Original Article

Multifactor Complex Containing B Element Binding Factor, BBF, and Repressors Regulate the Human α1(Ⅲ) Collagen Gene (*COL3A1*)

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Type Ⅲ collagen is found in fetal skin and blood vessels. Previously, we characterized the proximal promoter of the human α1(Ⅲ) collagen gene (*COL3A1*) using the human rhabdomyosarcoma cell line, A204, and NIH3T3 cells (Yoshino *et al.*, Biochim Biophys Acta, 2005). In the present study, we further analyzed this promoter using additional cell lines, namely a human embryonal rhabdomyosarcoma cell line (RD) and bovine vascular smooth muscle cells (vSMCs), both of which show high expression of type Ⅲ collagen. Using a luciferase assay, electrophoretic mobility shift assays (EMSA), and DNase footprinting assay, 2 types of multifactor complexes were shown to bind to the DNA region in the vicinity of the B element (−80 to −58), depending on the cell type. Next, we used cells stably transfected with a GFP-linked type Ⅲ collagen promoter fragment for analysis of promoter expression. Usually, transfected cells retained the characteristics of the original cells. However, in several clones derived from RD cells, promoter expression as well as cell shape changed to patterns characteristic of the A204 cell line. Nuclear factors expressed by these clones were also characteristic of the A204 line.

Key words: type Ⅲ collagen, promoter, transcription, DNA binding protein

T ype Ⅲ collagen is found in many stromal connective tissues, especially in fetal skin and blood vessels [1–3]. It is also expressed during the repair process of wound healing. Patients with type Ⅳ Ehlers-Danlos syndrome, which have fragile blood vessels and skin, often have abnormality in the *COL3A1* gene [4, 5]. The phenotype of homozygous mutant mice deficient in type Ⅲ collagen gene resembles the clinical manifestations in human patients. The phenotypic similarities extend to sudden death due to rupture of the large vessels [6]. Type Ⅲ col-

lagen is coexpressed with type Ⅰ collagen, which is the most abundant member of the collagen family. Type Ⅲ collagen is a component of striated fibrils, along with type Ⅰ collagen [7]. Type Ⅲ collagen may be present on banded collagen fibrils regardless of the fibril diameter [8]. The abundance ratio of type Ⅲ to type Ⅰ collagen varies in different tissues and distinct developmental stages. It seems likely that the transcriptional controls on the type Ⅲ collagen gene are distinct from those operative on the type I collagen gene [9].

Ruteshouser and de Crombrugghe analyzed the proximal promoter of the mouse α1(Ⅲ) collagen gene (*Col3A1*) [10, 11]. They identified a factor that binds to the region between −83 and −61. The fac-

tor was a heat-resistant polypeptide named "B element binding factor" (BBF), with a molecular weight of approximately 95 kDa. Recently, we characterized the proximal promoter of the human *COL3A1* gene [12]. The segment from −96 to −34 was necessary for the activation of transcription. Multiple proteins probably including BBF were found to form a DNA-protein complex at −79 to −63. The binding proteins existed in different combinations in different cell types. The environmental condition of the nucleus also had an effect on which proteins were noted.

In the present study, we further characterized the proximal promoter of the *COL3A1* gene using cells that highly express type Ⅲ collagen. We also examined collagen promoter regulation in cells stably transfected with a Green Fluorescent Protein (GFP)-linked type Ⅲ collagen promoter fragment. Taken together, our data show the complexity of the regulation of the *COL3A1* gene.

Materials and Methods

Northern blotting and RNase protection assay.　Total cellular RNAs were isolated by acid guanidium phenol chloroform (AGPC) extraction as described previously [13], and 20 μg of total RNA was electrophoresed in 0.8% agarose gel under denaturing conditions, blotted onto Hybond N nylon filter (Amersham Biosciences, Piscataway, NJ, USA) and hybridized with a human $\alpha 1(Ⅲ)$ collagen probe [14]. RNase protection assays were also carried as described previously [15].

Cells and cell culture conditions.　Human rhabdomyosarcoma A204, human fibrosarcoma HT1080, human embryonal rhabdomyosarcoma RD and mouse NIH3T3 cells were purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Smooth muscle cell explants from the medial layer of the aorta of a calf were prepared as described previously [16]. Smooth muscle cells in the fifth to tenth passages were used in these experiments. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 ℃ in a humidified atmosphere of 5% CO_2 in normal air.

Construction of chimeric luciferase reporter plasmids.　Constructs in which various promoter fragments were tagged with the luciferase reporter were previously described [12]. All constructs shared the same 3' end at +68 and had internal 10 bp deletions.

