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Nuclear factor Y (NF-Y) regulates the proximal promoter activity of the mouse collagen α1(XI) gene (*Col11a1*) in chondrocytes

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Abstract Type XI collagen, a heterotrimer composed of α1(XI), α2(XI), and α3(XI), plays a critical role in cartilage formation and in skeletal morphogenesis. However, the transcriptional regulation of α1(XI) collagen gene (*Col11a1*) in chondrocyte is poorly characterized. In this study, we investigated the proximal promoter of mouse *Col11a1* gene in chondrocytes. Major transcription start site was located at −299 bp upstream of the translation start site, and the proximal promoter lacks a TATA sequence but has a high guanine–cytosine (GC) content. Cell transfection experiments demonstrated that the segment from −116 to −256 is necessary for activation of the proximal *Col11a1* promoter, and an electrophoretic mobility shift assay showed that a nuclear protein is bound to the segment from −116 to −176 in this promoter. Additional comparative and in silico analyses demonstrated that an ATTGG sequence, which is critical for binding to nuclear factor Y (NF-Y), is within the highly conserved region from −135 to −145. Interference assays using wild-type and mutant oligonucleotide or with specific antibody revealed that NF-Y protein is bound to this region. Cell transfection experiments with reporter constructs in the absence of NF-Y binding sequence exhibited the suppression of the promoter activity. Furthermore, chromatin immunoprecipitation assay demonstrated that NF-Y protein is directly bound to this region in vivo, and overexpression of dominant-negative NF-Y A mutant also inhibited the proximal promoter activity. Taken together, these results indicate that the transcription factor NF-Y regulates the proximal promoter activity of mouse *Col11a1* gene in chondrocytes.

Keywords Type XI collagen · Chondrocyte · NF-Y · Transcription · Promoter

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Introduction

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. At the present, there are more than 46 distinct α-chains of collagen genes, which form at least 28 types of collagen proteins. They can be divided into several subgroups based on their structural and functional properties (Ricard-Blum 2012). Among them, type I, II, III, V, XI, XXIV, and XXVII collagens are classified into the group of fibril-forming collagens, based on their structural and functional features. They are divided into three subgroups, major (I, II, and III), minor (V and XI), and new (XXIV and XXVII) fibrillar collagens on the basis of their contents in tissues and phylogenetic analyses.

Vertebrate fibrillar collagens are either restricted to cartilage (II, XI, and XXVII) or widely distributed in non-cartilage tissues (I, III, V, and XXIV), and the quantitatively minor collagens regulate the diameter of the major collagen fibrils by participating in fibril assembly (Exposito et al. 2010; Fang et al. 2012). Cartilage is a highly specialized tissue and serves as a template for the growth and development of bones. It

contains an extensive extracellular matrix, which includes type II, IX, and XI collagen, and provides mechanical strength to resist compression in the joint (Mendler et al. 1989).

Type XI collagen is a fibrillar collagen mainly found in cartilage and consists of three polypeptide chains: α1(XI), α2(XI), and α3(XI). The α1(XI) and α2(XI) chains are genetically distinct products, whereas the a3(XI) chain is an overgylcosylated variant of the α1(II) chain, which is encoded by type II collagen gene (Ricard-Blum 2012). Type XI collagen co-polymerizes with type II collagen within collagen fibrils and is important for the regulation of the fibril diameter and function in connective tissue (Mendler et al. 1989). Therefore, the absence and/or mutation of α1(XI) chains results in abnormally thickened cartilage collagen fibrils. For example, chondrodysplasia mice (*cho* mice), which do not synthesize α1(XI) chains, show irregular collagen fibrils in their cartilage, causing a chondrodystrophic phenotype (Li et al. 1995). In the case of human diseases, mutation in the *COL11A1* gene has been identified in the Stickler and Marshall syndromes, which are clinically characterized by altered facial appearance, eye abnormalities, joint alterations, and hearing loss (Annunen et al. 1999; Myllyharju and Kivirikko 2001). Furthermore, fibrochondrogenesis, which is short-limbed skeletal dysplasia, is also caused by mutations in the *COL11A1* gene (Tompson et al. 2010). These observations suggest that the fidelity of *Col11a1* expression is intimately involved in maintaining normal structure and function of cartilage.

