

Multiple proteins are involved in the protein–DNA complex in the proximal promoter of the human $\alpha 1$ (III) collagen gene (*COL3A1*)

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Abstract

We have characterized the proximal promoter of the human $\alpha 1$ (III) collagen gene (*COL3A1*). Transient transfection assays using a series of chimeric constructs linked to the luciferase gene indicated that the segment from –96 to –34 is necessary to activate transcription. Electrophoretic mobility shift assays (EMSAs) showed that the multiple proteins form the DNA–protein complex in different combinations depending on the cell types. A competition assay using mutant oligonucleotides showed that the sequence 5'-GCTCTCATATTTTCAGAA-3' (–79 to –63 bp) is critical for DNA–protein complex formation. This sequence is contained in the B element of mouse $\alpha 1$ (III) collagen gene (*Col3a1*) reported by Ruteshouse and de Crombrugge (J. Biol. Chem., 1993). In the rhabdomyosarcoma cell line, A204, at least two proteins of 92–118 kDa and 40–52 kDa are involved in the DNA–protein complex bound to this motif.

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1. Introduction

Type III collagen is a member of the fibrillar collagen family [1–5]. The molecule is a homotrimer composed of a single type of α chain. It is widely distributed in soft connective tissues such as fetal skin and blood vessels. This molecule is also detected during the repair process of wound healing, reflecting the immaturity of tissues and increased vascularity. It is thought to play an important role in defining tissue architecture and mechanical properties, although its function is not entirely understood. Mutations in the

COL3A1 gene cause type IV Ehlers-Danlos syndrome, a disease leading to aortic rupture in early adult life [6–8]. The absence of type III collagen in a mutant mouse model causes rupture of the blood vessels [9]. Electron microscopic analysis of the vessels revealed that collagen fibrils were missing in the media of the aorta and were irregular in size in the adventitia of the aorta and skin.

Type III collagen is a component of the small argyrophilic collagen fibers that characterize reticular connective tissues [10]. Type III collagen is coexpressed with type I collagen in most tissues. Studies with monoclonal antibodies suggest that type III is a component of the striated fibrils, along with collagen type I [11]. Type III collagen can be present on banded collagen fibrils regardless of fibril diameter [12]. It can also form heterotypic fibrils with type I collagen. The expression of the ratio of type III to type I collagen varies spatially and temporally. It seems likely that transcriptional events are involved in the specific expression of the type III

Abbreviations: BBF, B element binding factor; AGPC, acid guanidium phenol chloroform; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; vSMC, vascular smooth muscle cells; ssDNA, salmon sperm DNA