Transient transfection and luciferase assays.　Transient transfection was performed by the calcium phosphate precipitation method as described previously [12]. Briefly, the cells were plated at a density of 1×10^5 cells in 24-well dishes with 400 μL of culture medium. After incubation for 1 d at 37 ℃, the cells were transfected with 1 μg of luciferase plasmid DNA plus 0.1 μg of *Renilla* pRL-SV40 vector (Promega, Madison, WI, USA) as an internal control. Six hours after transfection, the cells were rinsed with phosphate buffered saline (PBS), fed with fresh medium, and then further cultured for 2 d. 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, Wellesley, MA, USA) according to the manufacturer's protocol. The transcription activity of each chimeric construct was evaluated by
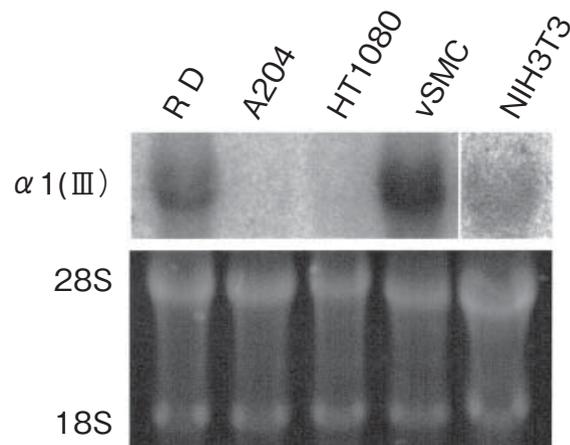
Fig. 1　Northern blot analysis to show *COL3A1* RNA expression in various cell types. Upper panel: RNAs were hybridized with an α1(Ⅲ) cDNA probe. Lower panel: the gel was stained with ethidium bromide to identify the locations of 28S and 18S rRNA. RD, human embryonal rhabdomyosarcoma cell line; A204, human rhabdomyosarcoma cell line; HT108, human fibrosarcoma cell line; vSMC, bovine vascular smooth muscle cell.
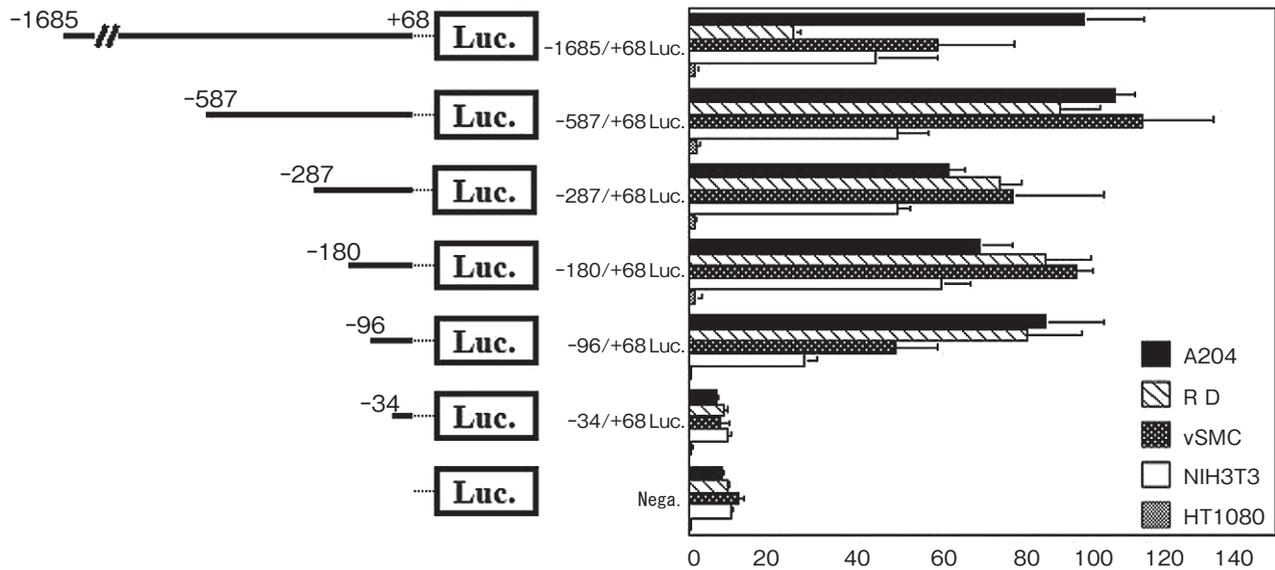
Fig. 2 Luciferase transcription activity of the *COL3A1* promoter using the 5'-deletion reporter constructs. Luciferase activities in A204, RD, vSMC, NIH3T3 and HT1080 cells were measured. The data for A204 cells have been previously published [12]. All the constructs were cotransfected with the pRL-SV40 vector as an internal control for transfection efficiency. Relative luciferase activities (%) were normalized to the activity of the − 1685 to + 68 *Luc* construct and transfected into A204 cells. Data are the means ± SD values of at least 3 independent experiments.
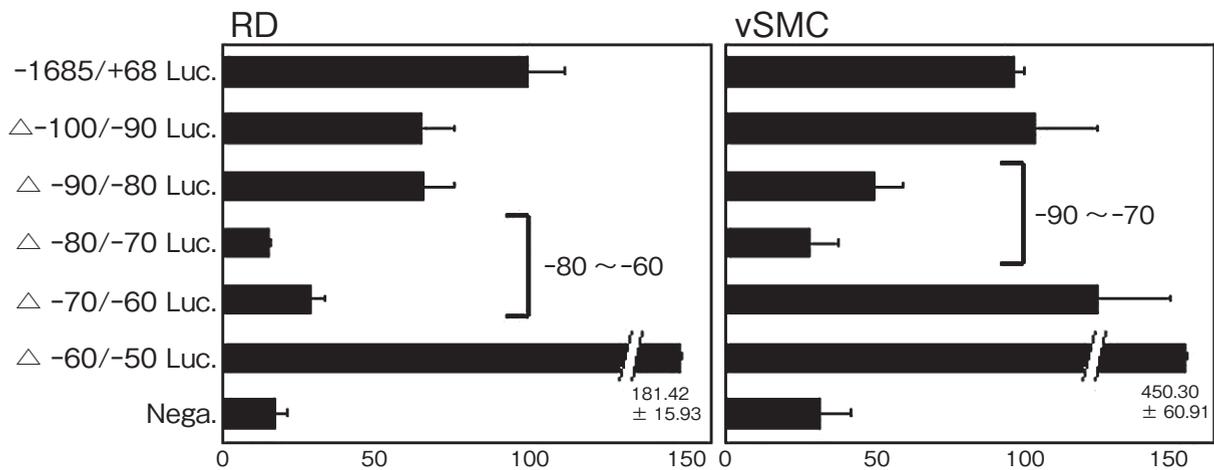
Fig. 3 Luciferase transcription activity of the *COL3A1* promoter in RD and vSMC cells using the internal deletion constructs. All the constructs were cotransfected with the pRL- SV40 vector as an internal control for transfection efficiency. Relative luciferase activities (%) were normalized against the activity of the − 1685 to + 68 Luc construct transfected into each cell. Data are the means ± SD values of at least 3 independent experiments.

