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The Transcription Factor CCAAT-binding Factor CBF/NF-Y Regulates the Proximal Promoter Activity in the Human α1(XI) Collagen Gene (*COL11A1*)*

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We have characterized the proximal promoter region of the human *COL11A1* gene. Transient transfection assays indicate that the segment from −199 to +1 is necessary for the activation of basal transcription. Electrophoretic mobility shift assays (EMSAs) demonstrated that the ATTGG sequence, within the −147 to −121 fragment, is critical to bind nuclear proteins in the proximal *COL11A1* promoter. We demonstrated that the CCAAT binding factor (CBF/NF-Y) bound to this region using an interference assay with consensus oligonucleotides and a supershift assay with specific antibodies in an EMSA. In a chromatin immunoprecipitation assay and EMSA using DNA-affinity-purified proteins, CBF/NF-Y proteins directly bound this region *in vitro* and *in vivo*. We also showed that four tandem copies of the CBF/NF-Y-binding fragment produced higher transcriptional activity than one or two copies, whereas the absence of a CBF/NF-Y-binding fragment suppressed the *COL11A1* promoter activity. Furthermore, overexpression of a dominant-negative CBF-B/NF-YA subunit significantly inhibited promoter activity in both transient and stable cells. These results indicate that the CBF/NF-Y proteins regulate the transcription of *COL11A1* by directly binding to the ATTGG sequence in the proximal promoter region.

The collagen superfamily, one of the extracellular matrix proteins, plays an important role, not only in stabilizing the tissues as structural components but also in regulating a variety of biological functions, such as development, differentiation, proliferation, and morphogenesis (1–3). Among them, types I, II, III, V, and XI collagens are included in the group of fibril-forming collagens, based on their structural and functional features, and are divided into two subgroups, major (I, II and III) and minor (V and XI) fibrillar collagen (4) on the basis of their contents in tissues.

Type XI collagen is a component of the collagen fibrillar network found in cartilage (5), and consists of three genetically distinct polypeptide chains: α1(XI), α2(XI), and α3(XI); the last of these is thought to be overglycosylated α1(II) chains (6). Although type XI collagen is a relatively minor collagen and is buried within the major collagen fibrils, it is important for the regulation of fibril diameter (7). Chondrodysplasia mice (*cho*), which do not synthesize α1(XI) chains, show irregular collagen fibrils in their cartilage (8). Contrary to the previous findings, the α1(XI) chain is not restricted to cartilage (9–14), and it was demonstrated that the α1(XI) chain could form a heterotrimer with the α2(V) chain in a 2:1 ratio in non-cartilaginous cells and tissues (15, 16). Although the precise function of this cross-type trimer remains unclear, the α1(XI) collagen gene is more broadly expressed than other collagen genes, showing that the α1(XI) collagen gene is the sole collagen gene to be expressed in both cartilaginous and non-cartilaginous tissues. This implies that the regulation of the α1(XI) collagen gene might be more complex than expected.

We have previously reported the structural and functional features of the human α1(XI) collagen gene (*COL11A1*) promoter (17). DNase I footprinting has mapped nine areas, FP1 to FP9, covering the region −541 to +1, where nuclear proteins probably bind. In characterizing the −541 to −199 segment, FP9-protein, which has homology to the GATA consensus motif and binds at −531 to −487, was recognized to be a distinct ~100-kDa polypeptide (18). It was expressed in mesenchymal cells, and its level of binding activity was positively correlated with the degree of cell differentiation in osteoblastic and skeletal muscle cell lines, *in vitro*. At −395 to −379, the core sequence of FP7 exhibits some homology with that of FPB[1] in *COL5A2*, and FP7-protein complexes can be competed with an excess of FPB oligonucleotides in an EMSA using nuclear extracts from the 1120 cell line (17, 19). Further studies using transfection experiments and EMSAs in A204 cells, however, indicated that the FPB-protein complexes identified were PBX, PREP and HOX proteins and therefore different from the FP7-binding proteins (20).

In this study, we have characterized the region downstream of the −541 to −199 region in the human *COL11A1* promoter. Transient transfection assays indicate that the segment from −199 to +1 is necessary for the activation of basal transcription, and EMSAs demonstrate that the ATTGG sequence within the −147 to −121 fragment is critical for binding nuclear proteins. Furthermore, we identify the CCAAT binding factor CBF/NF-Y as binding to this region.

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[1] The abbreviations used are: FPB, Foot Printing B; EMSA, electrophoretic mobility shift assay; C/EBP, CCAAT/enhancer binding protein; CBF, CCAAT binding factor; wt, wild type; CTF, CCAAT transcription factor; NF-Y, nuclear factor for Y box.

MATERIALS AND METHODS

Cells and Cell Culture—Human rhabdomyosarcoma cell line A204 (17, 18) was used in this study. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heart-inactivated fetal bovine serum (Sanko Junyaku, Tokyo) at 37 °C in humidified 5% CO_2/95% air.

Construction of Chimeric Plasmids—Essentially, all *COL11A1* promoter-luciferase gene constructs were derived from −1454 COL11A1/CAT plasmid that fused to the *CAT* gene as described previously (17). To obtain various 5′ deletion constructs, these fragments were generated by PCR using sets of oligonucleotide primers, which are *Sac*I site-linked 5′- and *Xho*I site-linked 3′-primers specific for the promoter sequence. These PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, WI), followed by digestion with *Sac*I and *Xho*I, and subcloned into the *Sac*I/*Xho*I site of pGL3-Basic vector.

The constructs that contained tandem fragments, PGL3(−199/−65)x2 and pGL3(−147/−121)x4, were chosen. Their DNA sizes were checked on agarose gel, and the direction of the constructs was confirmed by sequence.

For internal deletion and mutation constructs, site-directed mutagenesis was apply (21). The constructs were derived from pGL3−541/+271 plasmid.

For 6 base-substitution, we introduced *Eco*RI sequence, GAATTC, for substitution sites. The primers for 6 base-substitution constructs, pGL3−541E1, pGL3−541E2, pGL3−541E3, and pGL3−541E4, were as follows: E1: sense, 5′-gaattcTGGGTCTGACCCTCAGCCTG-3′; antisense, 5′-gaattcCGACTCTGGGGCGGCCCCAA-3′; E2: sense, 5′-gaattcCTCAGCCTGCTTGTCAGTTT-3′; antisense, 5′-gaattcACCCAATCACACGACTCTGG-3′; E3: sense, 5′-gaattcTGTGATTGGGTCTGACCCTC-3′; antisense, 5′-gaattcTGGGGCGGCCCCAAGCCCGC-3′; and E4: sense, 5′-gaattcCTGACCCTCAGCCTGCTTGT-3′; antisense, 5′-gaattcTCACACGACTCTGGGGCGG-3′ (the *Eco*RI sites for mutation are indicated by lowercase letters). PCR products were digested with *Eco*RI, followed by self-ligation. All mutagenesis plasmids were digested with *Sac*I and *Xho*I and re-cloned into the *Sac*I/*Xho*I site of pGL3-Basic vector.

