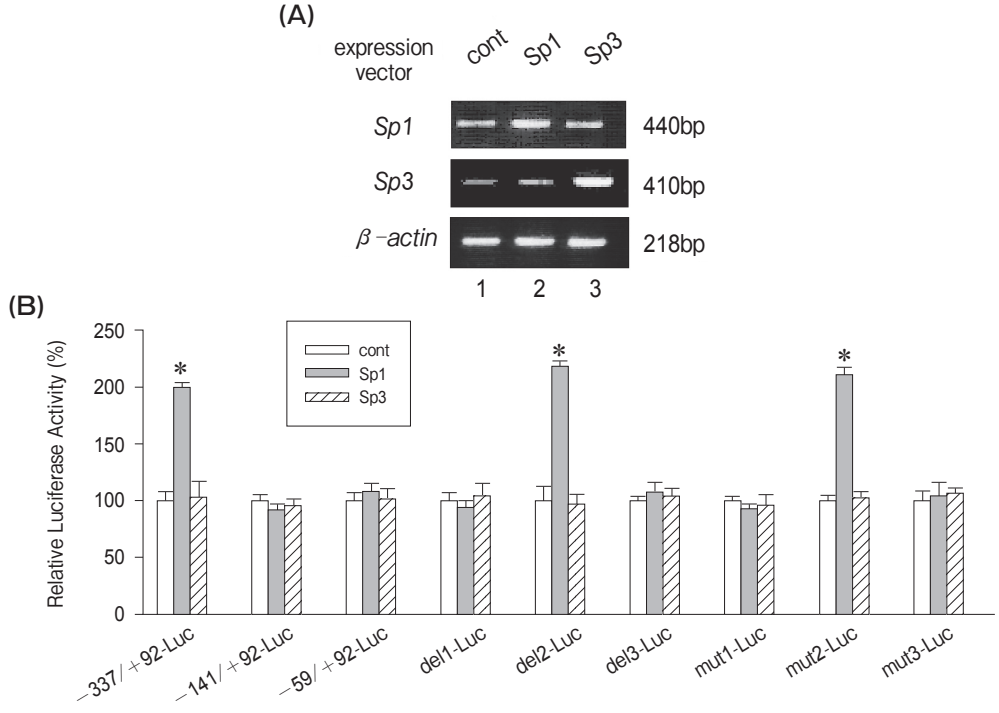

Fig. 6 A functional analysis by the overexpression of Sp1 and Sp3; **A**, RT-PCR was performed with total mRNA from ROS17/2.8 cells, which were transfected with the overexpression vector of Sp1 (lane 2) and Sp3 (lane 3). The empty vector was transfected as a control (lane 1); **B**, ROS17/2.8 cells were transfected with 0.5μg of pGL4.10 −337/+92-Luc, −141/+92-Luc, −59/+92-Luc, del1-Luc, del2-Luc, del3-Luc, mut1-Luc, mut2-Luc and mut3-Luc. Cells were cotransfected with 1.5μg of Sp1 expression vector (lane 2), Sp3 expression vector (lane 3), and an empty vector as a control (lane 1). All of the constructs were cotransfected with pRL-TK vector as an internal control for transfection efficiency. The relative luciferase activities (%) were normalized against the activity of the control. Data are the mean ± S.D. of 3 independent experiments. Asterisks indicate statistically significant results ($p<0.05$).

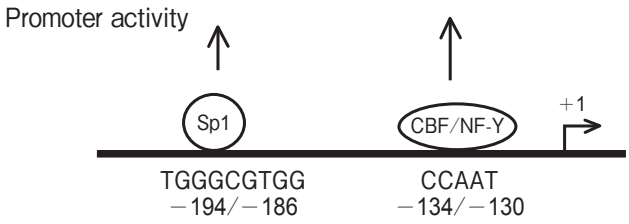

Fig. 7 Schematic illustration of the core promoter of *Col5a3* in osteoblastic cells. The binding sites of the Sp1 and CBF/NF-Y. The indicated sequences are critical for binding the factors.

ies have identified the Sp1, Sp3, and CBF/NF-Y binding factors for basal activity in both type I collagen genes [31–33]. Other transcription factors, such as c-Krox, BFCOL1, and IF-1, are also reported in the regulation of type I collagen [34–36]. Type III collagen expression patterns are similar to those of type I collagen. However, the transcription factors involved seem to be different from those of type I collagen genes [37–39]. Sox9 binds to and activates the collagen genes that are specifically expressed in the chondrocyte, such as *Col2a1* and *Col11a2* [40, 41]. L-Sox5 and Sox6, which share 50% identity with Sox9 in the Sox domain, are expressed downstream of Sox9 [42].

A previous report demonstrated that CBF/NF-Y and 2 repressors regulate the core promoter of the human *COL5A3* gene in schwannoma cells [22]. CBF/NF-Y is involved in transcriptional activity in osteoblastic cells in the mouse counterpart gene, as expected from the conservation of the CCAAT-motif and flanking sequence among species (Fig. 1B). CBF/NF-Y might be required to coordinate the expression of type I and V/XI collagen in noncartilaginous tissues [19, 25, 43–45]. However, no repressors were identified in the current study. This may be due to the difference in the binding sites of specific sequences (Fig. 1B) or cells. Instead, the current study found the Sp family to upregulate the gene in osteoblastic cells. Sp1 and Sp3 are closely related proteins with very similar structural features [46]. They bind to the GC box with the same specificity and affinity and regulate gene transcription [24]. Sp3 can either activate or repress the transcription of target genes depending on the cell type, based on the context of DNA binding sites, and the interactions with other nuclear factors [47]. Sp1 and Sp3 bind to the promoter in the current study. However, only Sp1 increased the activity of the *Col5a3* promoter in osteoblastic cells. Sp3 might therefore not affect the expression of the *Col5a3* gene.

Deletions and/or mutations that removed the binding sites of both Sp1 and CBF/NF-Y did not completely eliminate the promoter activity. This suggested that other factors or mechanisms are involved in the activation of the *Col5a3* gene in bone tissues. In the α1(I) collagen gene, Runx2/Cbfa1, which is regarded as the master transcription factor for osteoblast differentiation [48–50], bound at approximately −1347bp of the rat, mouse, and human genes of the promoter [51]. In the current study, although the binding of Runx2/Cbfa1 to the *Col5a3* promoter has not yet been demonstrated, such factors may therefore also be responsible for the activation of the *Col5a3* gene. Further study will provide some valuable insight regarding the involvement of type V collagen in bone formation.

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