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The Transcription Factor CCAAT-binding Factor CBF/NF-Y and Two Repressors Regulate the Core Promoter of the Human Pro-α3(V) Collagen Gene (*COL5A3*)*[S]

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To elucidate the mechanisms underlining α3(V) collagen chain expression, we performed an initial analysis of the structure and function of the core promoter of the human *COL5A3* gene. The core promoter, which lacks a typical TATA motif and has a high GC content, was defined within the −129 bp immediately upstream from the major transcription start site by transient transfection experiments. In this region, we identified four DNA-protein complexes, named A, B, C, and D, by a combination of DNase I footprinting and electrophoretic mobility shift assays. Electrophoretic mobility shift assays using mutant oligonucleotide revealed that the complexes A, B, C, and D bind to −122 to −117, the −101 to −96, the −83 to −78, and the −68 to −57 bp, respectively. The competition assays using consensus oligonucleotides and supershift assays with specific antibodies showed that complex A consists of CBF/NF-Y. In a chromatin immunoprecipitation assay, CBF/NF-Y protein directly bound to this region, *in vivo*. Functional analysis showed that CBF/NF-Y activated the gene, whereas the proteins of complexes B and C repressed its activity. Furthermore, overexpression of a mutant form of the CBF-B/NF-YA subunit, which forms CBF/NF-Y with CBF-A/NF-YB and CBF-C/NF-YC subunits, inhibited promoter activity.

Vertebrate collagens, a large family of extracellular proteins, are critically important for the formation and function of virtually every organ system (1). Among them, fibrillar collagen, which includes five different molecular types I, II, III, V, and XI, participates in the formation of fibrils with molecules packed in quarter-staggered arrays (2, 3). 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 (4–7). The collagen molecules are either homotrimers with α chains or heterotrimers with two or three different α chains. The predominant molecular form of type V is the heterotrimer $[\alpha1(V)]_2\alpha2(V)$ and is expressed in most tissues (8). Other forms of type V collagen include the $[\alpha1(V)]_3$ homotrimer that is synthesized in cultures of hamster lung cells (9) and the α1(V)α2(V)α3(V) heterotrimer that can be extracted from the placenta (10–12). Cross-type heterotrimer composed of α2(V) and α1(XI) chains is present in the rhabdomyosarcoma cell line A204 and bovine vitreous (13, 14). These findings suggest that type V and type XI chains constitute a single collagen type in which different combinations of chains associate in a tissue-specific manner. Recently, a fourth chain, α4(V), expressed in rat Schwann cells was reported (15). This α chain, which can form molecules with α1(V) and α2(V) chains, seems to be the counterpart of the mouse and human α3(V) chain (16).

Type V collagen, which is widely expressed spatially and temporally, is expressed in the mouse embryo as early as 11 days post-coitum (17). Altered production of type V collagen is associated with some connective tissue pathology, such as inflammation, some forms of cancer, and atherosclerosis (18–20). Characterization of the *cis*-acting elements and *trans*-acting nuclear factors that modulate correct patterns of gene expression is necessary for understanding physiological and pathological conditions. Among the collagen genes, the transcriptional regulation of type I collagen has been most extensively studied, showing common features of the proximal promoter and tissue-specific enhancer (21, 22). With regard to minor collagen, Penkov *et al.* (23) identified the nuclear factors binding to the proximal promoter of *COL5A2*. Their results indicate that members of the homeotic complex and TALE class of homeoproteins, PBX1/2, PREP1, and HOXB1, were involved in regulation of *COL5A2*. B-myb, a member of the myb gene family, indirectly repressed the *COL5A2* gene promoter by interacting with the positive factor that binds to the first exon (24). Lee and Greenspan (25) suggested that the GAGA boxes in the promoter and the first exon of the *COL5A1* gene affect the transcription. We have recently characterized the proximal promoter of the *COL11A1* (26) and *Col5a1* genes (27). In these studies, we demonstrated that the CCAAT binding factor, CBF/NF-Y, bound to the CCAAT motif in the proximal promoter and is involved in activation of the genes.

Recently, human and mouse full-length pro-α3(V) sequences were provided (16). These were shown to be closely related to the pro-α1(V) chain. *In situ* hybridization of mouse embryo detected its expression in epimysial sheaths of developing muscles and within nascent ligaments adjacent to forming bones and in joints, where it probably forms molecules with α1(V) and α2(V). In the present study, we cloned the human promoter DNA fragment and characterized the core promoter of the

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[S] The on-line version of this article (available at http://www.jbc.org) contains Table S1.

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α3(V) collagen gene. DNase I footprinting and DNA binding assays have demonstrated four nuclear protein binding sites in the promoter region. Cell transfection experiments showed that one works as an activator and two as repressors. Finally, functional analysis combined with oligonucleotide competition and supershift assays showed that CBF/NF-Y acts as a transcriptional activator of *COL5A3*.

MATERIALS AND METHODS

Cells and Cell Culture Conditions—Most of the cell lines used in this study were purchased from the American Type Culture Collection (ATCC) and the Japanese Collection of Research Bioresources (JCRB) cell banks. A204 cells (human embryonal rhabdomyosarcoma cell line, ATCC HTB-82), HT-1080 cells (human fibrosarcoma cell line, ATCC CCL-121), U251MG cells (human glioblastoma cell line, JCRB, IFO50288), IMR32 cells (human neuroblastoma cell line, ATCC CCL-1277), and A498 cells (human epithelial cell line, ATCC HB-44) were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (Sanko Junyaku, Tokyo, Japan) at 37 °C in a 5% CO_2/air environment. Jurkat cells (human T cell line, ATCC TIB-152) and YST-1 cells, a primary cell line of human Schwannoma cells (kindly provided by Dr. Y. Nagashima, Yokohama City University School of Medicine), were cultured in RPMI 1640 medium containing 10% fetal bovine serum in the same environment.

Isolation of RNA—Total RNA was isolated from cultured cells using Isogen (Nippon Gene Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. RNA was quantified by optical density (A_{260}) and stored at −80 °C until used.

Reverse Transcription-Polymerase Chain Reaction (RT[1]-PCR)—The expression of *COL5A3* mRNA in various cell lines was investigated by RT-PCR. Three micrograms of total RNA was reverse transcribed by random hexamer priming using SuperScript II reverse transcriptase (Invitrogen). The single-stranded cDNA was amplified by PCR using *COL5A3*-specific primer pairs and glyceraldehide-3-phosphate dehydrogenase primers. The primers are listed in Supplemental Materials Table S1.

PCR was carried out for 30 cycles using a step cycle of 94 °C for 30 s, 55 °C for 20 s, 72 °C for 30 s, followed by 72 °C for 7 min. The PCR products were analyzed by electrophoresis on a 4% ethidium bromide-stained agarose gel. The amplified fragment was eluted from the gel and subcloned into the pGEM-T Easy vector (Promega) for sequencing.

Screening Human BAC Library and Cloning of Genomic DNA Fragment—A human genomic BAC library was screened to obtain the *COL5A3* genomic fragment using the PCR protocol supplied by the manufacturer (Genome System, Inc., St. Louis, MO). The primers specific for the *COL5A3* used in the PCR-based screening are listed in Supplemental Materials Table S1.

The DNA from the PCR-positive BAC clone was purified and the sequences at both ends were determined by a direct sequencing method using Sp6 and T7 primers specific for the BAC cloning vector arms. The sequences were compared with those in the EMBO/GenBank™ data base. To obtain a smaller DNA fragment that contains the promoter region of *COL5A3*, the DNA was digested with ApaI because this restriction site was located just upstream of the initiating ATG codon. The resulting fragments were subcloned into the pBluescript (SK+) vector. The sublibrary was screened by colony hybridization.

Rapid Amplification of 5′-cDNA Ends (5′-RACE)—The 5′-RACE experiment was carried out according to the method described by Frohman *et al.* (28). Briefly, 10 μg of total RNA from A204 or YST-1 cells was reverse transcribed using the *COL5A3*-specific primer, RACE1 or RACE3 (Fig. 2*C*). Homopolymeric dC was added to the 3′-end of the first strand cDNA using terminal deoxynucleotidyl transferase in the presence of dCTP. The dC-tailed cDNA was amplified with PCR using a poly(dG) containing the 5′ adapter primer and a 3′ *COL5A3*-specific primer, RACE2 or RACE4, which was located just upstream of RACE1 or RACE3, respectively (Fig. 2*C*). PCR was carried out for 35 cycles using a step cycle of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min, followed by 72 °C for 8 min. The 5′-RACE products were purified by electrophoresis on a 4% ethidium bromide-stained agarose gel, and then subcloned into the pGEM-T Easy vector for sequencing.