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Table 1

Primers used for PCR procedures

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1. For generation of luciferase constructs of 5' stepwise deletion 1 (see Fig. 1)
- 587/+68: (sense; –587) 5'-ggagctcGGAAGCCATTCAAACATTGC-3'
 –287/+68: (sense; –287) 5'-ggagctcCATTGTATGTATTAACAA-3'
 –96/+68: (sense; –96) 5'-ggagctcTGAGGGATGGGTGCGGCTC-3'
 –34/+68: (sense; –34) 5'-ggagctcCGGTGCTGAAGGGCAGGAA-3'
 Common: (antisense; +68) 5'-gctcgagGTTCAAAGTAGCACCATCAA-3'
 (Lowercase letters underlined indicate tagged the *SacI* and *XhoI* sites in the sense and antisense primer for cloning.)
2. For generation of luciferase constructs of internal deletion 1 (see Fig. 3A)
- Δ–130/–100: (antisense, –130) 5'-ccgaattcTATAGAAGACACAAC-3'
 (sense, –100) 5'-ccgaattcACTGCTGAGGGGATG-3'
 Δ–100/–80: (antisense, –100) 5'-ccgaattcAAAAGAAATGATAAATATT-3'
 (sense, –80) 5'-ccgaattcGGCTCTCATATTTCA-3'
 Δ–80/–50: (antisense, –80) 5'-ccgaattcGCACCCATCCCCTCAGCAG-3'
 (sense, –50) 5'-ccgaattcGTGAGGGAAGCCAAACTTT-3'
 Δ–50/–20: (antisense, –50) 5'-ccgaattcTTCCAGCCCCTTTCTG-3'
 (sense, –20) 5'-ccgaattcGGCCAAAGCAAAGGAAT-3'
 Δ–20/+10: (antisense, –20) 5'-ccgaattcTTAAATAGGAAAAAGTTTGG-3'
 (sense, +10) 5'-ccgaattcTTTTATGACGGGCC-3'
 (Lowercase letters underlined indicate tagged *EcoRI* site in the primer for cloning.)
3. For generation of luciferase constructs of internal deletion 2 (see Fig. 3E)
- Δ–100/–90: (antisense, –100) 5'-ccgaattcAAAAGAAATGATAAATATT-3'
 (sense, –90) 5'-ccgaattcGGATGGGTGCGGCTC-3'
 Δ–90/–80: (antisense, –90) 5'-ccgaattcCCTCAGCAGTAAAAG-3'
 (sense, –80) 5'-ccgaattcGGCTCTCATATTTCA-3'
 Δ–80/–70: (antisense, –80) 5'-ccgaattcGCACCCATCCCCTCAGCAG-3'
 (sense, –70) 5'-ccgaattcTTTCAGAAAGGGGCT-3'
 Δ–70/–60: (antisense, –70) 5'-ccgaattcTATGAGAGCCGACC-3'
 (sense, –60) 5'-ccgaattcGGGCTGGAAAGTGAG-3'
 Δ–60/–50: (antisense, –60) 5'-ccgaattcCCTTCTGAAATATGAGAG-3'
 (sense, –50) 5'-ccgaattcGTGAGGGAAGCCAAACTTT-3'
 (Lowercase letters underlined indicate tagged *EcoRI* site in the primer for cloning.)
4. For generation of oligonucleotides used for EMSA (see Fig. 6A)
- Wild type: (wt sense; –96) 5'-aagcttGCTGAGGGGATGGGT-3'
 (wt antisense; –31) 5'-aagcttAAAAGTTTGGCTTCC-3'
 (Lowercase letters underlined indicate tagged *HindIII* site in the primer for cloning.)
- m1: (m1 sense, –79) 5'-cctgcagGCTCTCATATTTAGAAA-3'
 (m1 antisense, –86) 5'-cctgcagCATCCCCTCAGCAGTAAA-3'
 m2: (m2 sense, –73) 5'-cctgcagATATTTAGAAAAGGGGCT-3'
 (m2 antisense, –80) 5'-cctgcagCGCACCCATCCCCTCAGC-3'
 m3: (m3 sense, –67) 5'-cctgcagCAGAAAGGGGCTGAAAAG-3'
 (m3 antisense, –74) 5'-cctgcagGAGAGCCGCACCCATCCC-3'
 m4: (m4 sense, –62) 5'-cctgcagAGGGGCTGGAAAGTGAGG-3'
 (m4 antisense, –69) 5'-cctgcagAATATGAGAGCCGCACCC-3'
 m5: (m5 sense, –56) 5'-cctgcagTGGAAAGTGAGGGAAGCC-3'
 (m5 antisense, –63) 5'-cctgcagTTCTGAAATATGAGAGCC-3'
 (Lowercase letters underlined indicate tagged *PstI* site in the primer for cloning and generating mutation.)
-