The primers for pGL3−541CBFm, pGL3-del−147/−121, and pGL3-del−199/−65 were as follows: for CBF mutant construct pGL3−541CBFm: sense, GaaATacGggCTGACCCTCAGCCTGCTT; antisense, ccCgtATttCACGACTCTGGGGCGGCCC (mutated nucleotides are indicated by lowercase letters); for deletion construct pGL3-del-147/−121: sense, 5′-AGCCTGCTTGTCAGTTTCGC-3′; antisense, 5′-GGGGCGGCCCCAAGCCCGCC-3′; for deletion construct pGL3-del−199/−65: sense, 5′-GGCGGAGGAGGGGGCTGCCC-3′; antisense, 5′-GAGCAGGCCCAGCCCACCAA-3′.

Construct of the dominant-negative CBF-B/NF-YA was generated by reverse transcriptase PCR as described previously (22, 23). Two sets of primers were as follows: for 5′-CBF-B/NF-YAmut: sense, 5′-gtcgacGGAGGGACCATGGAGCAGTATA-3′; antisense, 5′-GCGGCCGCCTTCCGTGCCATGGCATGAC-3′, and for 3′-CBF-B/NF-YAmut: sense, 5′-GCGGCCGCAGGTGGACGATTTTTCTCTC-3′; antisense, 5′-tctagaGGGTTAGGACACTCGGATGATC-3′ (functional mutated nucleotides are indicated by lowercase letters.)

These PCR products were cloned into the pGEM-T Easy vector and subcloned into the *Sal*I/*Not*I site for 5′-CBF-B/NF-YA mut, followed by *Not*I/*Xba*I site for 3′-CBF-B/NF-YAmut into the empty vector. Finally, this dominant-negative form of CBF-B/NF-YA was cloned into pCXN2 mammalian expression vector (24). All constructs mentioned above were sequenced on an ABI 310 sequencer (Applied Biosystems) according to the manufacturer's protocol.

Transfection and Luciferase Assays—The cells were inoculated at a density of 2×10^5 per 35-mm dish 24 h before transfection. For transient transfection, each of plasmid DNA was transfected into these cells by using the calcium phosphate precipitation method followed by a 15% glycerol shock for 60 s (25). After an additional cultivation for 48 h, the transfected cells were harvested, lysed, centrifuged to pellet the debris and performed to luciferase assay as described below. For stable transfection, 1 μg of pGL3−199/+271 or pGL3(−147/−121)x4 luciferase construct with 50 ng of pMAM2-BSD selection vector were transfected into A204 cells by using the LipofectAMINE Plus reagent system (Invitrogen) and cultured for 48 h. These transfected cells were replated at a density of 1×10^3 per 100-mm dish with fresh medium containing 8 μg/ml of blasticidin (Funakoshi, Tokyo, Japan). After an additional cultivation for 2 weeks, the resistant colonies were isolated and further cultured in the present of blasticidin, followed by assayed the luciferase activity to select stable cell lines.

Luciferase activities were assayed by the Dual-LuciferaseTM Reporter Assay System according to the manufacturer's protocol (Promega, Madison, WI) using a luminometer (Lumat L.D. 9507; PerkinElmer Life Sciences). Five micrograms of firefly luciferase reporter construct and/or dominant-negative CBF-B/NF-YA expression vector were cotransfected with 0.25 μg of pRL-TK *Renilla reniformis* luciferase expression vector as an internal control for transfection efficiency. The pGL3-Basic and pGL3-Control vectors were used for each experiment as negative and positive controls, respectively. Relative luciferase activities (percentages) of each construct were normalized against the activity of pGL3-Control vector and results were expressed as mean ± S.E. of three to five independent experiments.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared according to Dignam *et al.* (26) with some modifications. All buffers contained the protease inhibitors leupeptin (2 μg/ml), aprotinin (2 μg/ml), pepstatin A (2 μg/ml), phenylmethylsulfonyl fluoride (0.5 mM), and dithiothreitol (1 mM). Cells (1×10^8) were resuspended in buffer (10 mM HEPES, pH 7.8, 10 mM KCl, 0.1 mM EDTA, and 0.1% Nonidet P-40), followed by incubation on ice for 10 min and homogenized. After centrifugation at 3000 rpm for 10 min, the cell pellets were resuspended in buffer (50 mM HEPES, pH 7.8, 420 mM KCl, 5 mM $MgCl_2$, 0.1 mM EDTA, and 20% glycerol) and rotated at 4 °C for 1 h. The supernatants with nuclear proteins were recovered by centrifugation at 24,000 × *g* for 30 min, and protein concentration of the nuclear extracts was determined by the Bradford's colorimetric reagent (Bio-Rad) using bovine serum albumin as a standard.

Wild-type and mutant probes derived from *COL11A1* promoter fragment were generated by PCR using each set of *Hin*dIII site-linked primers, and all PCR products were cloned into the pGEM-T Easy vector (Promega). Both sense and antisense oligonucleotides of the mutant fragments covering −147 to −121, CBFwt, CBFmt, NF-1, C/EBP, and GATA consensus overhanging *Hin*dIII sites at the ends were synthesized (Proligo Japan KK, Kyoto, Japan), annealed to make double-stranded oligonucleotides, digested with *Hin*dIII, and cloned into the *Hin*dIII site of pBluescript SK vector. The CBFwt, CBFmt, NF-1, C/EBP, and GATA consensus sequences, based on the data from Santa Cruz Biotechnology, Inc., used as competitors, were as follows: CBFwt, 5′-AGACCGTACGTGATTGGTTAATCTCTT-3′; CBFmt5′-AGACCGTACGAAATACGGGAATCTCTT-3′ (mutated nucleotides are underlined); NF-Y, 5′-TTTTGGATTGAAGCCAATATGATAA-3′; C/EBP, 5′-TGCAGATTGCGCAATCTGCA-3′; and GATA, 5′-CACTTGATAACAGAAAGTGATAACTCT-3′.

All plasmids were digested with *Hin*dIII and the digested fragments were radiolabeled with [α-^{32}P]dCTP using Klenow fragment to fill in the *Hin*dIII overhang sites. For EMSA, the binding reaction was performed for 30 min at 25 °C in 25 μl of reaction buffer (50 mM HEPES, pH 7.8, 250 mM KCl, 25 mM $MgCl_2$, 5 mM EDTA, and 50% glycerol) containing 20,000–30,000 cpm of labeled probe, 3 μg of poly(dI-dC), and 1–20 μg of nuclear extracts.

For competitors and antibody interference assay, unlabeled probes or antibody was added to the reaction mixture for 1 h at 4 °C before the addition of [α-^{32}P]dCTP labeled probe. These antibodies against CBF-A/NF-YB, CBF-B/NF-YB, CBF-C/NF-YC, C/EBP, NF-1, GATA-1, and pre-immune goat IgG were purchased from Santa Cruz Biotechnology. The DNA-protein complexes were separated on 4.5% nondenaturing polyacrylamide gel in 0.25× Tris-borate/EDTA with 2.5% glycerol at 4 °C and visualized by autoradiography using Bio Imaging Analyzer BAS-2000 (Fuji Film, Tokyo, Japan).