In Vitro Transcription and RNase Protection Assay—RNase protection assays were carried out to confirm the transcription initiation sites. A 252-bp genomic fragment, containing putative transcription initiation sites from the data of the 5′-RACE experiment, was generated by PCR. This PCR product was subcloned into pGEM-T Easy vector, followed by digestion with EcoRI, and subcloned into the EcoRI site of pBluescript (SK+) vector. After linearization with BamHI, the antisense riboprobe was synthesized using the *in vitro* transcription kit, MAXIscript (Ambion, Austin, TX), according to the manufacturer's instructions. This riboprobe was examined by 5% polyacrylamide gel containing 8 M urea, and the full-length riboprobe was recovered from the gel. RNase protection analysis was performed using a ribonuclease protection assay kit, RPAIII (Ambion), following the manufacturer's protocol. Briefly, 20 μg of total RNA isolated from A204 or YST-1 cells and 8×10^4 cpm of riboprobe were dissolved in 10 μl of hybridization buffer and incubated overnight at 42 °C. After digestion with the RNase A/T1 mixture, protected RNA fragments were separated on a 6% polyacrylamide gel containing 8 M urea. As a size marker, ^{32}P-end-labeled ϕX174 DNA/HaeIII marker was loaded onto the same gel. After the run had finished, the gel was transferred onto 3MM paper (Whatman) and dried under vacuum. The dried gel was visualized by autoradiography using a Bio Imaging Analyzer FLA-5000 (Fuji Film, Tokyo, Japan).

Construction of Chimeric Plasmids—The ApaI genomic fragment corresponding to the region from −1785 to +112 bp of the *COL5A3* was blunt-ended with T4 DNA polymerase and subcloned into the SmaI site of pGL3-Basic vector (Promega). 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 HindIII site-linked 3′ primers specific for the *COL5A3* sequence and the pGL3−1785/+109 plasmid as a template. These PCR products were subcloned into the pGEM-T Easy vector, followed by digestion with SacI and HindIII, and subcloned into the SacI/HindIII site of pGL3-Basic vector.

Internal deletion and substitution mutation constructs were generated by site-directed mutagenesis using the pGL3−310/+109 plasmid as a template. The primers used in the PCR amplification are listed in Supplemental Materials Table S1. The PCR products were digested with endonuclease, followed by self-ligation. All mutagenesis plasmids were digested with SacI and HindIII and re-cloned into the SacI/HindIII site of pGL3-Basic vector. Construction of the dominant negative CBF-B/NF-YA was generated by RT-PCR as previously described (26, 29).

Transient Transfection and Luciferase Assays—Transient transfection experiments were carried out using A204 and YST-1 cells. The cells were plated at a density of 2×10^5 per 35-mm dish ~18 h before transfection. For transient transfection, 1 μg of plasmid DNA was transfected into these cells by using the LipofectAMINE Plus reagent system (Invitrogen). Plasmid pRL-TK vector (Promega) was always cotransfected as an internal control for transfection efficiency. After an additional cultivation for 48 h, the transfected cells were harvested, lysed, centrifuged to pellet the debris, and subjected to luciferase assay. Luciferase activities were measured as chemiluminescence in a luminometer (Lumat LB 9507, PerkinElmer Life Sciences) using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's protocol. The cotransfection experiments with a mutant type of CBF-B/NF-YA subunit expression vector were performed as previously described (26, 27). All transfections were repeated in triplicate and the results were expressed as the mean ± S.D. of three independent experiments.

Preparation of Nuclear Extracts—Nuclear extracts from A204 and YST-1 cells were prepared according to the method of Dignam *et al.* (30). Briefly, cells, 1×10^8, were scraped in ice-cold phosphate-buffered saline (PBS), centrifuged for 5 min at $1,500 \times g$, 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 $3,000 \times g$ for 10 min at 4 °C, 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 °C for 1 h. After centrifugation at $24,000 \times g$ for 30 min at 4 °C, the supernatants were collected and stored at −80 °C until used. The protein concentration of the nuclear extracts was determined by Bradford assay (Bio-Rad) using bovine serum albumin as a standard.

DNase I Footprinting Assay—Probes for DNase I footprinting were generated by PCR amplification using sets of oligonucleotides that are SacI site-linked 5′ and HindIII site-linked 3′ primers specific for *COL5A3*, and all PCR products were subcloned into the pGEM-T Easy vector. These plasmids were digested with HindIII and radiolabeled

[1] The abbreviations used are: RT, reverse transcription; CBF/NF-Y, CCAAT-binding factor; RACE, rapid amplification of cDNA ends; EMSA, electrophoretic mobility shift assay; CHIP, chromatin immunoprecipitation assay; PBS, phosphate-buffered saline; ss, single-stranded; FP, footprint; wt, wild-type; CEBP, cAMP element-binding protein.

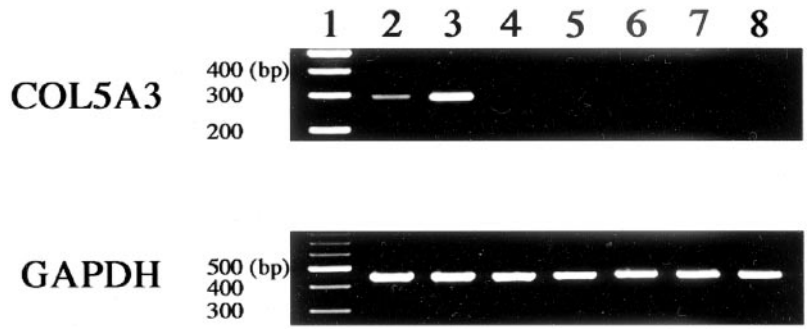

FIG. 1. **RNA expression of the *COL5A3* in various human cell lines.** RT-PCR analysis on total RNA from different cell lines. *Lane 1*, 100-bp ladder marker; *lane 2*, A204; *lane 3*, YST-1; *lane 4*, HT1080; *lane 5*, Jurkat; *lane 6*, U251MG; *lane 7*, IMR32; *lane 8*, A498. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was included as a positive control for the PCR.

with [α-^{32}P]dCTP using Klenow fragment to fill in the HindIII overhanging sites. After digestion with SacI, these probes were examined by a 4.5% non-denaturing polyacrylamide gel and 3′ end-labeled probes were recovered from the gel. DNase I footprinting was performed according to the protocol described by Galas and Schmitz (31) with some modifications. Briefly, the 3′ end-labeled probe (50,000 cpm) was incubated at 25 °C for 30 min with 50 μg of nuclear extracts (or bovine serum albumin as control) in a 100-μl reaction mixture containing 20 mM Tris-HCl (pH 7.9), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 μg of bovine serum albumin, and 4 μg of poly(dI-dC). After the addition of 5 units of DNase I (or 0.1 unit with bovine serum albumin as control) to the mixture, the incubation was continued for 5 min at 25 °C. The reaction was stopped by adding 100 μl of a stop buffer (200 mM sodium acetate, 20 mM EDTA, 1% SDS, 10 μg of yeast tRNA), and DNA fragments were subsequently extracted with phenol/chloroform and precipitated with ethanol before being loaded onto a 6% polyacrylamide gel containing 8 M urea. After electrophoresis, the gel was transferred onto 3 MM paper, dried, and exposed to a BioImaging Analyzer FLA-5000.

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 Klenow fragment to fill in the HindIII overhanging sites. The binding reaction was carried out for 30 min at 25 °C 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), and 5–15 μg of nuclear extracts. For the competition assays, double strand oligonucleotides containing a consensus CBF, CBF mutant, NF-1, CEBP, GATA-1, c-Myb, and MEF-1 binding site were generated by annealing equimolar complementary oligonucleotides. The consensus sequences are shown in Fig. 7*A*. For the supershift assay, anti-CBF-A/NF-YB, anti-CBF-B/NF-YA, anti-CBF-C/NF-YC, anti-NF-1, anti-CEBP, and anti-GATA-1 polyclonal antibodies and preimmune goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA) were purchased. For these interference assays, 5–100-fold molar excess of unlabeled competitor or 2 μg of antibody was added to the reaction mixture for 1 h at 4 °C before the addition of 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. Following completion of running, the gel was transferred onto 3MM paper and dried under vacuum. The dried gel was visualized by autoradiography using a BioImaging Analyzer FLA-5000.