collagen gene [13]. Ruteshouse and de Crombrughe identified two positive *cis*-regulatory elements, designated A and B, in the mouse $\alpha 1(\text{III})$ collagen promoter [14,15]. A factor related to Jun/AP-1 appeared to bind at the A element located between –122 and –106, whereas the factor binding to the B element located between –83 and –61 was a heat-resistant polypeptide, named the B element binding factor (BBF), with a molecular weight of approximately 95 kDa.

In the present study, we characterized the proximal promoter of the *COL3A1* gene. The segment from –96 to –34 was necessary for the activation of transcription. Multiple proteins in different combinations, depending on cell types, were found to form the DNA–protein complex at –79 to –63.

2. Materials and methods

2.1. DNA clone

The *COL3A1* clone containing the promoter fragment was provided by Dr. F Ramirez of the Medical College of Cornell University, New York, NY [16].

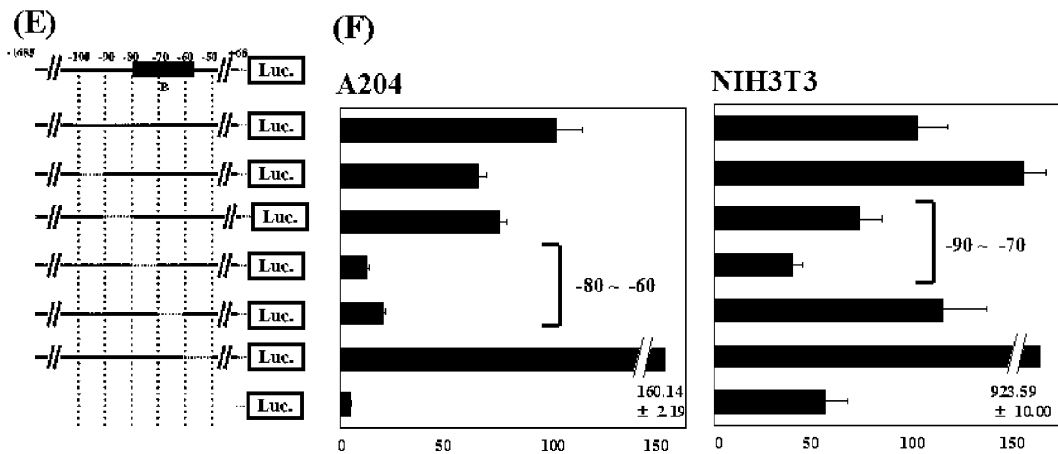
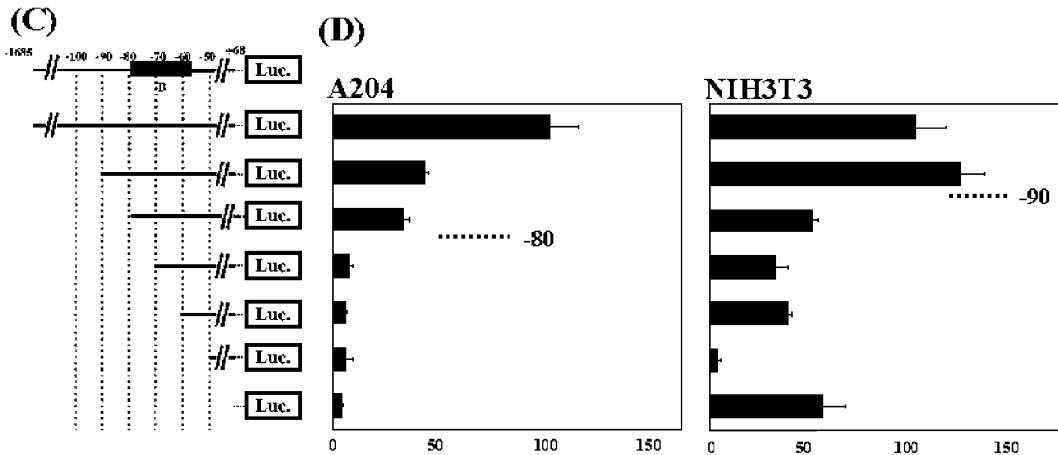
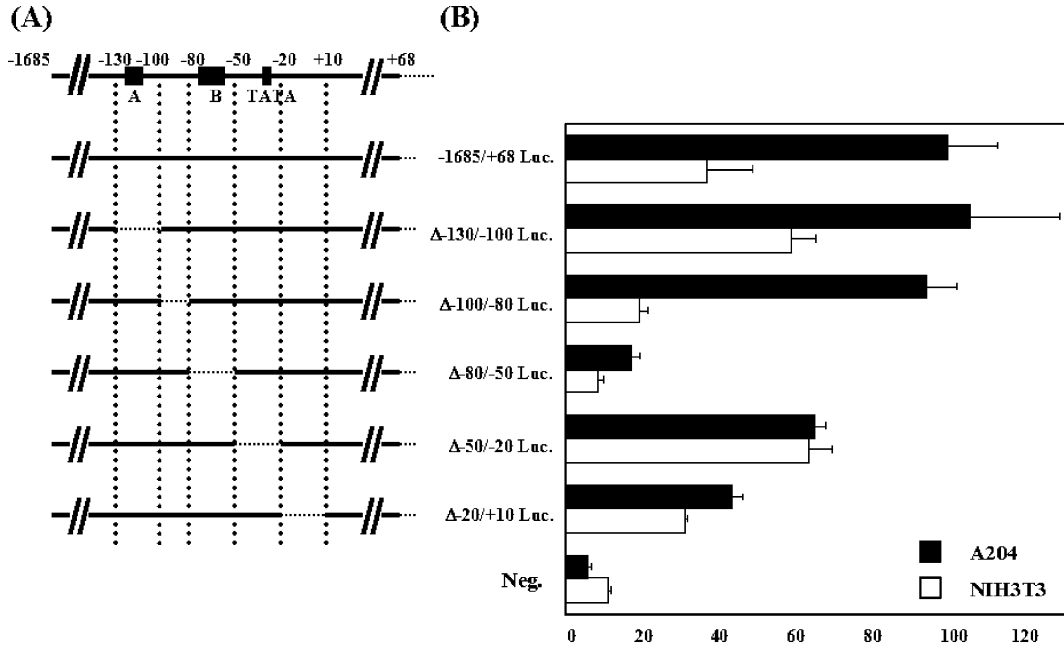
2.2. Cells and cell culture conditions

Human rhabdomyosarcoma A204 and mouse NIH3T3 cells were purchased from American Type Culture Collection (ATCC, Rockville, MD). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supple-

2.4. Transient transfection and luciferase assays

Transient transfection was performed by the calcium phosphate precipitation method with some modifications [19]. The cells were plated at a density of 1×10^5 cells in 24-

well dishes with 400 μ L of culture medium. After incubation for 1 day at 37 °C, cells were transfected with 1 μ g of luciferase plasmid DNA plus 0.1 μ g of *Renilla* pRL-SV40 vector (Promega) as an internal control. Six hours after transfection, cells were rinsed with phosphate buffer saline