Chondrogenesis is an important biological event in skeletal development and tissue formation. It starts from multipotent mesenchymal cells, and then these cells proliferate, followed by differentiating into chondrocytes. During these processes, the cells secrete chondrogenic matrices such as type II, IX, XI, and XXVII collagens around themselves. Therefore, to understand chondrocyte differentiation and cartilage formation, it is necessary to study the regulation of extracellular matrix (ECM) protein in cartilage (de Crombrugghe et al. 2000). Previous investigations also indicate that the components of ECM play important roles in maintaining the phenotype of chondrocytes and their correct organization (Mundlos and Olsen 1997a, b). Among several ECMs in cartilage, type II collagen, which is expressed in all chondroprogenitor cells and at high level in chondrocytes, is well characterized as a model gene of chondrogenic marker. Analyses of type II collagen gene revealed multiple regulatory elements targeted by the transcriptional activator and/or repressor such as Sox9 (Bell et al. 1997; Zhou et al. 1998), Sp1/3 (Ghayor et al. 2001; Renard et al. 2012), Egr-1 (Tan et al. 2003), c-Krox (Ghayor et al. 2000), and Ap2 (Niebler and Bosserhoff 2013) in both the proximal promoter and the first intron. In the type XI collagen gene, it has been reported that two chondrocyte-specific enhancer elements were identified in the proximal promoter and the first intron of the *Col11a2* gene and were regulated by Sox9 (Bridgewater et al. 1998; Liu et al. 2000). However, the transcriptional regulation of *Col11a1* gene in chondrocytes is poorly examined.

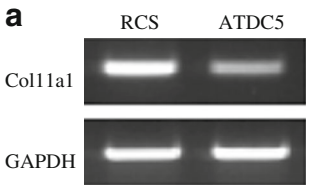
In this study, we have first characterized the proximal promoter region of mouse *Col11a1* gene in chondrocytes. Transient transfection assays revealed that the segment from −116 to −256 is necessary for the activation of basal transcription. Electrophoretic mobility shift assays (EMSAs) and chromatin immunoprecipitation (Chip) assay demonstrated that the transcription factor, nuclear factor Y (NF-Y), binds to the ATTGG sequence within the proximal promoter region in vitro and in vivo. Furthermore, cell transfection experiments in combination with DNA-binding assay demonstrated that NF-Y regulates the proximal promoter activity of *Col11a1* in chondrocytes.

Materials and Methods

Cell cultures. Rat chondrosarcoma (RCS) and mouse prechondrocyte ATDC5 cells were used in this study. RCS cells were kindly provided by Dr. Francesco Ramirez (Mount Sinai, School of Medicine, New York, NY), and ATDC5 cells were purchased from the RIKEN BRC through the National BioResource of the NEXT, Tokyo, Japan. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum at 37°C in humidified 5% CO_2 and 95% air.

RNA analysis. Total RNA was isolated from RCS and ATDC5 cells using Isogen (Nippon Gene, Tokyo, Japan) according to the manufacturer's instruction. Reverse transcriptions were carried out using ReverTra Ace reverse transcriptase (TOYOBO, Tokyo, Japan) with random and oligo(dT) primers. PCR was carried out using the single-stranded cDNA molecules and gene-specific primers. Amplification conditions included a cycle at 95°C for 2 min followed by 25–35 cycles at 95°C for 30 s, 56°C for 30 s, and 72°C for 1 min and a final elongation cycle at 72°C for 5 min. The PCR products were analyzed by electrophoresis on 1.5% agarose gel. The primers for Col11a1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: Col11a1 sense 5′-GACTACTCAGATGGCATG-3′ and antisense 5′-ACTTCCTGGTTTCTCCTT-3′ and GAPDH sense 5′-ACCACAGTCCATGCCATCAC-3′ and antisense 5′-TCCACCACCCTGTTGCTGTA-3′.

To determine the transcriptional start sites, the oligonucleotide-capping rapid amplification of cDNA ends (RACE) was performed using the GeneRacer kit according to the manufacturer's protocol (Invitrogen, Carlsbad, CA) with amplification primers corresponding to the sequences of mCol11a1R1 (5′-CTCCCTCTGACCTCTCTAG-3′) and mCol11a1R2 (5′-TGACGACTGTGAACCCAC-3′). Amplification products were subcloned into the pGEM-T Easy



b

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          ---------- ---------- ----gaggag gggggaagg
-240      agagggaaga tgaagggtgg ctggtgggct gggcctgcac
-200      agaagtctgg atcctccagg gagagactgg gcgggttcaa
-160      ggctgcgggg aggcctgtga ttgggtatga ccttcagtcg
                          CCAAT(ATTGG)box
-120      gcttgtcagt ttctcgggca cacaggaggc gggaggagtg
-80       ggcggaggag ggggctgcgc gagcctcttg tccagcccac

              1 11                                 1
              * **                                 *
-40       ccacagcatg aagcctttag gggtgctcag tgctttcggc