Purification of DNA Binding Protein Using DNA Affinity Latex Beads—DNA affinity latex beads were prepared as described previously (27). To purify DNA binding proteins, the DNA-latex beads were added to the nuclear extracts and incubated for 24 h at 4 °C. After centrifugation at 15,000 rpm for 5 min, the beads were washed three times with 0.1× buffer A (50 mM Tris-HCl. pH 7.9, 20% glycerol, 0.1 M KCl, 1 mM EDTA, 1 mM dithiothreitol, and 0.1% Nonidet P-40) containing 0.1 M KCl. To elute the binding proteins from the DNA-latex beads, the beads were resuspended in 0.4× buffer A containing 0.4 M KCl and incubated for 15 min at 4 °C. After centrifugation at 15,000 rpm for 5 min, the supernatant containing DNA binding proteins was recovered and concentrated by microcon-10 (Millipore).

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotech, Lake Placid, NY) according to the manufacturer's protocol. All solutions were contained with 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin A. A204 cells were inoculated at a density of 1×10^6 per 100-mm dish 16 h before formaldehyde cross-linking and chromatin immunoprecipitation. Cells were fixed with 1% formaldehyde for 15 min at 37 °C. After washing twice with PBS, cell pellets were resuspended in SDS lysis buffer,

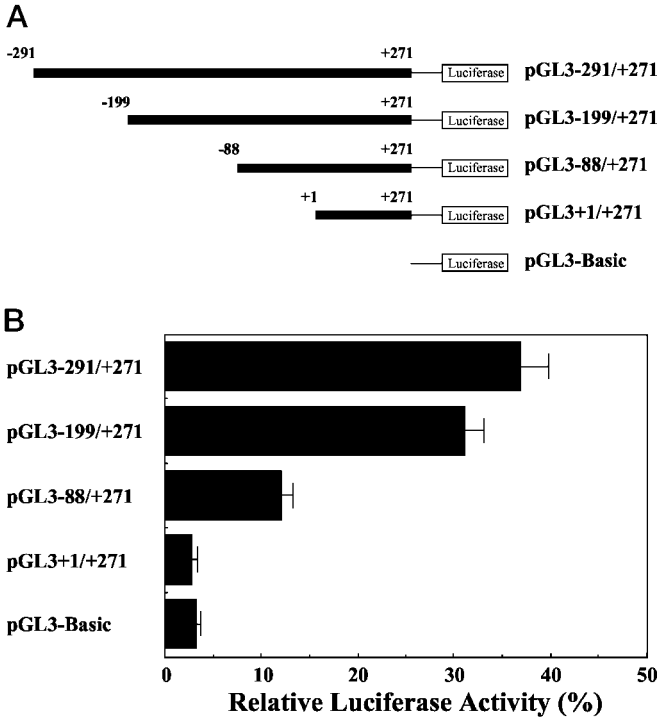

FIG. 1. **Deletion analysis of the human *COL11A1* promoter in the luciferase assay.** *A*, schematic representation of the deletion constructs of the human *COL11A1* promoter. *B*, 5′-deletion analysis of the human *COL11A1* promoter. A series of human *COL11A1* promoter-luciferase constructs were transfected into the cells with the pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities (percentages) were normalized against the activity of the pGL3-control vector, and results are expressed as mean ± S.E. of three independent experiments.

incubated for 10 min at 4 °C, and sonicated four times for 10 s using ultrasonic homogenizer VP-5S (TAITEC, Tokyo, Japan). After centrifugation, the supernatant was diluted in chromatin immunoprecipitation dilution buffer and then incubated overnight at 4 °C with anti-CBF-A/NF-YB, CBF-B/NF-YA, and CBF-C/NF-YC antiserum (Santa Cruz Biotechnology). Immune complexes were recovered by the addition of 60 μl of salmon sperm DNA/protein A-agarose slurry, followed by incubation for 2 h at 4 °C. After washing the beads with both low- and high-salt buffer, then LiCl buffer, and finally Tris/EDTA buffer, the immune complexes were eluted by incubation for 15 min at 25 °C with 200 μl of elution buffer (1% SDS, 100 mM $NaHCO_3$, and 1 mM dithiothreitol). To reverse the cross-linking of DNA, the elutes were added to 8 μl of 5 M NaCl and incubated for 4 h at 65 °C, followed by treatment with proteinase K for 1 h at 45 °C. DNA was recovered by phenol-chloroform extraction and ethanol precipitation, and then the pellets were resuspended in 50 μl of Tris/EDTA buffer. Quantitative PCR was carried out for 35 cycles using 5 μl of sample DNA solution, and PCR products were separated on 2% agarose gels in 1× Tris-acetate/EDTA.

RESULTS

Deletion Analysis of the Human COL11A1 Promoter—To delineate the proximal regulatory regions in the human *COL11A1* promoter, a series of chimeric constructs containing 5′-end deletions linked to the luciferase gene were generated, and then luciferase assays were carried out (Fig. 1*A*). The activity of each construct was compared with the longest construct, pGL3−291/+271. As shown in Fig. 1*B*, the promoter activity of pGL3−199/+271 was not significantly reduced compared with that of pGL3−291/+271. However, deletion to −88 produced a significant reduction in the transcriptional activity; furthermore, deletion to +1 produced activity similar to that of the pGL3-Basic negative control vector. This result indicates that the segment spanning the −199 to +1 region is important for basal transcriptional activity of the human *COL11A1* promoter.

Definition of the Nuclear Proteins Binding Site in the Proximal COL11A1 Promoter—On the basis of the aforementioned data, we examined the binding proteins that interact in this region. We prepared three overlapping oligonucleotides, GS1–GS3, covering the −199 to +1 region (Fig. 2*A*), and carried out EMSAs. As shown in Fig. 2*B*, ^{32}P-labeled GS2 probe, covering −147 to −65, and GS3 probe, covering −199 to −121, bound nuclear proteins extracted from A204 cells in a dose-dependent manner. Both probes were found to form DNA-protein complexes with the same mobility in an EMSA. To determine whether GS2 and GS3 oligonucleotides bind the same proteins, these probes were used with each other in a competition assay. The ^{32}P-labeled GS2-protein complexes could be competed away not only by the corresponding unlabeled probe (Fig. 2*C*, *lane 4*), but also by the unlabeled GS3 oligonucleotide (Fig. 2*C*, *lane 5*). Likewise, the ^{32}P-labeled GS3-specific band also disappeared when either unlabeled GS3 or GS2 oligonucleotides were added as competitors (Fig. 2*C*, *lanes 9* and *10*); however, an excess of the unlabeled GS1 (−88 to +1) oligonucleotides did not compete for the binding of ^{32}P-labeled GS2 and GS3 probes (Fig. 2*C*, *lanes 3* and *8*). To further dissect this protein-binding region, another three oligonucleotides covering the GS2 and GS3 regions were generated (Fig. 2*A*). Fig. 2*D* shows that ^{32}P-labeled GS4 probe, covering −147 to −121, formed DNA-protein complexes in a dose-dependent manner. However, neither ^{32}P-labeled GS5 nor GS6 probes, which do not possess the GS4 region, could bind protein. These results indicate that a 27-bp sequence from −147 to −121 in the proximal *COL11A1* promoter region is necessary to bind nuclear proteins. We subsequently carried out EMSAs using substitution mutation probes within the 27-bp sequence to narrow down the critical region for binding (Fig. 3*A*). The ^{32}P-labeled GSE1 and GSE4 probes, which contain 6 single bp substitutions, could not form DNA-protein complexes, whereas the ^{32}P-labeled GSE2, GSE3, and GSwt probes bound protein in a dose-dependent manner. In addition, Fig. 3*C* shows that an excess of the unlabeled GSE2 and GSE3 probes inhibited the binding activity in a manner similar to that of GSwt, whereas both GSE1 and GSE4 could not compete with the ^{32}P-labeled GSwt-specific band. To confirm a core-binding site, we further performed competition experiments using oligonucleotides with 2 single bp mutations. As shown in Fig. 3*D*, an excess of unlabeled GSM1, GSM2, GSM3, and GSM4 failed to inhibit the binding, whereas GSM5 and GSM6 blocked the binding of nuclear proteins (Fig. 3*C*). These results suggest that the GTGATTGG sequence within the 27-bp region was the critical binding site within the proximal *COL11A1* promoter.