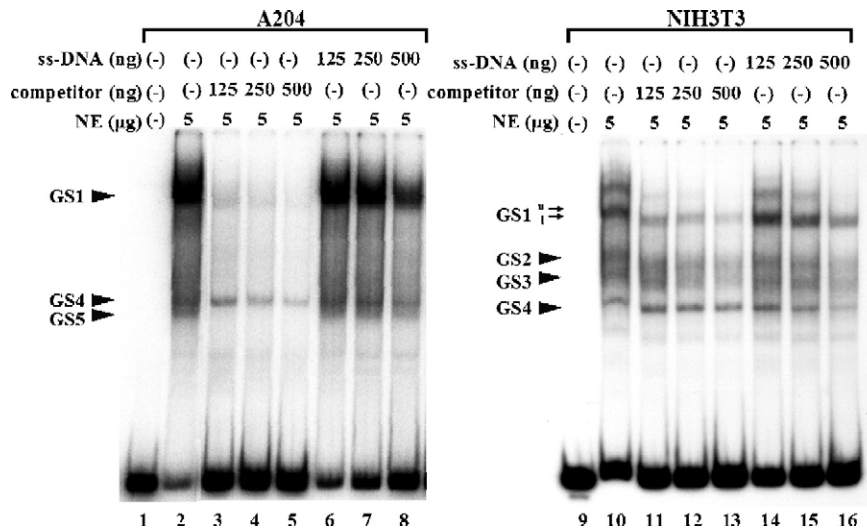


Fig. 4. EMSA analysis of the *COL3A1* promoter using the ^{32}P -labeled $-97/-31$ probe. The DNA–protein complexes GS1, GS2, GS3, GS4 and GS5 are indicated by arrows. Note that GS1 in NIH3T3 shows a doublet; upper (u) and lower (l) bands are indicated with arrows. The ^{32}P -labeled $-97/-31$ probe was incubated with nuclear extracts from A204 (lane 2) and NIH3T3 (lane 10). Lanes 1 and 9 are controls performed without nuclear extracts. The probe was also incubated with nuclear extracts in the presence of excess unlabeled $-97/-31$ nucleotide (lanes 3–5 and 11–13) and ssDNA (lanes 6–8 and 14–16).

(PBS), fed with fresh medium and then further cultured for 2 days. The cells were rinsed with PBS, harvested by scraping with passive lysis buffer (Promega) and then centrifuged to pellet the debris.

Luciferase assays were performed using the Dual-Luciferase reporter assay system (Promega) and their activities in the cell extracts were measured using a luminometer (Lumat LB 9507, PerkinElmer Life Sciences) according to the manufacturer's protocol. The transcription activity of each chimeric construct was evaluated by *Renilla* luciferase activity to normalize for the transfection efficiency. Results were expressed as means \pm S.D. of four to six independent experiments.

2.5. Preparation of nuclear extracts and EMSA

Nuclear extracts were prepared from the cells as described previously [17]. The concentration of protein was determined by Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as a standard.

Double-stranded wild-type and mutant oligonucleotides used for EMSA were obtained by PCR amplification using pBL-COL3A1 plasmid DNA as a template [17]. The mutation oligonucleotides in Fig. 6 were generated by

site-directed mutagenesis [17]. Primers used for PCR are listed in Table 1. Amplified PCR fragments were subcloned into pGEM-T Easy vector. After digestion with *Hind*III, DNA was radiolabeled with ^{32}P -dCTP (Amersham Bioscience) using Klenow fragment. EMSAs were carried out as previously described [17]. Briefly, labeled probes (4×10^4 cpm) were incubated with various amounts of nuclear extract (2.5–10 μg) for 30 min at 25 $^\circ\text{C}$ in the presence of 3 μg of poly(dI-dC). In the competition experiments, the excess of unlabeled DNA fragments were pre-incubated with nuclear extract for 30 min on ice. In some experiments, nuclear extracts were treated with heating at 95 $^\circ\text{C}$ for 5 min, 10% SDS and/or Triton X-100 before incubation with the DNA probe.

The DNA–nucleoprotein complex was separated from the free labeled probe by electrophoresis on a 4.5% polyacrylamide gel. The dried gels were exposed to a bioimaging plate and analyzed with a Fuji Bio Imaging Analyzer (BAS2000 or FAL5000, Fuji Film, Tokyo).

2.6. Estimation of molecular size of nuclear proteins

Nuclear extracts were run on 12.5% or 5% SDS-PAGE gels under reducing conditions and stained with Coomassie

Fig. 3. Luciferase assay of the proximal *COL3A1* promoter. (A) Schematic illustration of the internal deletion constructs of the *COL3A1* promoter. (B) Luciferase activity in A204 and NIH3T3 cells. All of the constructs were co-transfected with the pRL-TK vector as an internal control for transfection efficiency. Relative luciferase activities (%) were normalized against the activity of the $-1685/+68$ Luc construct that was transfected into A204 cells as 100%. Data are the means \pm S.D. of six independent experiments. (C) Schematic illustration of the 10-bp stepwise-deletion constructs of the *COL3A1* promoter. (D) Luciferase activity in A204 and NIH3T3 cells. 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 the $-1685/+68$ Luc construct that was transfected into A204 and NIH3T3 cells as 100%. Data are the means \pm S.D. of six independent experiments. (E) Schematic illustration of the 10-bp internal-deletion constructs of the *COL3A1* promoter. (F) Luciferase activity in A204 and NIH3T3 cells. 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 the $-1685/+68$ Luc construct that was transfected into A204 and NIH3T3 cells as 100%, respectively. Data are the means \pm S.D. of six independent experiments.