Chromatin Immunoprecipitation (CHIP) Assay—CHIP assays were performed using a chromatin immunoprecipitation assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's protocol. Briefly, A204 and YST-1 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, cells were removed from dishes in PBS containing 1 mM EDTA, and harvested by centrifugation at 1,000 × *g* for 1 min at 4 °C. The cell pellets were resuspended in SDS lysis buffer, incubated for 10 min at 4 °C, and sonicated four times for 10 s yielding DNA fragments 200–1000 bp in size. After centrifugation, the supernatant was diluted in CHIP dilution buffer, precleared with salmon sperm DNA/protein A-agarose slurry, and immunoprecipitated with the indicated antibodies overnight at 4 °C. Immunocomplexes were captured on the ssDNA/protein A-agarose slurry, and washed with low salt wash buffer, high salt wash buffer, and LiCl wash buffer and finally washed twice with TE buffer. The immunocomplexes were eluted by incubation for 15 min at 25 °C with 200 μl of elution buffer (1% SDS, 100 mM $NaHCO_3$, 1 mM dithiothreitol). To reverse the cross-linking of DNA, the elutes were treated with 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. The DNA fragments were extracted with phenol/chloroform and precipitated with ethanol. PCR were carried out for 40 cycles using a step cycle of 94 °C for 30 s, 55 °C for 20 s, 72 °C for 30 s, followed by 72 °C for 8 min. The PCR products were analyzed by electrophoresis on a 4% ethidium bromide-stained agarose gel.

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

RESULTS

RNA Expression of COL5A3 in Cultured Cells—Initially, we examined the expression of the *COL5A3* in various culture cells using the RT-PCR technique. As shown in Fig. 1, the predicted band of 295 bp was observed in YST-1 (strong) and A204 (weak) cells. The PCR products were verified by nucleotide sequence to confirm the specificity of the amplified *COL5A3* sequence. By contrast, the band was not detected in HT1080, Jurkat, U251MG, IMR32, and A498 cells. From these results, we decided to use A204 and YST-1 cell lines for the analysis of the *COL5A3* promoter.

Structural Analysis of the 5′ Portion of COL5A3—To obtain the *COL5A3* genomic fragment, we screened a human genomic BAC library by PCR, and isolated one positive clone, 218M16. Compared with the EMBO/GenBank™ data base, DNA sequences of both ends of the clone were identical with those of two clones, AC008742 and AC020931, in chromosome 19 of *Homo sapiens*. From the data base, this BAC genomic fragment, which was ~141 kb in size, contained the promoter region and both exons and introns 1–32 of *COL5A3* (Fig. 2*A*). To subclone a DNA fragment containing the promoter region, the BAC genomic fragment was digested with ApaI endonuclease and subcloned into the pBluescript (SK+) vector. The sublibrary was screened by colony hybridization and the insert DNA of the positive clone was sequenced using *COL5A3*-specific or vector-specific primers. As a result, a 1897-bp genomic fragment containing the 5′-flanking region of *COL5A3* was isolated (Fig. 2*B*).

To determine the transcription initiation sites of *COL5A3*, 5′-RACE experiments and RNase protection assays were performed. The 5′-RACE was carried out using total RNA derived from A204 and YST-1 cells. Sixteen independent clones, eight clones from A204 cells and eight clones from YST-1 cells, were selected and sequenced. As shown in Fig. 2*C*, the 5′-ends of the *COL5A3* cDNA were located at 113 to 119 bp upstream from the initiating ATG codon, and 13 of the 16 RACE products were generated from the same position, which suggested that the major transcription initiation site is located 119 bp upstream from the initiating ATG codon. On the basis of these data, we also performed RNase protection assays to confirm the transcription initiation sites. In this experiment, we used a 357-nucleotide riboprobe spanning nucleotides containing the putative transcription initiation site, and at least two specific protected bands were observed in both A204 and YST-1 cells

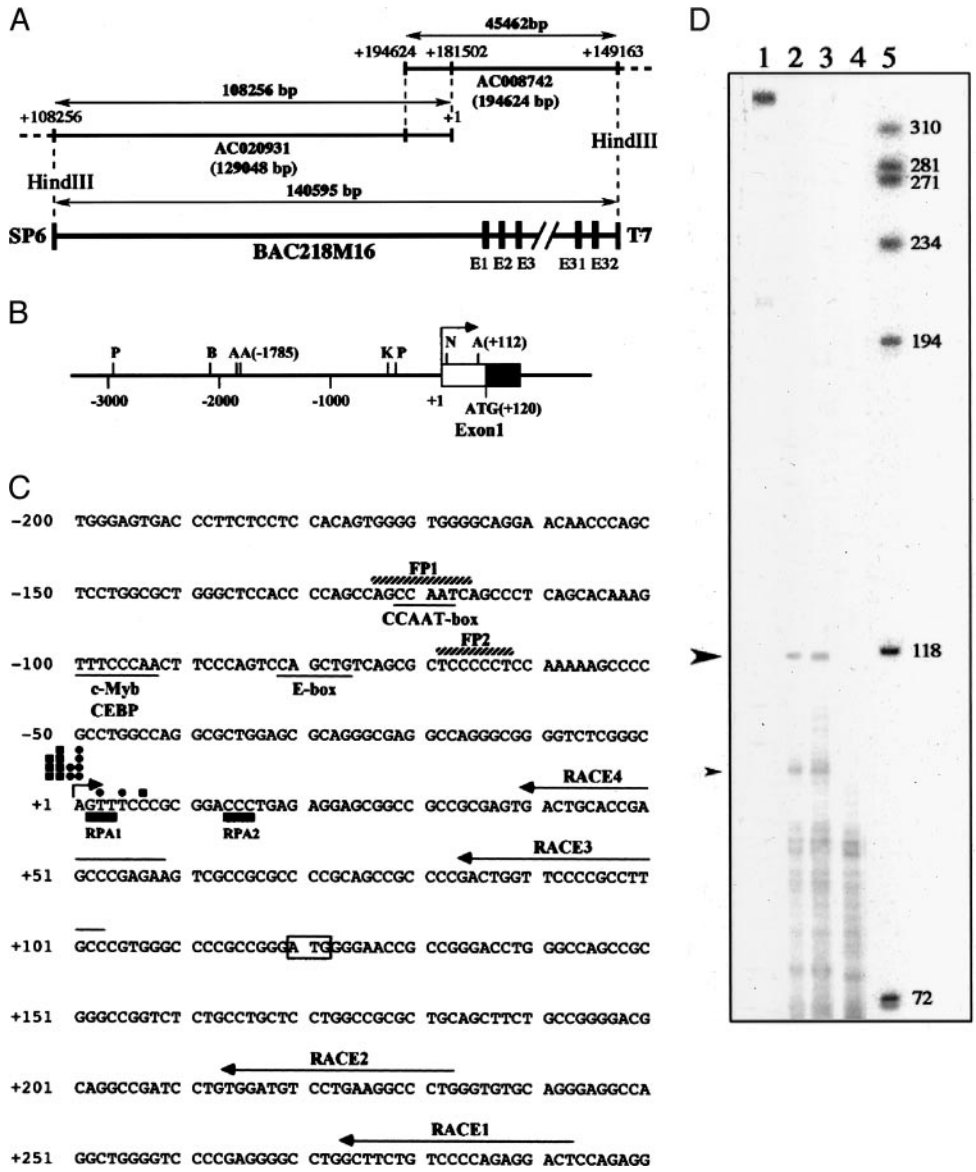

FIG. 2. **Structural analysis of the 5′ region of the *COL5A3*.** *A*, schematic representation of BAC clone containing *COL5A3*. AC020931 and AC008742 clones, which are assigned on human chromosome 19, are only shown with the portions that overlap with the BAC218M16 clone. E1, E2, E3, E31, and E32, indicated with *filled boxes*, are the exons of *COL5A3*. *SP6* and *T7* indicate primers on the vector arms. *B*, partial restriction map of *COL5A3*. *Open* and *closed boxes* represent untranslated and coding regions of exon 1, respectively. The transcription initiation site is represented by an *arrow*. Restriction endonuclease sites are shown as follows: *P*, PstI; *B*, BamHI; *A*, ApaI; *K*, KpnI; *N*, NotI. *C*, nucleotide sequence of the proximal promoter region and part of exon 1 of *COL5A3*. The 5′ ends of the RACE products are shown by the *black dots* (A204) and the *black boxes* (YST-1). RACE 1–4, indicated with *horizontal arrows*, are sites of the primers used for 5′-RACE experiments. The transcription initiation site and the translation initiator ATG codon are shown with a *short arrow* and a *box*, respectively. FP1 and FP2, indicated with *striped bars*, are the DNase I-footprinted sites. RPA1 and RPA2, indicated *under bars*, are the approximate 5′ ends of the RNase-protected bands. The putative sites where the factors bind are *underlined*. *D*, RNase protection analysis. *Lane 1*, undigested riboprobe; *lane 2*, RNA derived from A204 cells; *lane 3*, RNA derived from YST-1 cells; *lane 4*, yeast RNA (negative control); *lane 5*, ^{32}P-end-labeled ϕX174 DNA/HaeIII marker. *Arrows* indicate specific protected bands. The ladders seen in the lower portion of *lanes 2–4* are nonspecific.