Identification of DNA-Binding Proteins in the Proximal COL11A1 Promoter—To identify the nuclear binding proteins in the proximal *COL11A1* promoter, we searched the computer data base and found four candidate transcription factors: CCAAT binding factor CBF/NF-Y, CCAAT transcription factor NF-1/CTF, CCAAT/enhancer binding protein C/EBP, and GATA (Fig. 4*A*). An excess of the consensus oligonucleotides of CBF/NF-Y inhibited the binding of the ^{32}P-labeled GSwt probe (Fig. 4*B*, *lane 2*), whereas NF-1, C/EBF, GATA consensus, and CBFmt oligonucleotides could not abolish the binding activity observed (Fig. 4*B*, *lanes 3–6*). To further characterize the binding protein, we performed an interference assay using specific antibodies against CBF/NF-Y, NF-Y, C/EBP, and GATA1. As shown in Fig. 4*C*, the DNA-protein complex was only supershifted by anti-CBF antibodies, namely anti-CBF-A/NF-YB (Fig. 4*C*, *lane 3*), CBF-B/NF-YA (Fig. 4*C*, *lane 4*), and CBF-C/NF-YC (Fig. 4*C*, *lane 5*). Specific antibodies against the other proteins (Fig. 4*C*, *lanes 6–8*) and control IgG (Fig. 4*C*, *lane 9*) failed to inhibit the supershift (Fig. 4*B*).

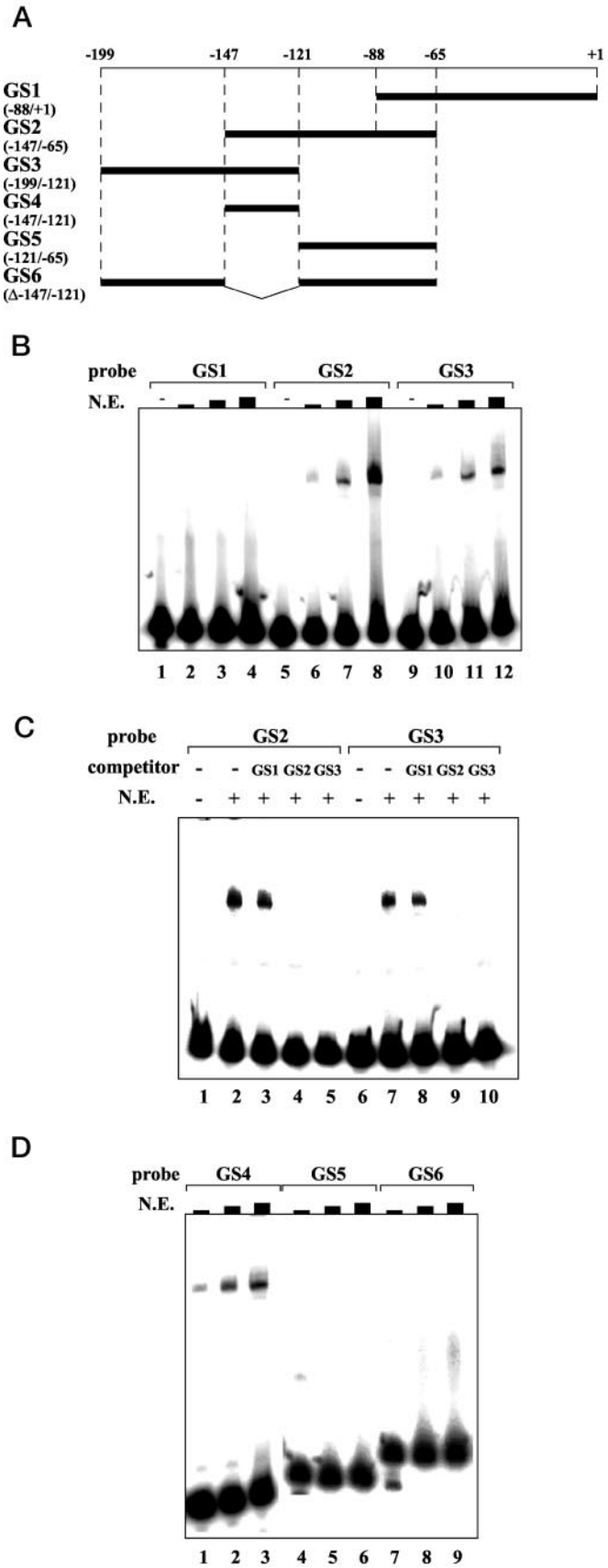

FIG. 2. **EMSA analysis of nuclear proteins in the proximal *COL11A1* promoter.** *A*, schematic representation of the probes used for EMSA. *B*, binding assay using overlapping probes in the *COL11A1* promoter. ^{32}P-labeled GS1 (*lanes 1–4*), GS2 (*lanes 5–8*), and GS3 (*lanes 9–12*) were incubated with nuclear extracts from A204 cells and separated on a 4.5% nondenaturing polyacrylamide gel. *C*, interference assays using an excess of competitor oligonucleotide. ^{32}P-labeled GS2 (*lanes 1–5*) and GS3 (*lanes 6–10*) were incubated with nuclear extracts from A204 cells in the presence of competitors, GS1 (*lanes 3* and *8*), GS2 (*lanes 4* and *9*), and GS3 (*lanes 5* and *10*). *D*, binding assays using dissected probes in the proximal *COL11A1* promoter. ^{32}P-labeled GS4 (*lanes 1–3*), GS5 (*lanes 4–6*), and GS6 (*lanes 7–9*) were incubated with nuclear extracts from A204 cells. *N.E.*, nuclear extracts.