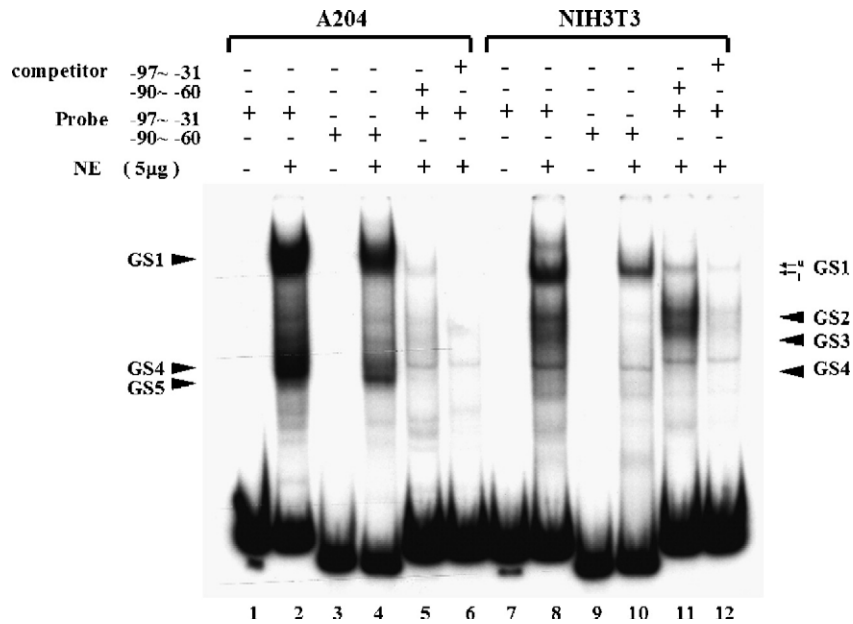


Fig. 5. EMSA analysis using the ^{32}P -labeled $-90/-60$ probe. The ^{32}P -labeled $-90/-60$ probe was incubated with nuclear extracts from A204 (lane 4) and NIH3T3 (lane 10). Control assays were performed using the $-97/-31$ probe (lanes 2 and 8). The probe was also incubated with nuclear extracts in the presence of excess unlabeled $-90/-60$ nucleotide (lanes 5 and 11) and $-97/-31$ (lanes 6 and 12). Lanes 1, 3, 7 and 9 were performed without nuclear extracts. Note that the GS2 and GS3 were not seen in NIH3T3 cells (lane 10).

brilliant blue. Gels were cut into slices, in which the bands could be estimated from the migration of the prestained molecular weight standards. Proteins in the nuclear extracts were eluted from each slice and renatured, essentially as previously described [15,20]. EMSAs were performed using the protein samples as mentioned above.

3. Results

3.1. Functional analysis of the COL3A1 promoter

Initially, the transcriptional activity of the $-1685/+68$ Luc plasmid, containing the human 1.7-kb upstream sequence, was examined in A204 and NIH3T3 (Fig. 1A). Transfection of $-1685/+68$ Luc plasmid yielded a high level of luciferase enzyme activity in both cells (Fig. 1B). This result suggests that the 1.7-kb promoter sequence contains basic regulatory elements. To narrow down the length of the minimum active promoter sequence, six constructs harboring progressive 5'-deletions of the 1.7-kb promoter fragment were generated (Fig. 1A). An essential loss of transcriptional activity was seen in $-34/+68$ Luc (Fig. 1B). It suggested that the basic machinery for the transcription of the type III collagen gene is contained in the region from -96 to -34 bp, in which the nucleotide sequences among human [16], mouse [21] and bovine (accession number AB189399) are very similar (Fig. 2).

Next, we generated 20-bp or 30-bp internal deletion constructs of $-1685/+68$ Luc to characterize the proximal promoter. The deletion construct of -80 to -50 bp showed low activity of the promoter in A204 cells, and those of -100 to -80 bp and -80 to -50 bp also showed low activity in

NIH3T3 (Figs. 3A and B). An internal deletion of -50 to -20 showed low activity in both cells. To delineate the mechanism of transcription in detail between -100 and -50 bp, a series of 5' stepwise deletions with 10 bp internal deletions of $-1685/+68$ Luc was generated. In the experiment, using the stepwise deletions of 10 bp, the promoter activity decreased at -80 bp in A204, and at -90 bp in NIH3T3 cells (Figs. 3C and D). Similarly, in the experiment using internal deletions of 10 bp, the promoter activity decreased at the region from -80 to -60 bp in A204 cells, and from -90 to -70 in NIH3T3 cells (Figs. 3E and F). Interestingly, the promoter activity was increased in both cells by the internal deletion of 10 bp in the region between -60 and -50 bp. A suppressor factor may be restricted to this region.