(Fig. 2*D*). The protected bands were about 115 and 100 nucleotides in size, and the larger protected band corresponded to the results of the 5′-RACE. Therefore, we concluded that *COL5A3* contains at least two transcription initiation sites and that the major transcription initiation site is located 119 bp upstream from the initiating ATG codon. The *COL5A3* promoter also 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′ end and internal deletions linked to the luciferase gene were transfected into A204 and YST-1 cells (Fig. 3*A*), and luciferase assays were carried out. The longest construct, pGL3−1785/+109, derived from a 1.9-kb genomic fragment of the 5′-flanking region of *COL5A3*, had strong transcriptional activity. The activity of each construct was compared with this construct. As shown in Fig. 3*B*, deletion from −1785 to −129 bp had no significant change on luciferase activity; however, deletion to −40 bp resulted in a significantly lower luciferase activity compared with that of pGL3−1785/+109, and deletion to the +3 bp showed almost complete loss of luciferase activity in both A204 and YST-1 cells. In addition, the deletion mutant of pGL3−310/+109(del−129/+2), which is deleted between −129 and +2, also showed a significantly lower luciferase activity in both cells. These results indicate that the region between the −129 and +2 bp is important for basal transcriptional activity of the *COL5A3* promoter.

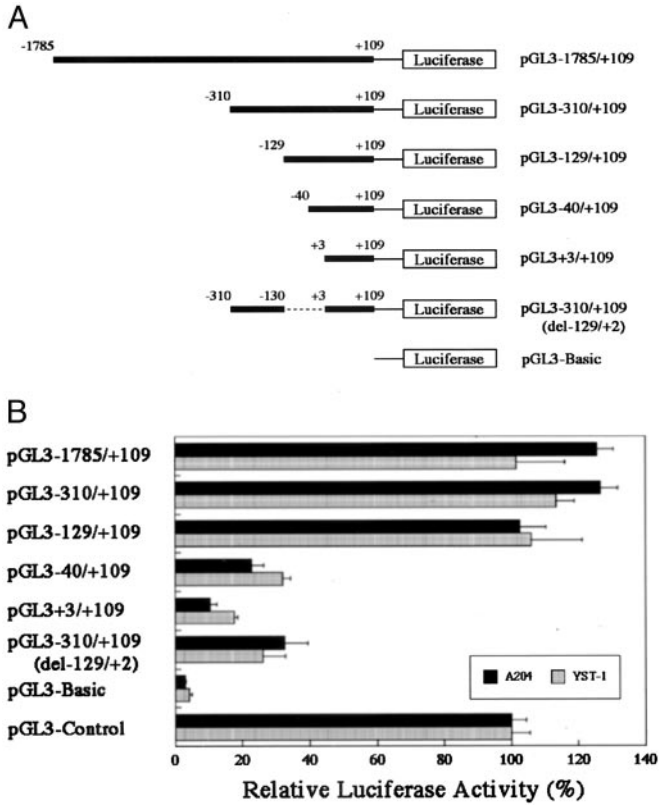

FIG. 3. **Luciferase assay of the *COL5A3* promoter.** *A*, schematic illustration of the 5′-deletion and internal deletion constructs of the *COL5A3* promoter. *B*, luciferase activity in A204 (*black* histogram) and YST-1 cells (*dotted* histogram). All of the constructs were cotransfected with the pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities (%) were normalized against the activity of pGL3-control vector. Data are the mean ± S.D. of three independent experiments.