          8 14  11
          * **  **
+1        ttttttttctt gaaacccttc atctgccctc agtctgaagg
          cagggtcagg cgtaggacgg gtcccggggt cgcagtctcc
+81       tgcgggccac cccaaaggga gaggaggggg cgagagacag
          gaaggaagca aaccatcaaa ttggaaaaaa aaaaattaga
+161      gccctttgac tttttttttt tttactccct ttcccttccg
          gatggctgtg tagcgaacgt aggcgacacc ttggaaggga
+241      caaagttggt ctgcagtggc aatttcgtgg gttcacagtc
                                          Col11a1-R2
+281      gtgagcgtgg ggcttggagA TGGAGCCCTG GTCCAGGTGG
         AAAACGAAAC GGTGGATCTG GGATCTCACC ATCTCAACCC
         TCGCCTTGAC TTTTCTCTTC CAAGCTAGAG AGGTCAGAGG
                                      Col11a1-R1
         AGgtaaggag
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Figure 1. RNA expression and determination of the transcription start site of *Col11a1* in chondrogenic cells. (*a*) Reverse transcription PCR analysis of *Col11a1* in RCS and ATDC5 cells. The ubiquitous glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was included as the normalizing control. (*b*) Nucleotide sequence of the proximal promoter region of mouse *Col11a1* gene. The oligo-nucleotide-capping RACE products are represented by *asterisk*, and total number of each clone is indicated by *Arabic numerals*. The transcriptional start site is designed as +1 and the predicted translation start codon is at position +300. The sequence of Exon1 is shown by a *capital letter*, and the primers used in the oligo-capping RACE experiments are *underlined.*

vector (Promega, Fitchburg, WI) and sequenced on an ABI3130 sequencer (Applied Biosystems, Carlsbad, CA).

Construction of chimeric plasmids. All *Col11a1* promoter-luciferase reporter gene constructs were derived from pBACe3.6 RP24-512D13 clone. To obtain various 5′ deletion constructs, these fragments were generated by using sets of oligonucleotide primers, which are KpnI site-linked 5′- and XhoI site-linked 3′-primers specific for the *Col11a1* sequence. Amplified products were cloned into the pGEM-T Easy vector (Promega, WI), followed by digestion with KpnI and XhoI, and subcloned into the KpnI/XhoI site of pGL3-basic vector (Promega, WI).

Internal deletion and nucleotide substitutions were generated by site-directed mutagenesis as described previously (Matsuo et al. 2003).

Cell transfection and luciferase assay. The cells were plated at a density of 1.5×10^5 cells per 35-mm dish 18 h before transfection. For transient transfection, RCS and ATDC5 cells were transfected with each of plasmid DNA by using the calcium phosphate-mediated precipitation methods with some modification (Chen and Okayama 1987) or by using the Lipofectamine plus reagent system according to the manufacturer's protocol (Invitrogen, CA).

Luciferase activity was assayed 24 h later by the Dual-Luciferase™ Reporter Assay system (Promega, WI) using a luminometer (Lumat, 9507, Berthold Technologies, Chennai, India). The cotransfection experiments with a dominant-negative NF-Y A subunit expression vector were performed as previously described (Matsuo et al. 2003; Nagato et al. 2004). The pRL-TK *Renilla reniformis* luciferase expression vector was used as an internal control for transfection efficiency. The relative luciferase acitivity of each construct was compared with the shortest construct, pGL3+1+68. Results were expressed as the mean ± SE of three independent experiments and evaluated by Student's *t* test.

DNA-nuclear protein binding assays. Nuclear extracts were prepared according to Dignam et al. (1983) with some modifications.

For the EMSA, wild-type and mutant oligonucleotide probes were genetrated by PCR amplification using HindIII site-linked primers. PCR products were subcloned into the pGEM-T Easy vector (Promega, WI), cleaved with HindIII, and radiolabeled with [α-^{32}P]dCTP using Klenow enzyme. DNA-nuclear protein binding reaction was carried out according to the published protocols (Matsuo et al. 2003, 2006). For competitors and antibody interference assays, unlabeled probes or antibodies were added to the reaction mixture and incubated for 1 h at 4°C before the addition of labeled probe. The anti-NF-Y A antibody (C-18) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

The DNA–protein complexes were separated in a 4.5% nondenaturing polyacrylamide gel in 0.25% TBE (89 mM Tris–borate, 2 mM EDTA) buffer and visualized by autoradiography using Bio Imaging Analyzer FLA-7000 (GE Healthcare, Buckinghamshire, UK). Chip assays were performed using a chromatin immunoprecipitation assay kit (Millipore, Burlington, MA) according to the published protocols. Quantitative PCR was carried out for 35 cycles using 5 μl of reaction, and amplification products were separated in 2% agarose gel in 1× TAE (40 mM Tris–acetate, 1 mM EDTA) buffer.