3.2. Identification of nuclear factor binding site in the proximal region of COL3A1 promoter

To identify nuclear factors in the proximal promoter, we performed a series of EMSAs. We prepared an oligonucleotide covering the region from -97 to -31 bp. Radiolabeled oligonucleotides were incubated with nuclear extracts from the cells and EMSAs were carried out. The ^{32}P -labeled probe bound several nuclear proteins in the cells (Fig. 4). The pattern of DNA-protein interaction differed in both cell types. GS1 and GS4 were seen in both cells, whereas GS2 and GS3 were seen in NIH3T3 cells, but were hardly seen in A204 cells. GS5 was only seen in A204. The band of GS1 in NIH3T3 cells was seen as a doublet (Fig. 4, lane 10; Fig. 6B, lane 9). Depending on the method of sample preparation, other complexes were sometimes seen. To examine the specificity of the bands, a competition assay was performed.

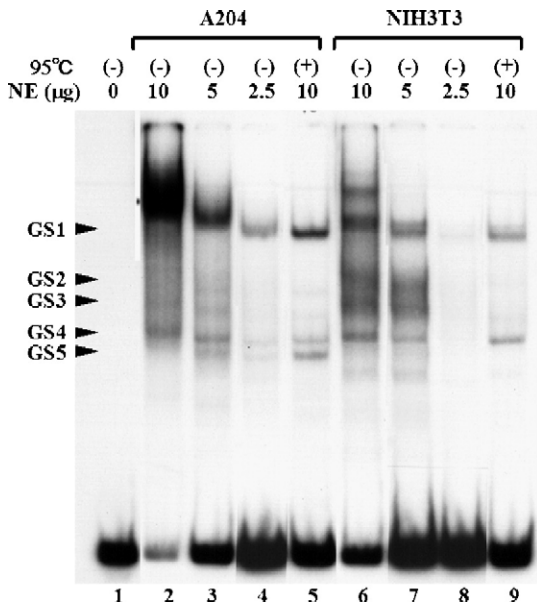


Fig. 7. The effect of the concentration of nuclear extract on the formation of the protein–DNA complex. The ^{32}P -labeled $-97/-31$ probe was incubated with nuclear extracts from A204 (lanes 2–5) and NIH3T3 cells (lanes 6–10). Lane 1 was performed without nuclear extracts. The concentration of nuclear extracts are 10 μg (lanes 2, 5, 6 and 9), 5 μg (lanes 3 and 7), and 2.5 μg (lanes 4 and 8). In lanes 5 and 9, the nuclear extract was treated with heating at 95 $^{\circ}\text{C}$ for 5 min before incubation with the probe.

(Fig. 5). However, GS2 and GS3 were not seen in NIH3T3 cells, suggesting that the proteins contained in the GS2 and GS3 complexes bind to the region from -59 to -31 bp.

To identify the binding site for the factors more closely, we prepared 6-bp mutant oligonucleotides in the region from -85 to -57 bp (Fig. 6A). In A204 cells, the mutant oligonucleotides, m2 and m3, failed to form, and m4 weakly formed GS1, GS4 and GS5 protein–DNA complexes (Fig. 6B, lanes 4–6). Similarly, in NIH3T3 cells, m2 and m3 failed to form, and m4 weakly formed protein–DNA complexes of the lower band of GS1 and GS4 (Fig. 6B, lanes 11–13). Next, mutant oligonucleotides were used as cold competitors (Fig. 6C). m2 and m3 hardly, and m4 partially, competed with isotope-labeled wild-type oligonucleotide, resulting in the formation of GS1, GS4 and GS5 in A204 cells (Fig. 6C, lanes 5–7), whereas m1 and m5 significantly, and wild-type completely, inhibited the formation of the GS1 and GS5 complexes. A similar tendency was seen towards blocking the formation of the lower band of GS1 in NIH3T3 cells (Fig. 6C, lanes 10–16). In both experiments, the data were consistent except for GS4. A competition assay showed that the sequence 5'-GCTCTCA-TATTCAGAA-3' (-79 to -63 bp) is critical for DNA–protein complex formation.

3.3. Characterization of the DNA-binding proteins

To characterize the DNA-binding proteins, we examined the influence of the concentration of nuclear extract, the heating and the detergent. Depending on the concentration,

the pattern of the bands changed. In high concentration, GS1 and GS5 formed complexes in A204 cells (Fig. 7, lane 2–4). The proteins of GS1, GS4 and GS5 in A204 cells and those of the lower band of GS1 and GS4 in NIH3T3 cells were resistant to heat (Fig. 7, lanes 5 and 9; Fig. 8, lane 3). However, following treatment with SDS after heating, the proteins of GS1, GS4 and GS5 in A204 cells completely lost their ability to bind DNA (Fig. 8, lane 4). Again, after adding an excess of Triton X-100, the proteins of GS1 and GS5, but not of GS4, gained the ability to bind. Similarly, proteins of the lower band of GS1 in NIH3T3 cells also gained the ability to bind following renaturation (Fig. 8, lane 6).