CBF/NF-Y Binds to the COL11A1 Proximal Promoter both in Vitro and in Vivo—To examine whether CBF/NF-Y binds directly to the *COL11A1* proximal promoter, we performed two *in vitro* and *in vivo* binding experiments. For the *in vitro* binding analysis, four tandem copies of the −147 to −121 GS4 oligonucleotide were generated and immobilized on latex beads. These were then used for the purification of binding proteins from A204 cell nuclear extracts, and the purified protein was subsequently used in EMSA analyses. As shown in Fig. 5, the ^{32}P-labeled GS4 probe bound to the affinity-purified proteins (*lane 4*) at levels similar to those of the crude extracts (*lane 1*), and the bands were supershifted by anti-CBF-A/NF-YB (*lane 6*), CBF-B/NF-YA (*lane 7*), and CBF-C/NF-YC (*lane 8*) antibodies. Furthermore, the affinity-purified proteins strongly bound to the CBF/NF-Y consensus oligonucleotides (*lane 9*) and were clearly supershifted by anti-CBF/NF-Y antibodies (*lanes 10–12*). Next, we performed a chromatin immunoprecipitation analysis to examine whether CBF/NF-Y binds to the *COL11A1* proximal promoter *in vivo*. Protein-DNA complexes were immunoprecipitated with antibodies, the cross-links reversed, and the recovered DNA fragments were monitored by PCR using primers for the −291 to +1 region of the *COL11A1* promoter. DNA fragments immunoprecipitated with polyclonal antibodies against CBF/NF-Y could be amplified by PCR using the indicated primers (Fig. 6, *lanes 5* and *6*) as well as the positive control (*lane 2*), whereas those immunoprecipitated with normal goat IgG (*lane 4*) or without antibody (*lane 3*) could not. These results indicate that CBF/NF-Y binds directly to the ATTGG region in the *COL11A1* proximal promoter.

Functional Analysis of CBF/NF-Y in the Proximal COL11A1 Promoter—To confirm our findings, we carried out a functional assay of CBF/NF-Y in the proximal *COL11A1* promoter. We examined by luciferase assay whether CBF/NF-Y could function in our system as an effective transcription factor. The pGL3−199/−65 exhibited transcriptional activity at ~10% of the relative luciferase activity, compared with that of pGL3 +1/+271 (~2–4%), and the activity of two copies of the −199/−65 fragment doubled the activity (~20%) compared with that of one copy. Furthermore, pGL3(−147/−121)x4 exhibited higher promoter activity (~30%) than pGL3−199/−65 and pGL3(−199/−65)x2 constructs. A series of substituted and deleted luciferase mutation constructs were transfected into A204 cells and analyzed for luciferase activities. The activities of the constructs containing a mutated or deleted CBF/NF-Y binding site, pGL3−541E1, −541E4, CBFm, del−147/−121, and del−199/−65, were suppressed, whereas in pGL3−541E2 and -E3 constructs, the activities did not change compared with that of pGL3−541/+271. Finally, we tested the dominant-negative effect using a CBF-B/NF-YA subunit, which was overexpressed in both transient and stable transfected cells. For the transient cell experiment, the dominant-negative CBF-B/NF-YA expression vector pCXN-CBF/NF-Ymut or empty vector pCXN was cotransfected into A204 cells with pGL3(−147/−121)x4 or pGL3−199/+271 luciferase constructs. For the stable cell experiment, pGL3(−147/−121)x4 or pGL3−199/+271 luciferase construct was also cotransfected into A204 cell with a pMAM2-BSD selection vector. After selection with blasticidin, the resistant clones were isolated and used in luciferase assays as stable cell lines. Overexpression of dominant-nega-

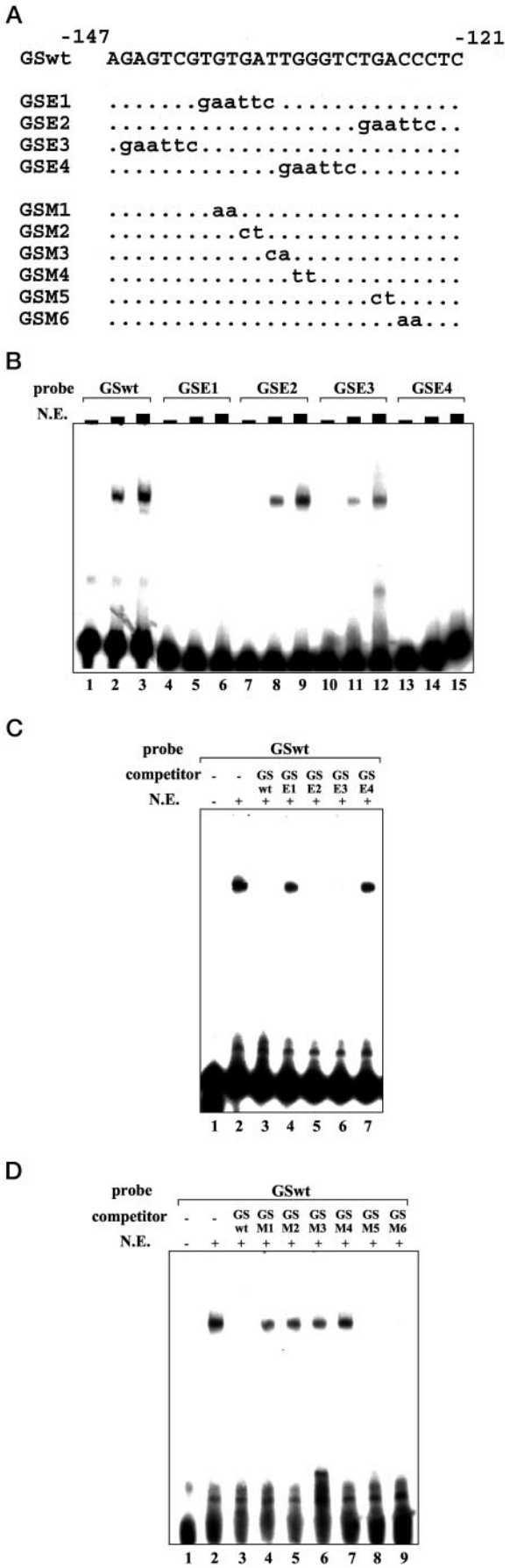

FIG. 3. **Definition of the nuclear protein-binding region in the proximal *COL11A1* promoter by EMSA.** *A*, oligonucleotide sequences of various probes used for EMSA. GSwt is the sequence from −147 to −121 in the proximal *COL11A1* promoter. In the mutated probes, identical and mutated nucleotides are indicated by *dots* and *lowercase letters*, respectively. *B*, binding assay using substituted mutation probes. ^{32}P-labeled GSwt (*lanes 1–3*), GSE1 (*lanes 4–6*), GSE2 (*lanes 7–9*), GSE3 (*lanes 10–12*), and GSE4 (*lanes 13–15*) were incubated with nuclear extracts from A204 cells and separated on a 4.5% nondenaturing polyacrylamide gel. *C*, interference assays using 6 single bp substituted mutation probes as competitors. ^{32}P-labeled GSwt was incubated with nuclear extracts from A204 cells in the presence of competitors, GSwt (*lane 3*), GSE1 (*lane 4*), GSE2 (*lane 5*), GSE3 (*lane 6*), and GSE4 (*lane 7*). *D*, interference assays using 2 single bp substituted mutation probes as competitors. ^{32}P-labeled GSwt was incubated with nuclear extract from A204 cells in the presence of competitors GSwt (*lane 3*) and GSM1-6 (*lane 4–9*).

tive CBF-B/NF-YA significantly suppressed the proximal *COL11A1* promoter activities in both transient (Fig. 7*C*, *top*) and stable cells (Fig. 7*C*, *bottom*). From these results, it was concluded that CBF/NF-Y binds directly to the proximal *COL11A1* promoter and activates the basal transcriptional activity.