Results

RNA expression and determination of the transcriptional start site of mouse Col11a1 gene in chondrogenic cells. Initially, we examined the expression of *Col11a1* transcript in cultured

chondrogenic cells using the RT-PCR analysis. In this study, we used rat RCS and mouse ATDC5 cell lines, which represent late (mature) and early (immature) stage of chondrocyte differentiation, respectively. As shown in Fig. 1*a*, the amplified PCR products were observed in both cells, and the level of Col11a1 expression in RCS cells was higher than in ATDC5 cells.

Next, to determine transcriptional start sites of mouse *Col11a1* gene in chondrogenic cells, oligo-nucleotide-capping RACE experiment was carried out using total RNA derived from mouse ATDC cells. Twenty independent RACE products were cloned and sequenced in this study. As shown in Fig. 1*a*, the transcriptional start sites of mouse *Col11a1* gene were broadly distributed approximately 300 bp upstream (−292 to −335) from the ATG translation initiation codon. Among them, 8 of the 20 RACE products were generated from the same position, which suggested that the major transcription start site is located 299 nucleotides immediately upstream of the ATG codon. This gene has no canonical TATA sequence and has a high GC content (Fig. 1*b*).

Deletion analysis of the proximal Col11a1 promoter in mouse chondrocytes. To delineate the proximal regulatory regions in the mouse *Col11a1* promoter in chondrogenic cells, a series of chimeric constructs containing 5′-end deletions linked to the luciferase gene were generated as shown in Fig. 2*a*, and then, luciferase assays were carried out. The promoter activity of each construct was compared with the shortest construct, pGL+1+68.

As shown in Fig. 2*b*, the longest construct, pGL−551+68, had strong transcriptional activity, and deletion from nucleotides −366 to −256 bp had no significant reduction in the luciferase activity. However, deletion of nucleotides between −256 and −116 bp led to a dramatic reduction (65–75%) in the transcriptional activity compared with pGL−256+68, and further deletion of nucleotides +1 (pGL+1+68) had slightly reduced the luciferase activity. These results indicated that the region between −116 and −256 bp was important for proximal transcriptional activity of the mouse Col11a1 promoter in chondrogenic cells.

Determination of nuclear protein binding site and identification of DNA-binding protein in the proximal Col11a1 promoter. To identify nuclear protein binding site on the proximal promoter region of *Col11a1*, we prepared two overlapping probes (G1, −117−196, and G2, −177−256) covering the proximal promoter region from −117 to −256 and carried out an EMSA. As shown in Fig. 3, ^{32}P-labeled G1 probe-bound nuclear protein was extracted from the RCS cells in a dose-dependent manner; however, G2 probe did not bind the DNA–protein complexes. This result indicated that a 60-bp sequence from −117 to 176 in the proximal promoter region is necessary to bind nuclear proteins.

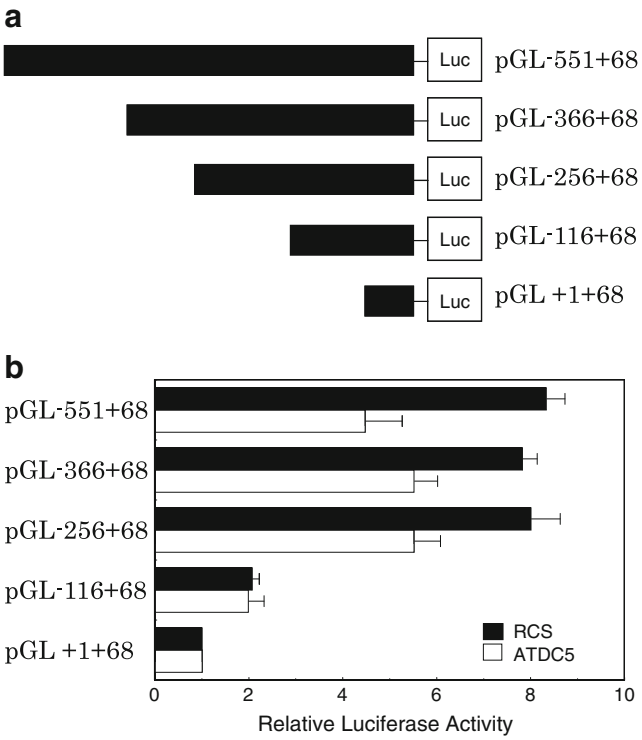

Figure 2. Determination of the proximal promoter sequence of mouse *Col11a1* gene. (*a*) Schematic representation of the deletion constructs of mouse *Col11a1* promoter. (*b*) 5′-Deletion analysis of mouse *Col11a1* promoter. A series of mouse *Col11a1* promoter-luciferase constructs were transfected into the cells with the pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities were normalized against the activity of the pGL+1+68 vector, and the results are expressed as mean + SE of five independent experiments.