Finally, to estimate the molecular sizes of the DNA-binding proteins, the nuclear extract from A204 was fractionated by SDS-PAGE (Figs. 9A and B). After the proteins had been recovered from the gel and renatured, EMSAs were performed. The molecular sizes of GS1 and GS5 in A204 cells were estimated to be 92–118 kDa and 40–52 kDa, respectively (Fig. 9C, lanes 4 and 6). Other DNA binding proteins, designated as the GS6 complex, were also estimated at 36–40 kDa (Fig. 9C, lane 7).

4. Discussion

In the present study, we characterized the proximal promoter of the *COL3A1*. The 1.7-kb genomic fragment that covers the -1685 to $+68$ region has strong transcriptional activity. Transient transfection assays using a series of

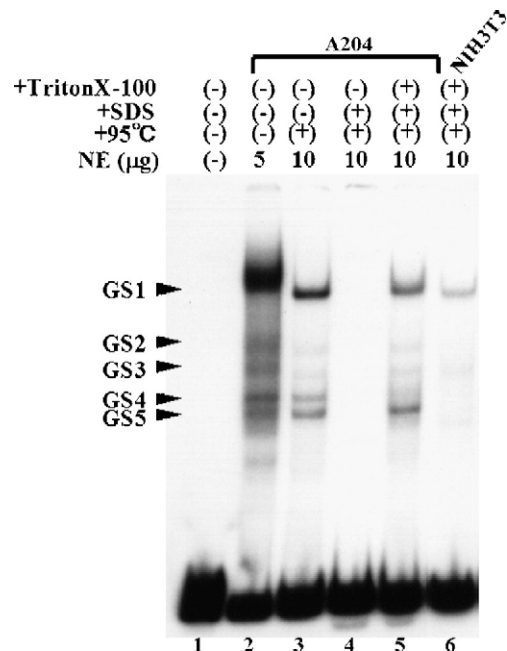


Fig. 8. The effect of various conditions for the DNA–protein complex. The ^{32}P -labeled $-97/-31$ probe was incubated with nuclear extracts from A204 (lanes 2–5) and NIH3T3 cells (lane 6). Lane 1 was performed without nuclear extracts. The nuclear extracts were treated with heating (lane 3), heating and 10% SDS (lane 4), and heating, 10% SDS and then renaturation with Triton X-100 (lanes 5 and 6), before incubation with the probe.

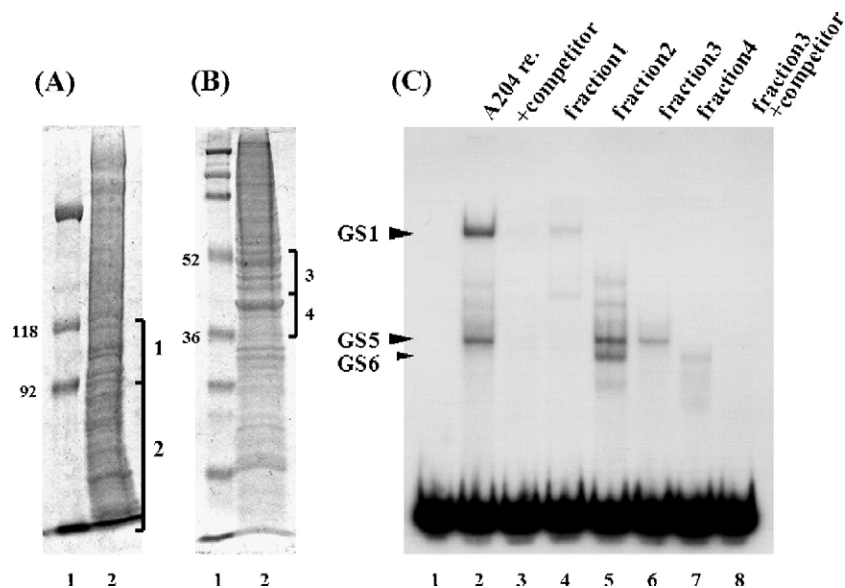


Fig. 9. EMSAs using the nuclear extracts that were fractionated with SDS-PAGE gels. The nuclear extract from A204 cells was run on 12.5% (A) or 5% (B) SDS-PAGE gel under a reducing condition and stained with Coomassie Brilliant Blue. Proteins in the nuclear extract were eluted from four slices (fractions 1–4) and renatured as described [15,20]. Lanes 1 and 2 in Panels (A) and (B) are protein size marker and sample lane, respectively. (C) EMSAs were performed. The samples are renatured nuclear extract (lane 2), renatured nuclear extract with competitor (lane 3), fraction 1 (lane 4), fraction 2 (lane 5), fraction 3 (lane 6), fraction 4 (lane 7) and fraction 3 with competitor (lane 8). Lane 1 was performed without nuclear extracts. Note that an additional DNA–protein complex, GS6, is seen in lanes 5 and 7.