DISCUSSION

To understand the transcriptional regulation of the human α1(XI) collagen gene (*COL11A1*), previous studies have characterized the structure and function of the −541 to −199 segment of the promoter (17, 18). In this study, we further characterized the proximal promoter region corresponding to a downstream region, the −199 to +271 segment, by transfection assay using a luciferase reporter gene and EMSA. We demonstrated that the segment spanning from −199 to +1 was necessary for the basal transcriptional activity of *COL11A1* (Fig. 1) and also demonstrated that the ATTGG sequence, an inverted CCAAT motif, was a critical binding site for nuclear proteins (Figs. 2 and 3). This pentanucleotide sequence is present in ~30% of eukaryotic promoters, and several CCAAT-binding proteins have been reported, including c/EBP, CTF/NF-I, CDP (CCAAT displacement protein), and CBF/NF-Y. Among these proteins, we showed that CBF/NF-Y bound to the ATTGG sequence in the proximal promoter region of *COL11A1* gene by EMSA using consensus oligonucleotides and specific antibodies (Fig. 4), and demonstrated direct binding to this region both *in vitro* and *in vivo* (Figs. 5 and 6). Furthermore, functional analysis by transfection with luciferase constructs showed that an increase in CBF/NF-Y binding produced high transcriptional activity, whereas the lack of binding activity, and the overexpression of a dominant-negative form, decreased the activity (Fig. 7). These results indicate that CBF/NF-Y plays an important role in the transcriptional regulation of the proximal promoter in the human *COL11A1* gene.

In collagen genes, CBF/NF-Y binds to the ATTGG sequence located between −100 and −96 in the α1(I), and between −84 and −80 in the α2(I) promoter, and stimulates their basal transcriptional activities, but it is not responsible for the transcriptional regulation of other collagen genes (28, 29). It is plausible that the structures and mechanisms on both proximal promoters are very similar, because this molecule is composed of a large number of heterotrimeric $[\alpha1(I)]_2\alpha2(I)$ or a small number of homotrimeric $[\alpha1(I)]_3$ molecules. It is also the most abundant and is widely expressed in vertebrate tissues, except for cartilaginous tissues. In fact, both α1(I) and α2(I) proximal promoters have a TATA box and a CCAAT box, and other putative transcription factors, such as c-krox, BFCOL1, IF-1 and Sp1, in addition to CBF/NF-F, are regulated on both proximal promoters (30). However, other major fibrillar collagens, such as the α1(II) and α1(III) genes, have no CCAAT box in the presence of a TATA box; thus CBF/NF-Y is not necessary for their transcriptional regulation (31–33). The expression pattern of type II collagen is clearly distinguished from that of type

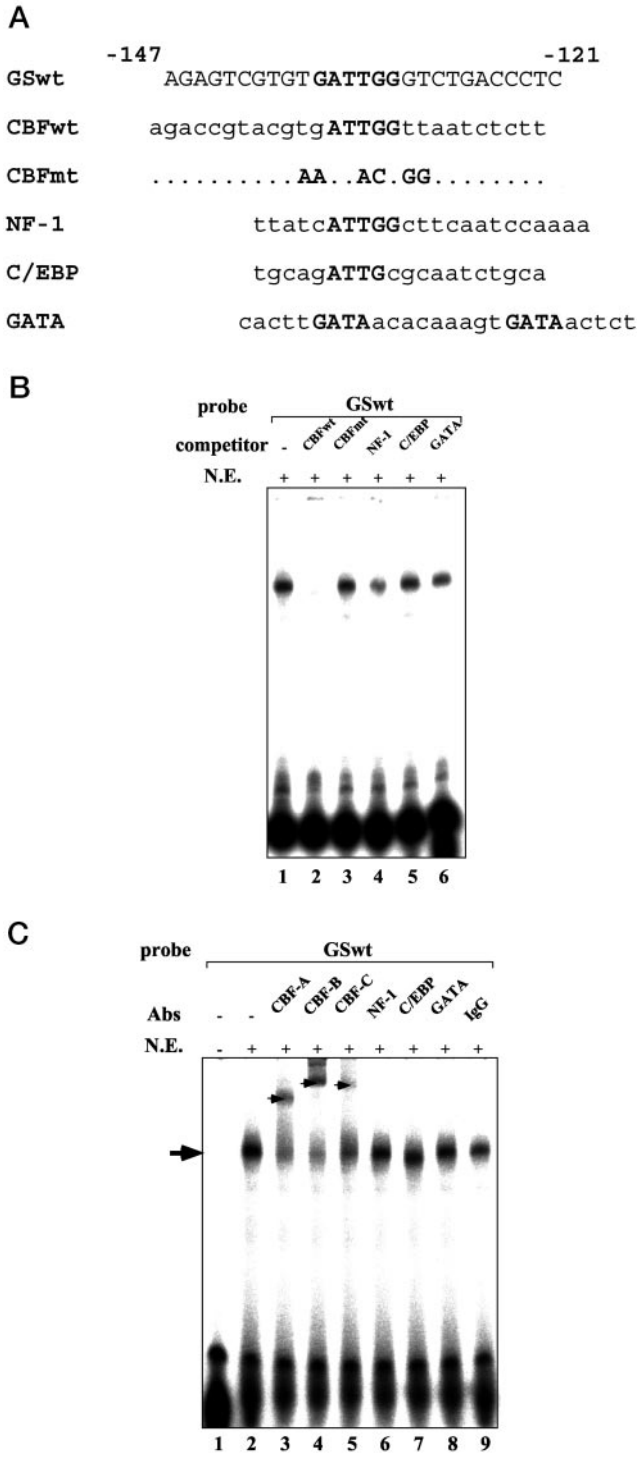

FIG. 4. **Identification of DNA-binding proteins in the proximal *COL11A1* promoter by EMSA.** *A*, oligonucleotide sequences of various consensus probes used for EMSA. GSwt is the sequence from −147 to −121 in the proximal *COL11A1* promoter and CBF, NF-1, C/EBP, and GATA consensus sequences are based on the oligonucleotide data sheets of Santa Cruz Biotechnology Inc. For CBFmt consensus sequences, identical and mutated nucleotides are indicated by *dots* and *underlines*, respectively. *B*, interference assays using various consensus oligonucleotides as competitors. ^{32}P-labeled GSwt was incubated with nuclear extracts from A204 cells in the presence of competitors, CBFwt (*lane 2*), CBFmt (*lane 3*), NF-1 (*lane 4*), C/EBP (*lane 5*), and GATA (*lane 6*). *C*, interference assays using specific antibodies against various transcription factors. ^{32}P-labeled GSwt was incubated with nuclear extracts from A204 cells in the presence of various antibodies, anti-CBF-A/NF-YB (*lane 3*), anti-CBF-B/NF-YA (*lane 4*), anti-CBF-C/NF-YC (*lane 5*), anti-NF-1 (*lane 6*), anti-C/EBP (*lane 7*), anti-GATA1 (*lane 8*) and control IgG (*lane 9*). *Arrowhead* and *arrows* indicate the DNA-protein complexes and the supershifted bands, respectively.