Next, we carried out the comparative sequence analysis of mouse *Col11a1* proximal promoter region with rat and human genes and then performed in silico analysis using TFSEARCH (www.cbrc.jp/research/db/TFSEARCH) and Genomatix (www.genomatix.de) to identify putative transcription factor binding sites.

As shown in Fig. 4*a*, the nucleotide sequence between mouse and rat gene is very similar (97%, rat vs mouse), while that of human gene is different (70%, human vs mouse). Most highly conserved region is 11 bp nucleotide sequence located at −135 to −145 (underlined). Furthermore, two in silico analyses indicated that this conserved region has a CCAAT (ATTGG sequence) box which is a potential binding site for the transcription factor NF-Y (as shown in Fig.1*b*). We therefore focused on the characterization of NF-Y in the proximal *Col11a1* promoter.

To examine whether NF-Y binds to this region, we performed an interference assay using wild-type or mutant oligonucleotide. An excess of the wild-type (NF-Y wt) probe inhibited the binding of the ^{32}-P-labeled G1 probe (Fig. 4*b*, lane 3), whereas the mutant (NF-Y mut) probe failed to interfere with DNA–protein complex formation (Fig. 4*b*, lane 4). To further characterize the binding protein, another

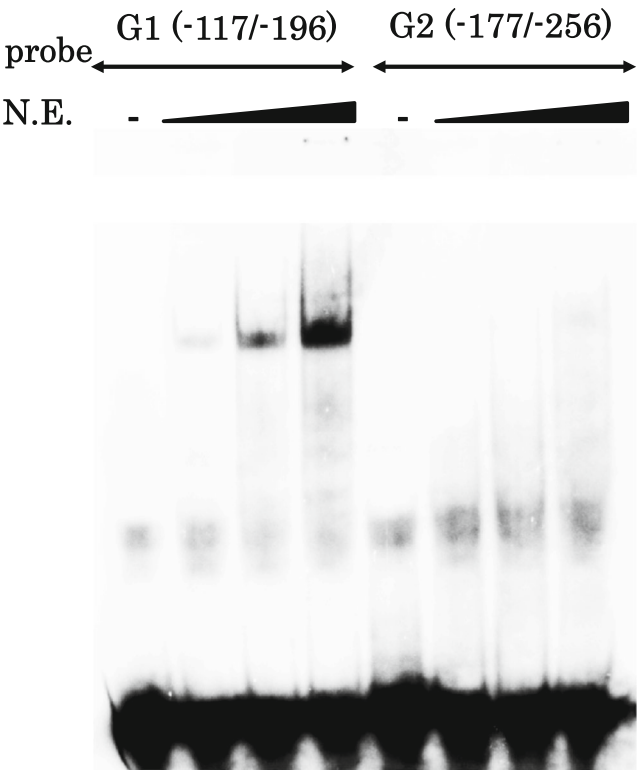

Figure 3. Identification of nuclear protein binding region in mouse *Col11a1* proximal promoter. EMSA analysis using overlapping probe in the *Col11a1* proximal promoter. ^{32}P-labeled G1 (−117 to −196) and G2 (−177 to −256) were incubated with nuclear extract from RCS cells and separated on a 4.5% nondenaturing polyacrylamide gel.

interference assay using specific antibody against NF-Y was carried out. As shown in Fig.4*b* (lane 6), the DNA–protein complex was supershifted by anti-NF-Y antibody, whereas control IgG failed to supershift the DNA–protein complex (Fig. 4*b*, lane 5).

Finally, we performed Chip assays to clarify whether NF-Y binds to the mouse *Col11a1* proximal promoter in vivo. As shown in Fig. 4*c*, DNA fragments immunoprecipitated with NF-Y antibody could be amplified by PCR with a set of P1/P2 primers as well as input, whereas those immunoprecipitated with normal IgG could not. Consistent with the EMSA data, Chip assay also provided evidence that the transcriptional factor NF-Y directly binds to the ATTGG sequence in the proximal promoter of mouse *Col11a1* gene.

Functional analysis of NF-Y in the proximal Col11a1 promoter. To confirm whether NF-Y regulates the proximal promoter activity of mouse *Col11a1* gene in chondrocytes, we carried out cell transfection experiments using internal deletion and/or mutation luciferase constructs. As shown in Fig. 5*a*, both internal deletion and mutation constructs had significantly reduced the luciferase activity (approximately 40–60%). Consistent with these data, overexpression of dominant-negative NF-Y A mutant subunit also suppressed the proximal promoter activity in both cells (Fig. 5*b*). Taken together, our data indicate that the transcription factor NF-Y regulates the proximal promoter activity of mouse *Col11a1* gene in chondrocytes.