chimeric constructs linked to the luciferase indicated that the segment from -96 to -34 is necessary for the activation of transcription. In particular, the region from -80 to -60 seems to be important for the activation in A204 cells, and that of the -90 to -70 NIH3T3 cells. A competition assay using normal and mutant oligonucleotides showed that the sequence $5'$ -GCTCTCATATTCAGAA- $3'$ (-79 to -63 bp) was critical for forming DNA–protein complexes in both cells. In NIH3T3 cells, this sequence was not consistent with the data of the luciferase assay, in that the -90 to -70 fragment, rather than the -79 to -63 fragment, was essential. We do not know the reason, but it may reflect the effect of cell type on the complexity of the regulation of this gene. This sequence is contained in the B element of the mouse gene reported by Ruteshous and de Crombrughe [15]. The DNA-binding protein of GS1 and GS5 in A204 cells, and the lower band of GS1 in NIH3T3 cells specifically bound to this region (Fig. 10). The proteins of GS1 and GS5 were heat-resistant and their molecular sizes were estimated at 92 – 118 kDa and 40 – 52 kDa, respectively (Fig. 9). By fractionation using SDS-PAGE, the small protein of the GS6 complex was also identified. However, we do not know whether it is specific for the sequence. The protein forming the GS1 complex is identical or similar to BBF reported by Ruteshous and de Crombrughe in terms of the molecular size and the character of heat resistance [15]. The protein of GS1 itself was found in many cells (data not shown). Therefore, it might be a ubiquitous protein. The complex of proteins, including this protein, could contribute to the specificity of gene expression. They also reported another upstream element, the A element, in the mouse gene.

However, we could not find an essential loss of transcriptional activity in the deletion of the element (Fig. 1). Three nucleotides in the A element region are different in the human and the mouse (Fig. 2). Therefore, the element may be important for the mouse gene not the human gene.

Initially, type III collagen was studied with regard to the tissue expression and function using biochemical and immunohistochemical techniques. It occurs in heterotypic fibrils with type I collagen such as fetal tissues and blood

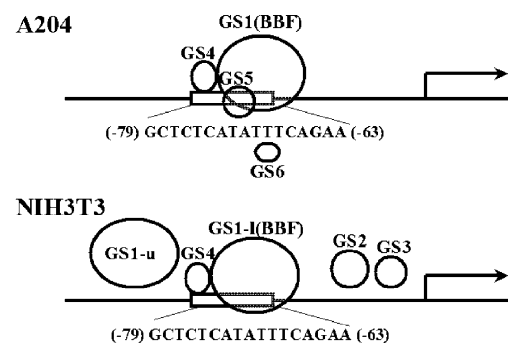


Fig. 10. Schematic summary of the DNA binding proteins in the proximal promoter of *COL3A1*. The proteins of the GS1 and GS5 complexes in A204, and the GS1-l complex in NIH3T3 cells specifically bind to the *COL3A1* promoter. The sequence $5'$ -GCTCTCATATTCAGAA- $3'$ (-79 to -63 bp) is critical for their binding. The protein of GS1 in A204 and the GS1-l complex in NIH3T3 cells is identical or similar to BBF reported by Ruteshous and de Crombrughe [15]. We do not know whether the protein of GS6 specifically binds to the region. The protein of GS4 non-specifically binds to the gene, but may tend to bind this sequence from the data shown in Figs. 6B and C. The proteins of the GS2 and GS3 complexes in NIH3T3 might bind to the region of -59 to -31 bp (Fig. 5, lane 10). Other proteins are known to non-specifically bind to the gene.

vessels. Both collagens are coordinately expressed, but the expression of the ratio varies spatially and temporally. It has been thought that the specific transcriptional events are involved in type III collagen gene. More than 10 years ago, Ruteshouse and de Crombrugge intensively characterized the basal promoter activity of *Col3a1*. However, the transcriptional mechanism of the type III collagen gene was poorly characterized. On the other hand, the study of the regulation of type I collagen has progressed during this period. The transcription of type I collagen genes is controlled by a series of complex interactions of positive and negative transcription factors. A number of regulatory elements that are required for constitutive expression have been identified, including the ubiquitous factors such as Sp1/Sp3 and CBF/NF-Y for basal activity of these promoters [22–25]. Other putative transcription factors, such as c-Krox, BFCOL1 and IF-1, are also involved in the transcriptional regulation of type I collagen genes [26–28].

The sequence of the B element is inconsistent with the consensus motif of CBF/NFY and Sp1. Indeed, competition and supershift assays excluded the possibility of CBF/NFY and Sp1 (data not shown). The Ku antigen, which has two components, Ku80 (80 kDa) and Ku (70 kDa), bound to the proximal promoter of *COL3A1* gene after induction with lysyl oxidase [29]. We tried to show binding of the Ku antigen at the basal level expression, but the protein–DNA complexes of GS1 to GS5 were not supershifted by the specific antibodies of the Ku antigen (data not shown). In addition to the proteins binding to the –79 to –63 region, our experiment suggests that there may be a repressor that binds downstream of this region (Fig. 3F). Transcription of type III collagen genes might be controlled by the interaction of positive and negative transcription factors. It seems to be more complicated than that of the type I collagen gene. The identification of the factors in the –79 to –63 region or the repressor should provide further information as to the regulation of type III collagen expression.

Acknowledgements

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