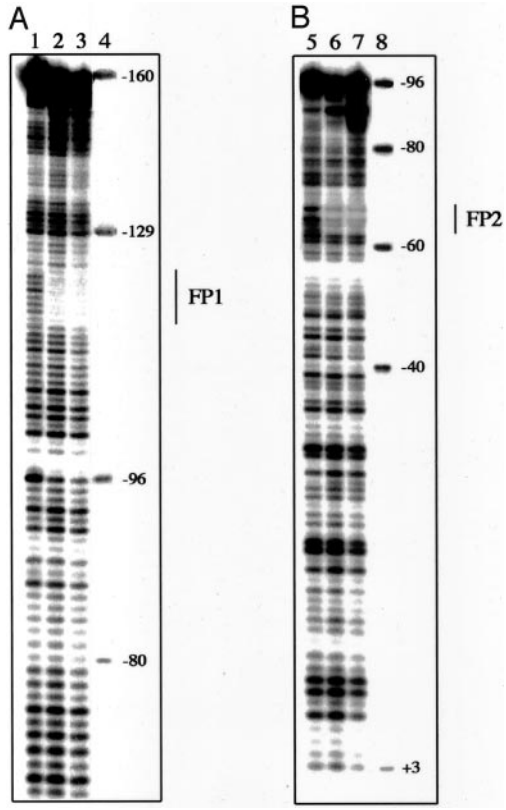

FIG. 4. **DNase I footprinting analysis of the proximal promoter region of the *COL5A3*.** A 3′-end labeled probe of −160/−51 (*A*) and −96/+30 (*B*) were used. These probes were incubated with bovine serum albumin (*lanes 1* and *5*), A204 nuclear extracts (*lanes 2* and *6*), and YST-1 nuclear extracts (*lanes 3* and *7*). Undigested 3′-end labeled nucleotides of −160/−51, −129/−51, −96/−51, and −80/−51 (*lane 4*), and −96/+30, −80/+30, −60/+30, −40/+30, and +3/+30 (*lane 8*) were loaded on the same gel as a size marker. The protected regions, FP1 and FP2, are indicated by *vertical bars*.

```
                -160                                                                  -101
-160/-101wt     ACAACCCAGC TCCTGGCGCT GGGCTCCACC CCAGCCAGCC AATCAGCCCT CAGCACAAAG
                                                          FP1
-160/-101m1     ---------- ---------- ---tctaga- ---------- ---------- ----------
-160/-101m2     ---------- ---------- ---------- --------tc taga------ ----------

                -115                                                          -71
-115/-71wt      GCCCT CAGCACAAAG TTTCCCAACT TCCCAGTCCA GCTGTCAGCG
-115/-71m3      ----- ----------c tcgag----- ---------- ----------
-115/-71m4      ----- ---------- -----ctcga g---------- ----------
-115/-71m5      ----- ---------- ---------- -gaattc--- ----------
-115/-71m6      ----- ---------- ---------- -------gaa ttc-------

                -80                                                   -51
-80/-51wt       GCTGTCAGCG CTCCCCCTCC AAAAAGCCCC
                                  FP2
-80/-51m7       ------gaat tc--------- ----------
-80/-51m8       ---------- --gaattc-- ----------
-80/-51m9       ---------- --------ga attc------
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FIG. 5. **The probes used for EMSA experiments.** Oligonucleotide sequences of wild-type and mutant probes. *FP1* and *FP2* indicate the DNase I footprinted sites. *Bars* in the mutant probes show the same nucleotide sequences as those of wild-type.

Identification of Nuclear Factor Binding Sites in the Core Promoter of the COL5A3—To identify transcription factor binding sites on the proximal promoter region of *COL5A3*, we initially performed DNase I footprinting. Two different probes, which contain the region from the −160 to −51 bp (Fig. 4*A*) and from the −96 to +30 bp (Fig. 4*B*), were used in this experiment. As shown in Fig. 4, *A* and *B*, two protected regions, FP (footprint) 1 in the −124 to −117 bp, and FP2 in the −69 to −63 bp regions, were observed in the *COL5A3* promoter.

To further investigate the binding of nuclear proteins to the proximal *COL5A3* promoter, we carried out EMSA experiments. From the footprinting data, we prepared three overlapping oligonucleotides covering the region between −160 and −51 bp (Fig. 5). The −160/−101wt probe, covering the −160 to −101-bp region, which contains the FP1 site, could bind nuclear protein extracted from A204 and YST-1 cells in a dose-dependent manner (Fig. 6*A*). The band was competitively inhibited by corresponding excess unlabeled oligonucleotide (Fig. 6*B*). We prepared two substitution oligonucleotides, −160/−101m2 and −160/−101m1 (Fig. 5). The former was mutated at the FP1 site and the latter at a site unrelated to FP1 region. As shown in Fig. 6*A*, the −160/−101m2 probe could not form a DNA-protein complex, whereas the −160/−101m1 probe could in A204 and YST-1. Furthermore, this DNA-protein complex A disappeared completely in the presence of 100-fold excess of unlabeled −160/−101m1 probe, but not in the presence of excess unlabeled −160/−101m2 probe (Fig. 6*B*).

Although a protected region was not observed in DNase I footprinting between positions −115 and −71 bp, the −115/−71wt probe could bind nuclear proteins extracted from A204 and YST-1 cells, and three and two DNA-protein complexes were observed in A204 cells and YST-1 cells, respectively (Fig. 6*C*, *lanes 1* and *10*). To clarify whether these complexes were specific or not, we carried out EMSA experiments using unlabeled −115/−71wt probe and salmon sperm DNA (ssDNA) as competitors. The three DNA-protein complexes in A204 cells were competed out in the presence of the unlabeled −115/−71wt probe in a dose-dependent manner (Fig. 6*C*, *lanes 2–5*), whereas the upper DNA-protein complex was abolished in the presence of only a 5-fold excess ssDNA (Fig. 6*C*, *lane 6*). The

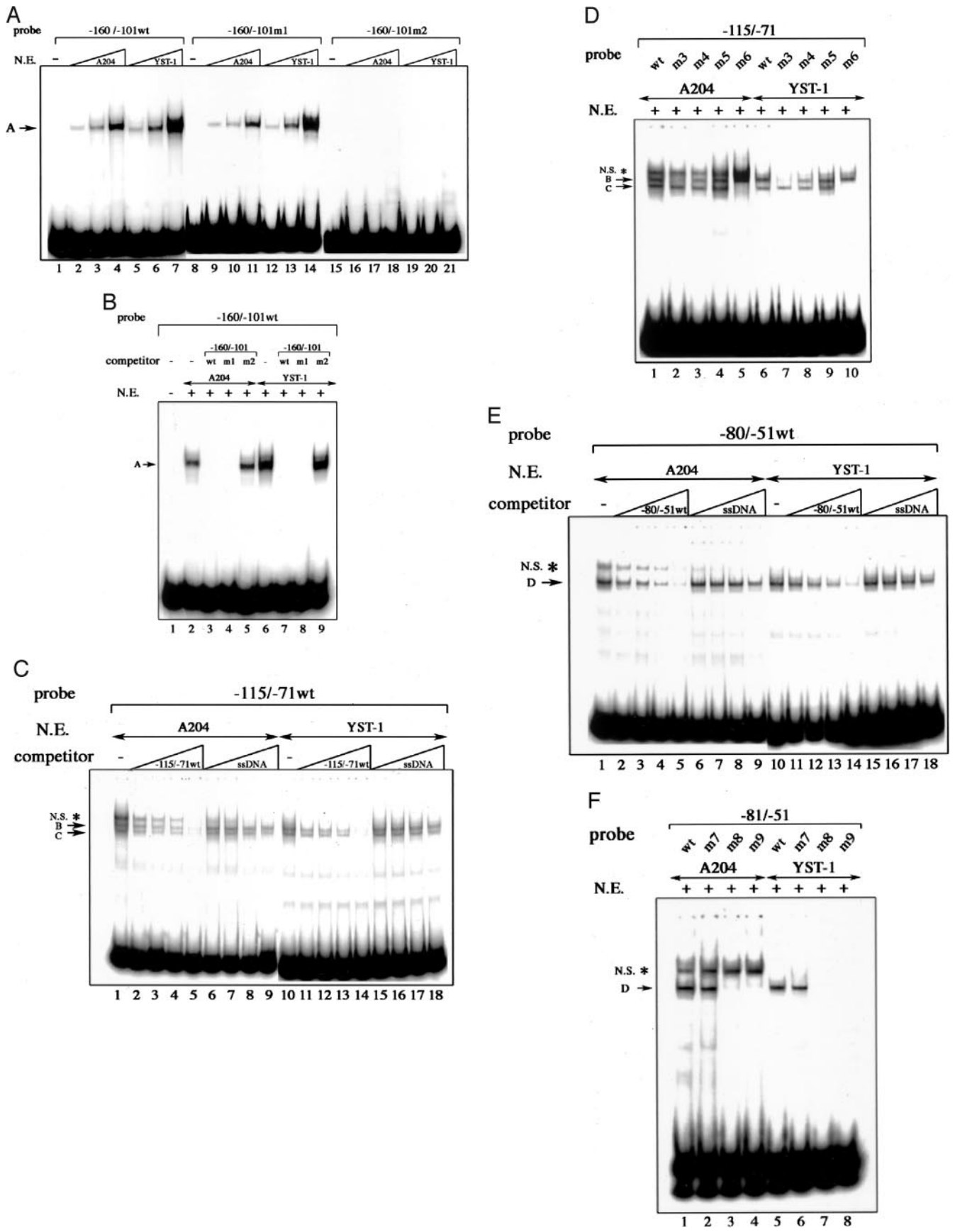

FIG. 6. **EMSA analysis of the *COL5A3* promoter.** *A*, the ^{32}P-labeled −160/−101wt (*lanes 2–7*), −160/−101m1 (*lanes 9–14*) and −160/−101m2 (*lanes 16–21*) probes were incubated with nuclear extracts from A204 and YST-1 cells. The DNA-protein complex is indicated by *A* with an *arrow*. *Lanes 1*, *8*, and *15* were performed without nuclear extracts. *B*, competition analysis using an excess of unlabeled oligonucleotides. The ^{32}P-labeled −160/−101wt probe was incubated with nuclear extracts from A204 and YST-1 cells in the presence of 100-fold excess unlabeled −160/−101wt (*lanes 3* and *7*), −160/−101m1 (*lanes 4* and *8*), and −160/−101m2 (*lanes 5* and *9*) probes. Control assays were performed without nuclear extract

middle and lower complexes remained even in the presence of 100-fold excess ssDNA (Fig. 6*C*, *lane 9*). We concluded that the middle and lower DNA-protein complexes are specific but the upper complex is nonspecific in A204 cells. On the other hand, the two DNA-protein complexes of YST-1 cells did not disappear in the presence of ssDNA (Fig. 6*C*, *lanes 15-18*). These results showed two nuclear protein binding sites between the −115 and −71 bp of the *COL5A3* promoter. To verify these nuclear protein binding sites, we prepared substitution mutation probes, −115/−71m3, -m4, -m5, and -m6 (Fig. 5), and carried out EMSA experiments. As shown in Fig. 6*D*, the −115/−71m3 and −115/−71m6 probes could not form the DNA-protein complexes B and C, respectively.