Discussion

To understand the transcriptional regulation of mouse a1(XI) collagen gene (*Col11a1*) in chondrocytes, we first identified the transcriptional start site by oligo-capping RACE experiments. As shown in Fig. 1*b*, this promoter has no canonical TATA motif and a high (GC) content, resulting in multiple transcriptional start sites of mouse *Col11a1* gene. These structural properties in the proximal promoter are almost identical to those of the other minor fibrillar collagen genes, except for *Col5a2* (Fang et al. 2012). Consistent with these properties, molecular phylogenetic analyses revealed that vertebrate fibrillar collagens are divied in the three independent clades: the type A clade consists of α1(I), α2(I), α1(II), and α2(V), the type B clade contains minor collagens, α1(V), α3(V), α1(XI), and α2(XI), with the exception of α2(V), and the type C clade consists of newly identified collagens, α1(XXIV) and α1(XXVII) (Wada et al. 2006). These observations suggest that the proximal promoter and the exon structures of minor collagen genes are evolutionally conserved.

Cell transfection experiments in combination with DNA-binding assays demonstrate that mouse *Col11a1* gene has an ATTGG sequence (a CCAAT box) in the proximal promoter region, and NF-Y regulates the proximal promoter activity of *Col11a1* gene in chondrocytes. NF-Y is a heterometric transcription factor composed of three subunits, NF-Y A, NF-Y B, and NF-Y C, and is specifically binds to the CCAAT (or ATTGG) motif. This transcription factor is able to regulate a wide range of genes, both ubiquitous and/or tissue-specific genes (Mantovani 1999). In fibrillar collagen genes, the previous reports demonstrated that NF-Y regulates the proximal promoter activity of α1(I), α2(I), α1(V), and α3(V) genes in fibroblasts and/or osteoblasts, which are widely distributed in non-cartilage tissues (Collins et al. 1997; Lindahl et al. 2002; Sakata-Takatani et al. 2004; Wu et al. 2010). Furthermore, we previously reported that NF-Y regulates the proximal promoter activity in the human *COL11a1* gene in non-cartilage cells (Matsuo et al. 2003). On the other hand, α1(II) and α2(XI) genes, which are restrictedly expressed in cartilage, have no CCAAT motif in these promoter, and therefore, NF-Y is not necessary for their transcriptional regulation in chondrocytes. However, this study showed that NF-Y also regulates the proximal promoter activity of mouse *Col11a1* gene in chondrocytes. These results indicate that NF-Y regulates the proximal *Col11a1* promoter activity in both cartilage and non-