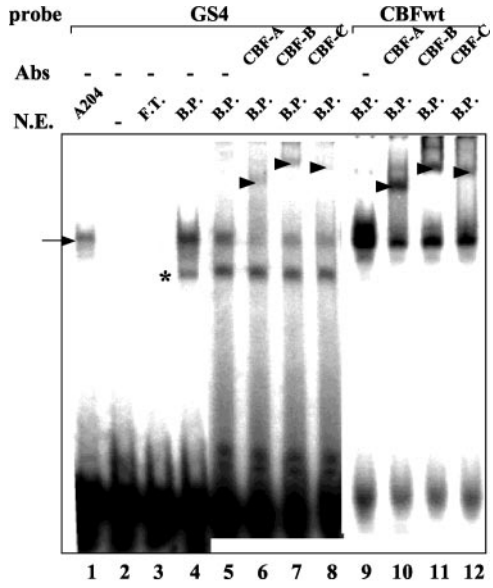

FIG. 5. **Affinity-purified proteins bind to CBF/NF-Y consensus oligonucleotides and anti-CBF/NF-Y antibodies, *in vitro*.** Four tandem copies of the −147 to −121 fragment were immobilized on latex beads, and used to purify DNA-binding proteins from A204 crude nuclear extracts. ^{32}P-labeled GS4 (*lanes 1–8*) and CBFwt consensus oligonucleotides (*lanes 9–12*) were incubated with A204 crude nuclear extract (*lane 1*), without nuclear extract (*lane 2*), flow-through fraction (*lane 3*), or affinity-purified fraction (*lanes 4–12*) in the presence of anti-CBF-A/NF-YB (*lanes 6* and *10*), anti-CBF-B/NF-YA (*lanes 7* and *11*), and anti-CBF-C/NF-YC (*lanes 8* and *12*) antibodies. *Arrows*, *arrowheads*, and an *asterisk* indicate the DNA-protein complexes, the supershifted bands, and the nonspecific bands, respectively. *A204*, crude A204 nuclear extracts; *F.T.*, 0.1 M KCl flow-through fraction; *B.P.*, 0.4 M KCl-eluted binding proteins.

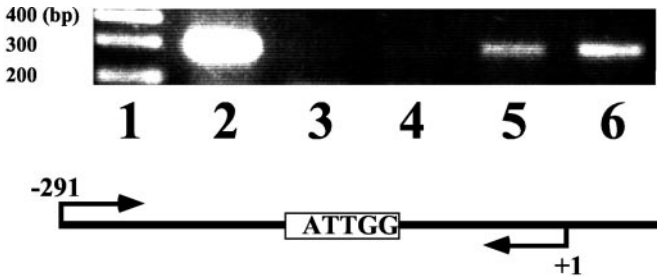

FIG. 6. **CBF/NF-Y binds the ATTGG region in the *COL11A1* proximal promoter, *in vivo*.** Chromatin immunoprecipitation analysis was performed to monitor the binding of CBF/NF-Y to the *COL11A1* proximal promoter, *in vivo*. Protein-DNA complexes were incubated with polyclonal antibodies against CBF-A/NF-YB, CBF-B/NF-YA, and CBF-C/NF-YC, and isolated by immunoprecipitation (*lanes 5* and *6*). Positive control was prepared before immunoprecipitation (*lane 2*) and negative controls were isolated by immunoprecipitation with normal goat IgG (*lane 4*) or without antibody (*lane 3*), respectively. All immunoprecipitated DNA fragments were analyzed by PCR with the indicated primers, and a 100-bp ladder was used as a molecular weight marker (*lane 1*).

I collagen, as described previously, and it has been reported that the 5′-flanking proximal region of the α1(II) gene has a weak promoter activity, and the transcription of *COL2A1* is regulated by several enhancers or silencers (34). Although type III collagen is coordinately expressed with type I collagen in most non-cartilaginous tissues, the ratio of type I to type III collagen varies in many tissues and during development, and further analysis of α1(III) proximal promoter indicates that two distinct positive factors, other than CBF-NF-Y, regulate the transcription of type III collagen (33). These observations in major fibrillar groups suggest that the mechanisms for basal transcriptional regulation of types II and III collagen may be