The position from −80 to −51 bp contained the FP2-protected site. As shown in Fig. 6*E*, the −80/−51wt probe could bind nuclear proteins extracted from A204 and YST-1 cells (*lanes 1* and *10*), and two and one DNA-protein complexes were observed in A204 cells and YST-1 cells, respectively. From the competition analysis, the upper DNA-protein complex observed in A204 cells was abolished in the presence of a 10-fold excess of ssDNA (*lane 7*), although the lower complex was not competed out in the presence of 100-fold excess ssDNA (*lane 9*). We concluded that the lower band, DNA-protein complex D, existed between the −80 and −51 bp. As shown in Fig. 6*F*, −80/−51 m7 mutation probe (Fig. 5) could form the DNA-protein complex D, but −80/−51m8 and -m9 probes could not. These results were consistent with the result of the DNase I footprinting analysis.

Identification of Nuclear Factors in the Core Promoter of COL5A3—To identify the nuclear binding proteins in the core promoter of the *COL5A3*, we performed a computer analysis (transfac.gbf.de/TRANSFAC/). The FP1 site, where a protein binds to form complex A, has the CCAAT motif that is recognized by transcription factors such as CBF/NF-Y, NF-1, CEBP, and GATA-1 (Figs. 2*C* and 7*A*). The binding site for DNA-protein complex B contained putative transcription factor-binding sites for c-Myb and CEBP and that of complex C was consistent with E-box (Figs. 2*C* and 7*A*). However, at the FP2 site, where a protein binds to form complex D, a known factor could not be identified. As shown in Fig. 7*B*, an excess of the consensus oligonucleotides of CBFwt inhibited the binding of the −160/−101wt probe, whereas NF-1, CEBP, GATA-1 consensus, and CBFmt oligonucleotides could not abolish the binding activity. On the other hand, c-Myb and CEBP, and MEF-1 consensus oligonucleotides could not compete out complexes B and C, respectively (Fig. 7*C*).

To confirm the binding of CBF/NF-Y to the CCAAT motif, we performed an interference assay using specific antibodies. As shown in Fig. 8, DNA-protein complex A was only supershifted by anti-CBF/NF-Y antibodies, namely anti-CBF-A/NF-YB, CBF-B/NF-YA, and CBF-C/NF-YC, but not by other specific antibodies against NF-1, CEBP, and GATA-1, and control IgG. To examine whether CBF/NF-Y binds directly to *COL5A3*, we performed a CHIP assay. DNA-protein complexes were immunoprecipitated with antibodies, the cross-links reversed, and recovered DNA fragments were monitored by PCR using primers for the −200 to +109 bp region of the *COL5A3* gene. As shown in Fig. 9, DNA fragments immunoprecipitated with polyclonal antibodies against CBF/NF-Y could be amplified by PCR using the indicated primers (*lane 5*) as well as the positive control (*lane 2*), whereas those immunoprecipitated with normal IgG (*lane 4*) or without antibody (*lane 3*) could not. These results indicate that CBF/NF-Y binds specifically to the CCAAT motif in the *COL5A3* core promoter, *in vivo*.

The CBF/NF-Y and Two Repressors Regulate the Core Promoter of COL5A3—From the DNase I footprinting and EMSA analysis, four nuclear protein binding sites were identified between −160 and −51 bp. To clarify whether these nuclear protein binding sites were functional, a series of substituted mutations of a luciferase construct (Fig. 10*A*) were transfected into A204 and YST-1 cells and luciferase assays were carried out. The activity of each construct was compared with that of pGL3−310/+109wt. As shown in Fig. 10*B*, the activity of the pGL3−310/+109m2 construct was suppressed, whereas those of pGL3−310/+109m3 and -m6 constructs increased in both A204 and YST-1 cells. On the other hand, the activities of the pGL3−310/+109 m8 and m9 constructs did not change compared with that of the pGL3−310/+109wt construct. These results show that the nuclear protein binding factor of DNA-protein complex A, namely CBF/NF-Y, activates the *COL5A3* promoter and those of complex B and C suppress. Consistent with these data, overexpression of the dominant-negative CBF-B/NF-YA subunit, which interacts with the CBF-A/NF-YB and CBF-C/NF-YC subunits to form CBF/NF-Y (27), suppressed the promoter activity in both A204 and YST-1 cells by ~30% ($p < 0.05$) (Fig. 11).

DISCUSSION

The cloned 1.9-kb genomic fragment from the 5′-flanking region of *COL5A3* had strong transcriptional activity. The promoter has multiple transcriptional start sites, lacks the canonical TATA box around −30 bp from the major transcriptional start site, and has a high GC content. These structural features are characteristic for the promoter of housekeeping genes. To define the proximal regulatory regions in the *COL5A3* promoter, a series of chimeric constructs containing progressive 5′ end and internal deletions linked to the luciferase gene were transfected into A204 and YST-1 cells. The region from −129 to +2 bp was necessary for the basal transcriptional activity of *COL5A3*. With the combination analysis using footprinting and EMSA, we identified four nuclear protein binding sites located between the −160 and −51 bp. Among them, we demonstrated that CBF/NF-Y bound to the CCAAT motif in the −122 to −117-bp region and acts as an activator, and the factors located in the −101 to −96 bp and in the −83 to −78-bp regions act as repressors.

Transcription of collagen genes is controlled by a series of complex interactions of positive and negative transcription factors. A number of regulatory elements required for constitutive and inducible expressions have been identified in the promoter of both type I collagen genes (21, 22). The previous studies have identified the ubiquitous factors, Sp1 and Sp3, and CBF/NF-Y