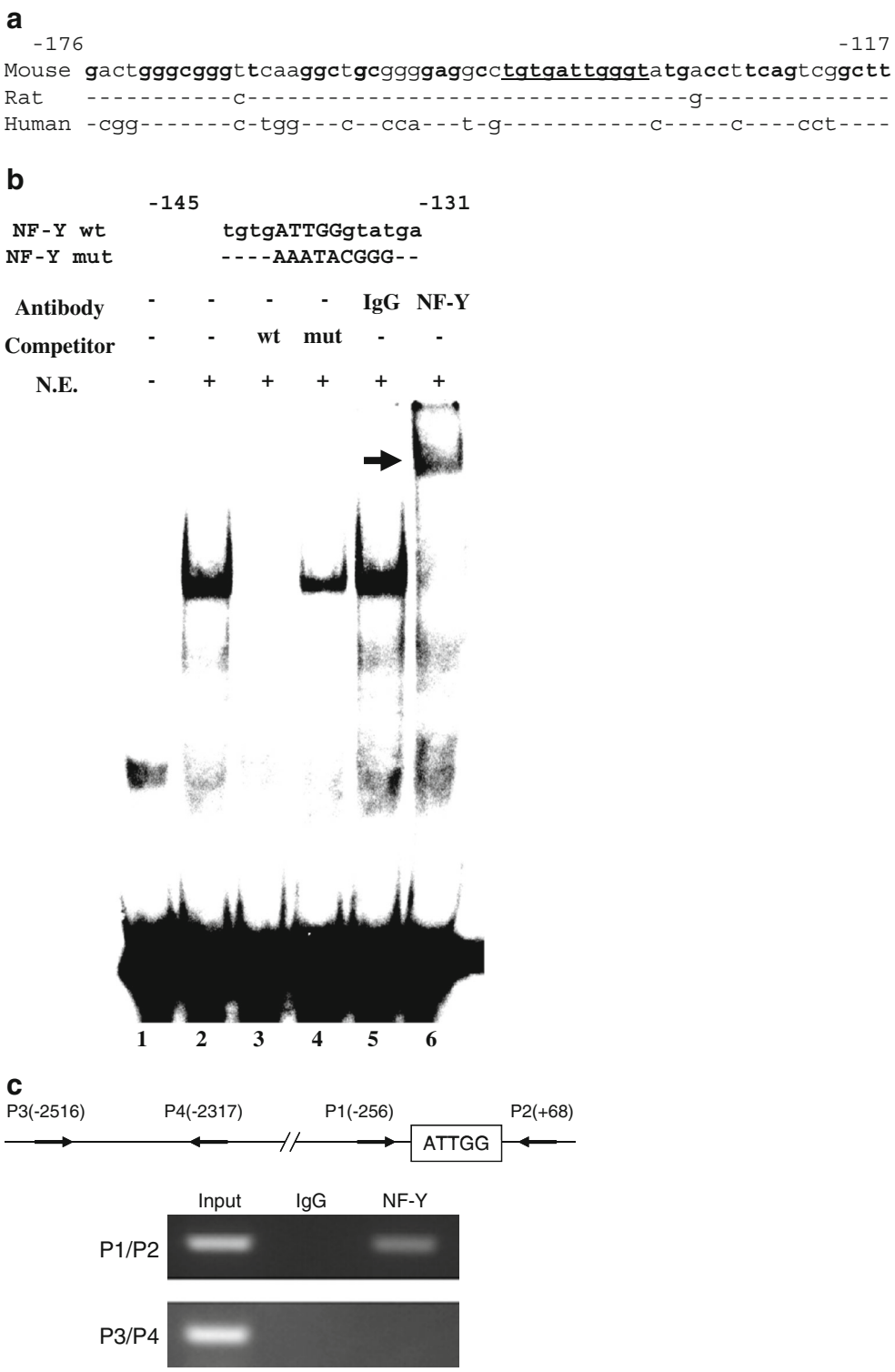

Figure 4. Identification of DNA-binding protein in mouse *Col11a1* proximal promoter. (*a*) Comparative analysis of mouse *Col11a1* proximal promoter sequence (−176 to −117) with rat and human gene. *Bold letter* represents conservation in all species, and most highly conserved sequence is *underlined* (11 bp, −145 to −135). This region has a CCAAT (ATTGG) box as shown in Fig.1*b*. (*b*) Interference assays using consensus oligonucleotide and specific antibody. ^{32}-P labeled G1 probe was incubated with nuclear extract from RCS cells in the presence of competitors, NF-Y wt (*lane 3*) or NF-Y mut (*lane 4*), and in the presence of antibody, anti-NF-Y (*lane 6*) or control IgG (*lane 5*). *Arrow* indicates the supershifted band. (*c*) Chip analysis of the relevant *Col11a1* proximal promoter sequence using NF-Y antibody. Positive control is input and negative control is normal IgG, respectively. All immunoprecipitated DNA fragments were analyed by PCR with the indicated primers. The P1/P2 primers are located in the relevant proximal promoter of mouse *Col11a1* gene, and P3/P4 primers are located approximately 2.0 kb upstream region as negative control, respectively.

cartilage tissues. As compared to other fibrillar collagen genes, the regulation of α1(XI) collagen gene might be complex than expected because the expression pattern of α1(XI) collagen gene is more broad than others, in both cartilaginous and non-cartilaginous tissues. Additional findings of NF-Y have been reported that two CCAAT boxes were identified in