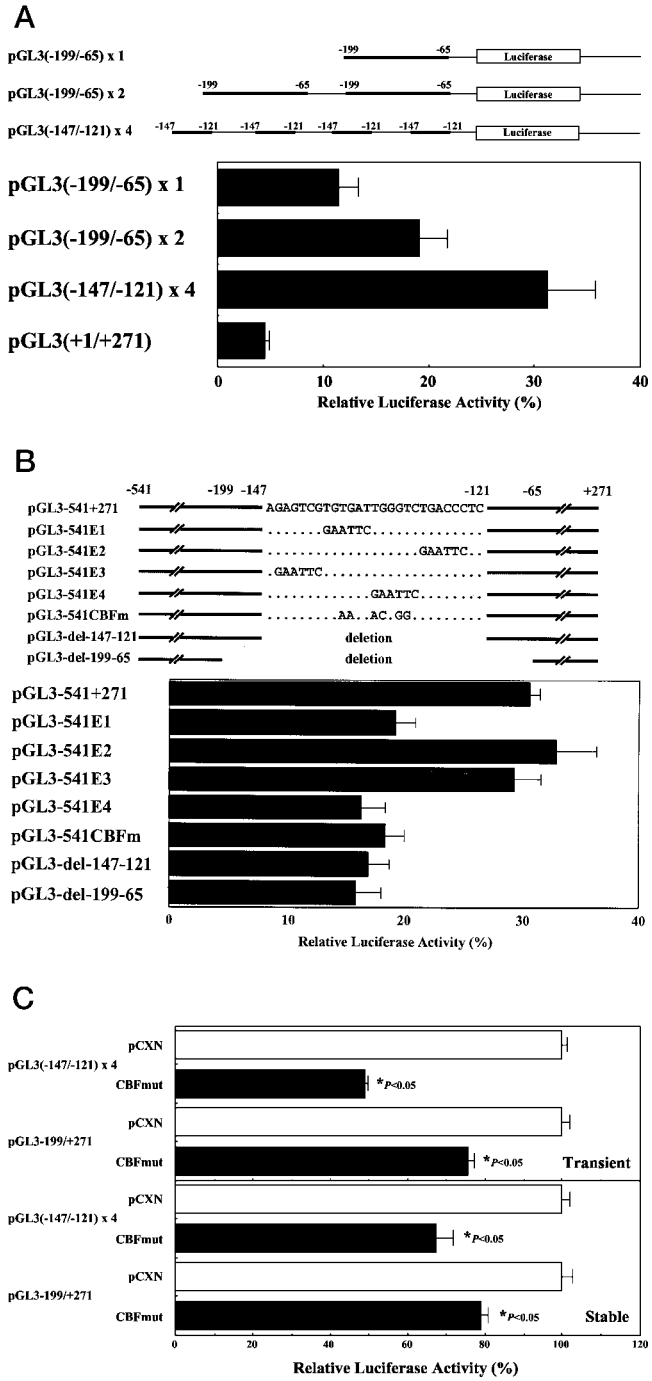

FIG. 7. **Functional analysis of CBF/NF-Y in the proximal *COL11A1* promoter.** *A*, one or two copies of −199/−65 and four tandem copies of the −147/−121 region containing the CBF/NF-Y binding site activate the luciferase activity. *B*, loss of CBF/NF-Y binding site suppresses the activity. Underline indicates CBF/NF-Y binding site. A204 cells were transfected with the constructs together with pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities (%) were normalized against the activity of a pGL3-control vector, and results are expressed as mean ± S.E. of three independent experiments. *C*, overexpression of dominant-negative CBF/NY-Y suppresses the proximal *COL11A1* promoter activity in both transient and stable cells. For the transient cell experiment (*top*), dominant-negative CBF/NF-Y expression pCXN-CBF/NFYm or empty vector pCXN was cotransfected into A204 cells with pGL3(−147/−121)x4 or pGL3(−199/+271) luciferase construct by lipofection. For the stable cell experiment (lower), pCXN-CBF/NFYm or pCXN vector was transfected into the (−147/−121)x4 cells or the −199/+271 cells by lipofection. Relative luciferase activities (percentages) were normalized against the activity of pCXN empty vector and results are expressed as mean ± S.E. of three independent experiments. *p* values were significantly different at < 0.05.

distinct from those of type I collagen genes in regard to CBF/NF-Y.

In the case of type XI collagen genes, previous studies indicated that the structure of the proximal promoter regions in the α1(XI) and α2(XI) genes exhibited the features of housekeeping genes, which have several GC boxes and no TATA box (17, 35–37). Our results first demonstrated that a functional CCAAT box exists in *COL11A1* gene by directly binding to the proximal promoter region, whereas the 5′ flanking region of *COL11A2* has no CCAAT box. In the transgenic mice experiments, the 742-bp promoter segment of *Col11a2*, located upstream of the retinoid X receptor gene, is able to direct transcription in most cartilaginous tissues (38). Further studies indicated that *Col11a2* is regulated by several cis-acting elements, the cartilage-specific element (−530 to −501), a neural tissue-specific element (−501 to −454), and a basal promoter element (39) and a Kruppel-associated box zinc-finger protein repress the cartilage-specific promoter activity of *Col11a2* (40). In addition, structural analysis of the two genes indicates that the *COL11A2* promoter region shares limited homology (<43% identity) with the *COL11A1* gene except for two homologous regions located within −93 to −49 (60%) and −301 to −285 (65%) of the *COL11A2* (40). This indicates that the functional cis-acting elements in both proximal promoters are not the same as each other, as described previously (17, 18, 39, 40).

Type XI collagens also form a heterotypic molecule that is composed of $[\alpha1(XI)]_2\alpha2(V)$ in non-cartilaginous tissues (9–14). Previous studies indicated that two cis-acting elements, Foot Printing A (−114 to −95) and FPB (−149 to −95), were responsible for the regulation of the human *COL5A2* proximal promoter (19), and the sequence of FPB in the *COL5A2* gene exhibited some homology with FP7 in *COL11A1* (17). However, the latest study demonstrated that FPB-binding proteins in the *COL5A2* gene differed from FP7-binding proteins in the *COL11A1* gene, identified as PBX, PREP, and HOX proteins (20). We preliminarily examined whether FPA-binding protein was in fact CBF/NF-Y by EMSA. However, the band of FPA complexes was different from that of CBF/NF-Y complexes, with a distinct mobility by EMSA (data not shown), thus indicating that CBF/NF-Y proteins may not be required for the basal promoter of the *COL5A2* gene. However, our recent study indicated that CBF/NF-Y is also responsible for the basal promoter activity of the α1(V) collagen gene.[2] These observations suggest that CBF/NF-Y plays an important role in the basal transcriptional regulation of some fibrillar collagens (α1(XI) and α1(V) in addition to α1(I) and α2(I), as described previously) that are expressed in non-cartilaginous tissues.

Transcriptional regulation of various eukaryotic genes requires both sequence-specific transcription factors proximal to the transcription start site and cis-acting elements at a distance to the genes (41). CBF/NF-Y was originally identified as the proteins bound to the MHC class II conserved Y box in Ea promoters (42), the yeast CYC1 UAS CCAAT box (43), and the α2(I) collagen gene (44) in the proximal promoter region. CBF/NF-Y is one of the most ubiquitous transcription factors, found in a variety of eukaryotes, and it is highly conserved throughout evolution (45). In unrelated promoters, CBF/NF-Y binds to CCAAT (40%) and ATTGG (60%) sequences in both TATA-containing and TATA-less promoters (46) and regulates on a case-by-case rather than an all-or-none basis (43–45, 47–49). We demonstrated that CBF/NF-Y bound to the −147 to −121 region but could not find any protein-DNA complex in the −199 to +271 region. These data are not consistent with the previous

[2] K. Sakata-Takatani, N. Matsuo, T. Tsuda, and H. Yoshioka, unpublished data.

experiments using DNA footprinting techniques, in which three areas, FP1 (−63 to −51), FP2 (−111 to −95), and FP3 (−171 to −159), were protected using the nuclear extracts from a primary line of smooth muscle cells, called 1120 (14). We do not know the reason for this discrepancy so far. It may be because of the different techniques or cells used or there may be some other reasons. However, other factors may also be involved in generating a basal promoter activity, because pGL3–88/+271 has still promoter activity (Fig. 1*B*).

One of the other mechanisms of transcriptional regulation is control by cis-acting elements, such as enhancers and silencers. Sox9, one of the HMG-box-containing transcription factors, binds to a 48-bp chondrocyte-specific enhancer element in the α1(II) collagen genes (50, 51) and is required for chondrocyte differentiation and for the expression of chondrocyte-specific collagen, such as α1(II), α2(IX), and α2(XI) genes (52, 53). In osteoblast differentiation and bone formations, Cbfa1/Runx2 is one of the positive regulators of type I collagen expression (54, 55). These findings suggest that the specific transcriptional mechanisms of some collagen genes, at least in part, are regulated by tissue-specific transcription factors, such as SOX9 and Cbfa1. Although the α1(XI) gene is expressed in both cartilage and bone (10, 11, 13), the tissue-specific cis-acting elements have yet to be identified. Thus, the identification of tissue-specific transcription factors deserves further study to fully understand the mechanism of transcriptional regulation in the α1(XI) collagen gene.

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