and competitor (*lane 1*), and with nuclear extracts but without competitor (*lanes 2* and *6*). *C*, competition analysis using an excess of unlabeled oligonucleotides. The ^{32}P-labeled −115/−71wt probe was incubated with nuclear extracts from A204 and YST-1 cells in the presence of 5-, 10-, 20-, and 100-fold excess of unlabeled −115/−71wt oligonucleotide (*lanes 2–5* and *11–14*) and ssDNA (*lanes 6–9* and *15–18*) as competitors. The specific DNA-protein complexes are indicated by *B* and *C* with *arrows*, and nonspecific complex (*N.S.*) is indicated by an *asterisk*. *D*, binding assay using 6-bp substituted mutation probes. The ^{32}P-labeled −115/−71wt, -m3, -m4, -m5, and -m6 probes were incubated with nuclear extracts from A204 (*lanes 1–5*) and YST-1 cells (*lanes 6–10*). *E*, competition analysis using an excess of unlabeled oligonucleotides. ^{32}P-Labeled −80/−51wt probe was incubated with nuclear extracts from A204 and YST-1 cells in the presence of 5-, 10-, 20-, and 100-fold excess of unlabeled −80/−51wt probe (*lanes 2–5* and *11–14*) and ssDNA (*lanes 6–9* and *15–18*) as competitors. The specific DNA-protein complex is indicated by *D* with an *arrow*, and the nonspecific complex (*N.S.*) is indicated by an *asterisk*. *F*, binding assay using 6-bp substituted mutation probes. The ^{32}P-labeled −80/−51wt, -m7, -m8, and -m9 probes were incubated with nuclear extracts from A204 (*lanes 1–4*) and YST-1 cells (*lanes 5–8*).

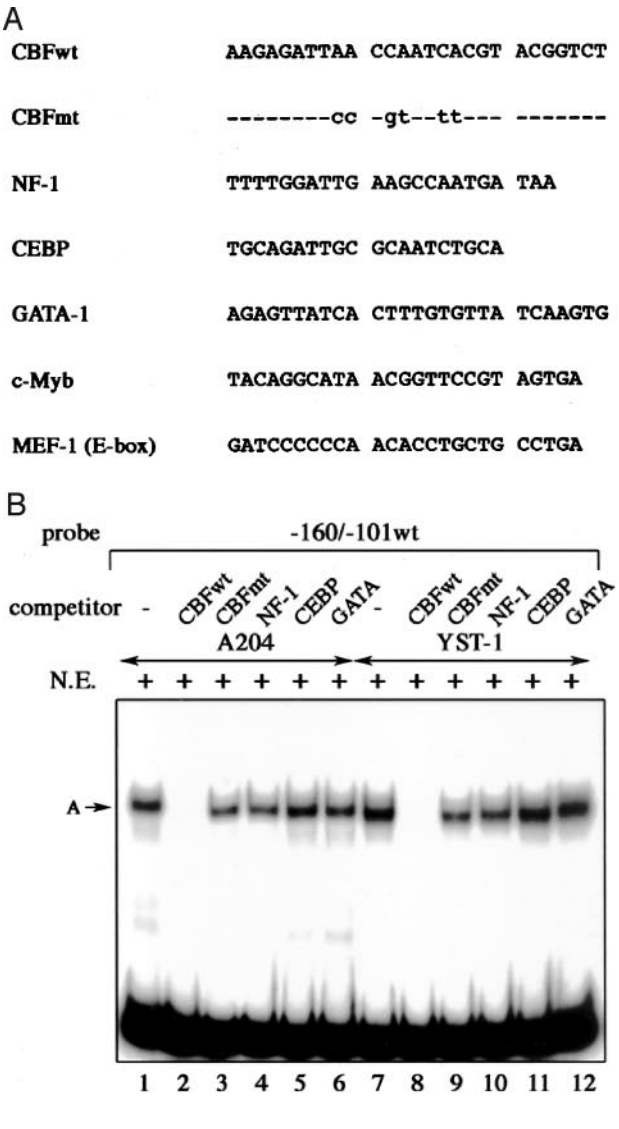

FIG. 7. **Competition analysis using various consensus oligonucleotides.** *A*, consensus sequences of competitors. *Bars* in CBFmt show the same nucleotide sequences with those of the wild-type. *B*, competition analysis using various consensus oligonucleotides as competitors. The ^{32}P-labeled −160/−101wt probe was incubated with nuclear extracts from A204 and YST-1 cells in the presence of 100-fold excess unlabeled CBFwt (*lanes 2* and *8*), CBFmt (*lanes 3* and *9*), NF-1 (*lanes 4* and 10), CEBP (*lanes 5* and *11*), and GATA-1 (*lanes 6* and *12*). *Lanes 1* and *7* were performed without competitor. *C*, competition analysis using various consensus oligonucleotides as competitors. The ^{32}P-labeled −115/−71wt probe was incubated with nuclear extracts from A204 and YST-1 cells in the presence of 100-fold excess competitors, ssDNA (*lanes 2* and *8*), −115/−71wt (*lanes 3* and *9*), c-Myb (*lanes 4* and *10*), CEBP (*lanes 5* and *11*), and MEF-1 (*lanes 6* and *12*). *Lanes 1* and *7* were performed without competitor.

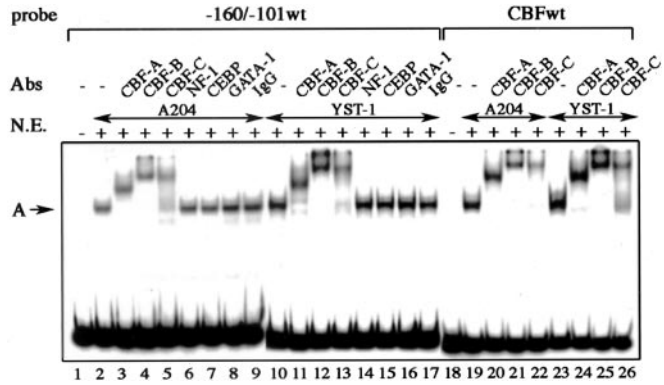

FIG. 8. **Supershift analysis using specific antibodies against various transcription factors.** The ^{32}P-labeled −160/−101wt probe (*lanes 1–17*) and CBFwt consensus oligonucleotide probe (*lanes 18–26*) were incubated with nuclear extracts from A204 and YST-1 cells in the presence of various antibodies, anti-CBF-A/NF-YB (*lanes 3*, *11*, *20*, and *24*), anti-CBF-B/NF-YA (*lanes 4*, *12*, *21*, and *25*), anti-CBF-C/NF-YC (*lanes 5*, *13*, *22*, and *26*), anti-NF-1 (*lanes 6* and *14*), anti-CEBP (*lanes 7* and *15*), anti-GATA-1 (*lanes 8* and *16*), and control IgG (*lanes 9* and *17*). Control assays were performed without nuclear extract and antibody (*lanes 1* and *18*), and with nuclear extracts but without antibody (*lanes 2*, *10*, *19*, and *23*).

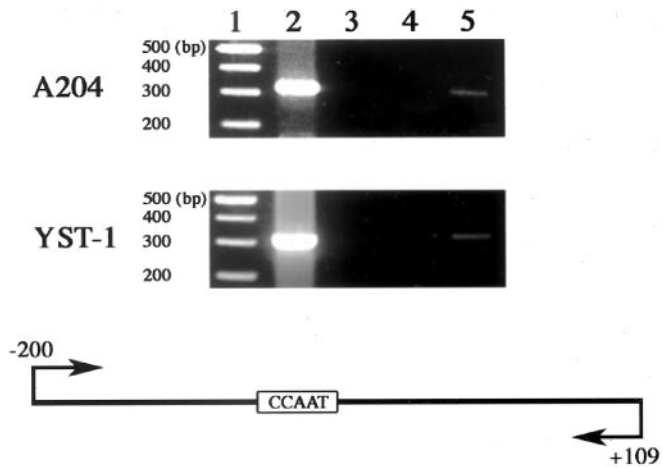

FIG. 9. **Chromatin immunoprecipitation analysis.** A CHIP assay was performed to confirm the binding of CBF/NF-Y to the *COL5A3* promoter, *in vivo*. *Lane 1*, 100-bp ladder marker; *lane 2*, input; *lane 3*; immunoprecipitation without antibody; *lane 4*, immunoprecipitation with normal IgG; *lane 5*, immunoprecipitation with polyclonal antibodies against CBF-A/NF-YB, CBF-B/NF-YA, and CBF-C/NF-YC. All immunoprecipitated DNA fragments were analyzed by PCR with the indicated primers.

binding factors for basal activity of these promoters (32–37). Other putative transcription factors such as c-Krox, BFCOL1, and IF-1 are also involved in the transcriptional regulation of type I collagen genes (38–40). In *COL5A3*, we demonstrated that CBF/NF-Y and two repressors are involved in the regulation of the core promoter. CBF/NF-Y was originally identified as the proteins bound to the major histocompatibility complex class II conserved Y box in Ea promoters and the yeast CYC1

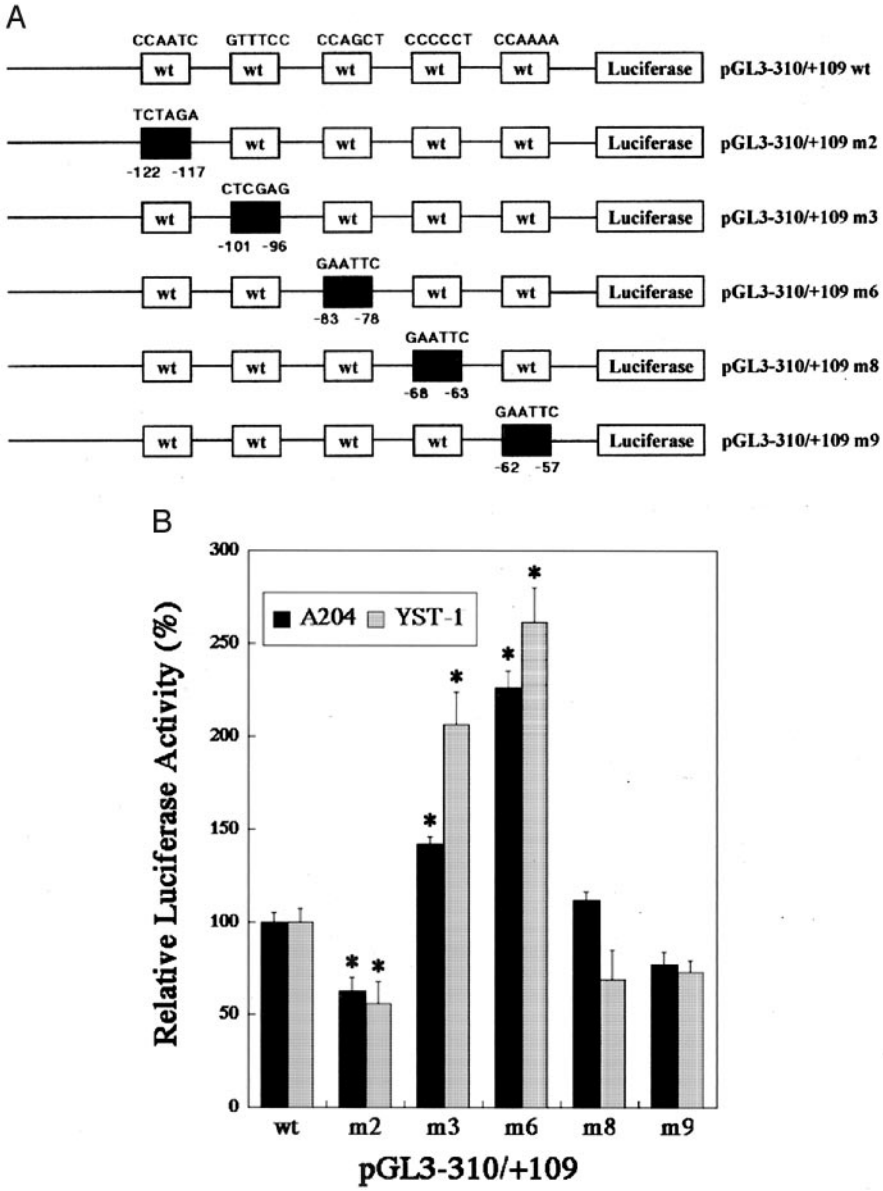

FIG. 10. **Functional analysis using substitution mutation constructs of the *COL5A3* promoter activity.** *A*, schematic illustration of constructs that are mutated in putative nuclear protein binding sites in the *COL5A3* proximal promoter. *B*, luciferase activities in A204 and YST-1 cells transfected with the pGL3−310/+109wt and substitution mutation 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 pGL3−310/+109wt construct. Data are the mean ± S.D. of three independent experiments. *Asterisks* indicate statistically significant results ($p < 0.05$).

UAS CCAAT box (41, 42). It was purified independently using affinity columns containing the Y box, the α2(I) collagen CCAAT, and the α-globin CCAAT sequence (36, 43, 44). CBF/NF-Y is a ubiquitous heterotrimer composed of three subunits, CBF-A/NF-YB, CBF-B/NF-YA, and CBF-C/NF-YC, all necessary for DNA binding (45). CBF/NF-Y requires the perfect pentanucleotide, CCAAT, except in a few genes (46). For CBF/NF-Y to bind to the CCAAT motif, three 5′ (C, Pu, Pu) and five 3′ (C/G, A/G, G, A/C, G) flanking nucleotides also appear to be important (Fig. 12*A*). These 5′- and 3′-flanking sequences are conserved in *COL5A3* except one for nucleotide at the 3′-flanking region.