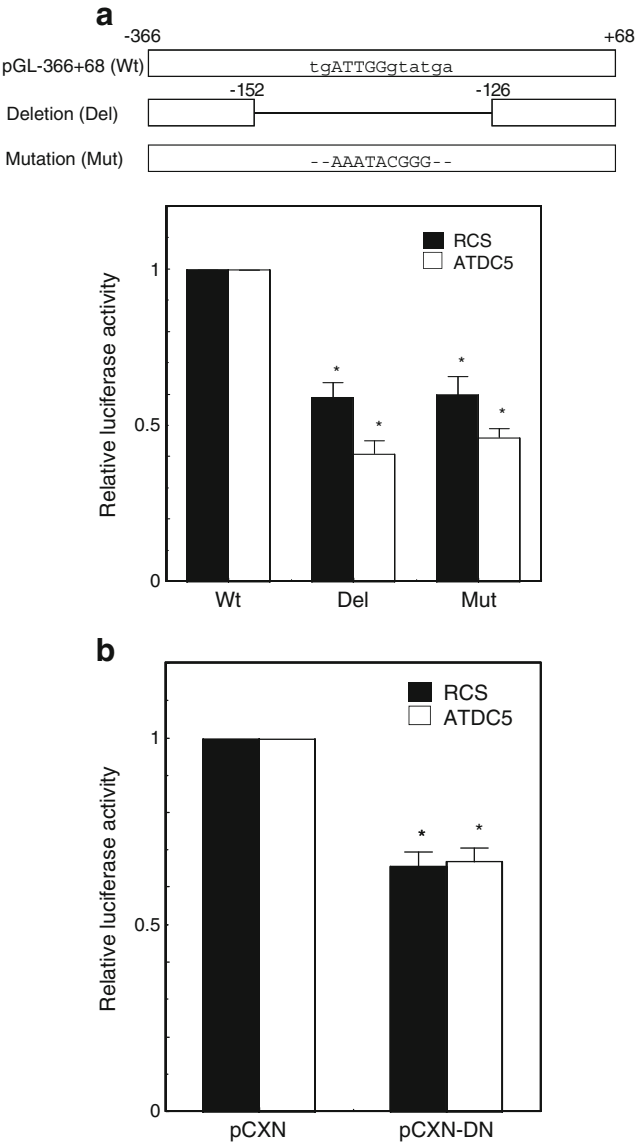

Figure 5. Functional analysis of NY-F in the proximal *Col11a1* promoter. (*a*) Deletion and/or mutation analysis of the mouse *Col11a1* proximal promoter. Deletion and/or mutation luciferase constructs were transfected into the cells with the pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities were normalized against of Wt (pGL−366+68) vector, and the results are expressed as mean + SE of five independent experiments. $p<0.01$ compared with pGL−366+68 (Wt). (*b*) Effect of overexpression of dominant-negative NF-Y on the mouse *Col11a1* promoter activity. Dominant-negative NF-Y expression vector (*pCXN-DN*) or pCXN empty vector was cotransfected into the cells with pGL−366+68 luciferase construct. Relative luciferase activities were normalized against the activity of pCXN empty vector, and the results are expressed as mean + SE of five independent experiments. $p<0.01$ compared with pCXN.

the human and mouse *Sox9* promoters, and NF-Y regulates the proximal promoter activity of *Sox9* gene through its interaction with the CCAAT boxes (Colter et al. 2005). Furthermore, NF-Y B affects the expression of *Col2a1*, *Sox9*, *Runx2*, and *Dlx2* to promote cartilage development in zebrafish (Chen et al. 2009). These studies suggest that NF-Y may regulate, at least in part, chondrocyte differentiation, cartilage formation, and skeletal development in addition to the regulation of collagen gene expression.

One of other mechanisms of the transcriptional regulation is controlled by *cis*-acting element, such as enhancers and silencers. In cartilage, Sox9, one of the HMG-box containing transcription factors, is essential for chondrocyte differentiation and skeletal development (Akiyama 2008). As described previously, cartilage collagens are composed of type II, IX, and XI collagens. In the α1(II) gene, it has been reported that a 48-bp chondrocyte-specific enhancer element is identified, and the expression of *Col2a1* gene is regulated by Sox9 (Bell et al. 1997; Zhou et al. 1998). The α2(XI) gene has two chondrocyte-specific enhancer elements in the proximal promoter and the first intron and is regulated by Sox9 as the *Col2a1* gene (Bridgewater et al. 1998; Liu et al. 2000). Type IX collagen gene also has Sox9 binding elements in the proximal promoter, resulting in the activation of the proximal promoter by Sox9 (Zhang et al. 2003; Genzer and Bridgewater 2007). On the other hand, there is no direct evidence that Sox9 regulates the expression of α1(XI) collagen gene. However, Lincoln et al. have shown that Sox9 is required for heart valve development as well as *Col11a1* and *Col5a1*, suggesting that it is possible that Sox9 may regulate the expression of *Col11a1* and *Col5a1* genes (Lincoln et al. 2006, 2007). Recently, it has been reported that lymphocyte enhancer-binding factor 1 indirectly activates the proximal promoter *Col1a1* gene in osteoblasts and suppressed terminal osteoblast differentiation (Kahler et al. 2008). However, the chondrocyte-specific *cis*-acting elements have not been identified in α1(XI) gene yet. Thus, further study on the identification of tissue-specific transcription factor would clarity the mechanism of transcriptional regulation in the α1(XI) collagen gene.

In conclusion, we first characterized the proximal promoter region of mouse *Col11a1* gene in chondrocytes. Our results demonstrated that NF-Y was directly bound to this region and regulated the proximal promoter activity of mouse *Col11a1* gene in chondrocytes.

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