One of the repressors might bind to the E-box, located at −83 to −78 bp, in the core promoter region of *COL5A3*. The factor binding to the E-box usually works as an activator, such as MyoD (47). One of the factors for the specific expression of mouse *Col1a1* in tendon fibroblasts binds to the E-box located between −3.2 and −2.3 kb (48). The E-box in the 3′-flanking region is also involved in the stimulation of transcription of *Col1a1* (49). The E2-box, a subset of the E-box, has a *cis*-element containing both activator and repressor binding sites. The δEF1, which is a zinc finger protein, bound to −307 to −100 bp in the promoter of rat *Col2a1* gene, and repressed its transcription (50). Seki *et al.* (51) reported that, in the *snail* and *slug*, the C2H2 type zinc finger protein suppressed the promoter activity of *Col2a1*, and that four E-box up to the −1,000 bp from the transcription start site were responsible with the additive efficiency. Other putative suppressors bound to −101 to −96 bp were also examined with the competition assay using the oligonucleotide motifs of known factors. The results showed neither c-Myb nor CEBP, which were candidate factors predicted from a computer search. c-Myb could transactivate the *COL1A2* gene by binding at around −1,025 bp, whereas B-Myb, which has a similar structure, could not (52). B-Myb repressed the transcription of both type I collagen genes (53) and *COL5A2* (24). The Myb family may have a role in the regulation of *COL5A3*.

Heterotrimer α1(V)α2(V)α3(V) molecules were originally extracted from human placenta (10–12). The α3(V) chain was also isolated from synovial membrane, gingival, skin (11), and uterus (54). *In situ* hybridization of mouse embryo detects α3(V) expression in the epimysial sheaths of developing muscles and within nascent ligaments adjacent to forming bones and in joints (16). Chernousov *et al.* (55) reported the isolation of heparin sulfate-binding collagenous protein, p200, which is expressed by Schwann cells in developing peripheral nerves.

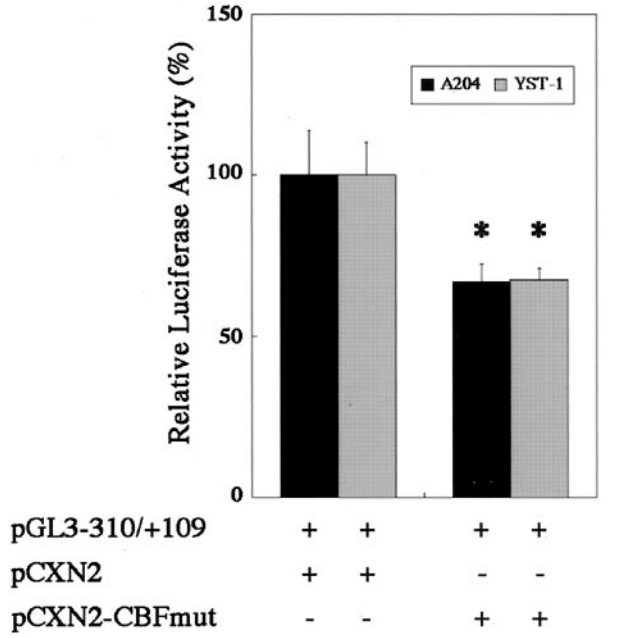

FIG. 11. **Effect of overexpression of dominant negative CBF-B/NF-YA on the *COL5A3* promoter activity.** Cells were cotransfected with 0.5 μg of pGL3−310/+109 construct and 1.5 μg of mutant CBF/NF-Y expression vector (*pCXN2-CBFmut*) or empty vector (*pCXN2*). Relative luciferase activities (%) were normalized against the activity of overexpressed pCXN2 empty vector and the results are the mean ± S.D. of three independent experiments. *Asterisks* indicate statistically significant results ($p < 0.05$).

A

Consensus	5'-	C	Pu	Pu		C	C	A	A	T	C/G	A/G	G	A/C	G -3'
α3 (V)		C	A	G		C	C	A	A	T	C	A	G	C	C
α1 (V)		C	A	G		C	A	A	A	T	C	A	G	A	G
α1 (I)		C	A	G		C	C	A	A	T	C	A	G	A	G
α2 (I)		C	C	A		C	C	A	A	T	C	G	G	A	G

B

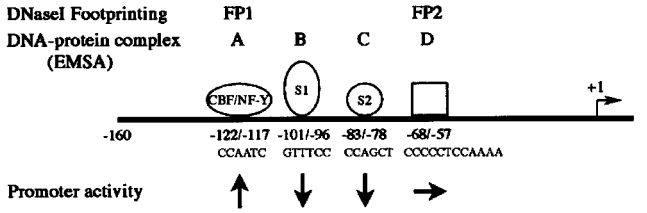

FIG. 12. **The flanking sequence of the CCAAT motif in the collagen promoter, and schematic of the core promoter of *COL5A3*.** *A*, the sequences are forward orientation in α3(V) and reverse orientation in α1(V), α1(I), and α2(I) collagen. The nucleotides that differ from the consensus sequence are indicated with *underlines*. *B*, schematic illustration of four nuclear protein binding sites and transcriptional activities in the *COL5A3* core promoter. The indicated sequences are critical for the binding of nuclear proteins.

They named the rat α4(V) chain that shows 82% amino acid sequence identity to human α3(V) chain (15). In our experiment, human α3(V) chain is expressed in the Schwann cell line, YST-1. The expression of α3(V) chain is less abundant compared with α1(V) chain, but distributed in many more tissues than previously expected. The $[\alpha1(V)]_2\alpha2(V)$ form is the only one whose tissue distribution and supramolecular organization has been extensively studied. It is usually co-expressed with type I collagen and forms type I/V heterotypic fibrils. The nature of the α1(V)α2(V)α3(V) form has remained relatively obscure. Our previous study suggested that CBF/NF-Y is required for the coordinated expression of the α1(V) gene and both type I collagen genes (26). In this study, we showed that it binds to the *COL5A3* promoter. Penkov *et al.* (23) identified PBX1/2, PREP1, and HOXB1 proteins as an FPB-bound complex in the proximal *COL5A2* promoter. Although they did not recognize a *cis*-acting element that binds CBF/NF-Y, it may exist in some region of the *COL5A2* promoter.

In fibrillar collagen genes, the expression of the type II collagen gene is clearly different from those of type I collagen genes. The promoter of the α1(II) gene has a weak activity, and the tissue-specific expression is regulated by the enhancer region in the first intron, where the Sox family binds (56). Although type III collagen is coordinately expressed with type I collagen in fetal tissues and blood vessels, the mechanism of regulation seems to be different from those of type I collagen genes. Two distinct positive factors, one of which is a heat-resistant protein of ~95 kDa, bind in the proximal promoter of the *Col3a1* gene (57). CBF/NF-Y itself is a ubiquitous transactivating factor; therefore, it may be utilized for the broad expression in noncartilaginous tissue such as type I and V collagen genes. However, it is difficult to explain the decrease in promoter activity from −129 to −40 bp by the sole involvement of CBF/NF-Y because the dominant negative mutant of CBF-B/NF-YA was partially suppressed (Fig. 11). Other activators that could not be identified under the conditions of our system might bind in this region. On the contrary, we showed two repressors in this region instead of activators (Fig. 12*B*). The identification of factors other than CBF/NF-Y should provide further information on the type V collagen expression, followed by characterization of different roles for the isoforms of type V collagen.

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