means of the *Renilla* luciferase activity to normalize for the transfection efficiency. Results were expressed as the means ± SD of at least 3 independent experiments.

Preparation of nuclear extracts and EMSA. Nuclear extracts were prepared from various cells as described previously [17]. The concentration of protein was determined by Bio-Rad

protein assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin as a standard.

Double-stranded oligonucleotides used for EMSA were described previously [12]. Isotope-labeled probes (4×10^4 cpm) were incubated with various amounts of nuclear extract (5–10 μg) for 30 min at 25 ℃ in the presence of 3 μg of poly (dI-dC).

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 an imaging analyzer (BAS2000, Fuji Film, Tokyo, Japan).

DNase I footprinting assay.   DNase I footprinting was performed according to the protocol described by Galas *et al.* [18] with some modifica-

tions. Briefly, the 3' end-labeled probe (50,000 cpm) was incubated at 25 ℃ for 30 min with 50 μg of nuclear extracts (or BSA as control) in a 100 μl reaction mixture containing 20 mM Tris-HCl (pH 7.9), 3 mM MgCl₂, 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5 μg of BSA, and 4 μg of poly (dI-dC). After the addition of 5 U of DNase Ⅰ (or 0.1 U with BSA as control) to the mixture, the incubation was continued for 5 min at 25 ℃. The reaction was stopped by adding 100 μl of a stop buffer (200 mM sodium acetate, 20 mM EDTA, 1% SDS, 10 μg 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 analyzed with the imaging analyzer.

Stable transfection of promoter-GFP constructs.   A fragment covering the −1685 to +68 bp region of the type Ⅲ collagen promoter was excised from a −1685 to +68-luciferase gene construct, and subcloned into GFP vectors containing either a neomycin-resistance gene or a blasticidin-
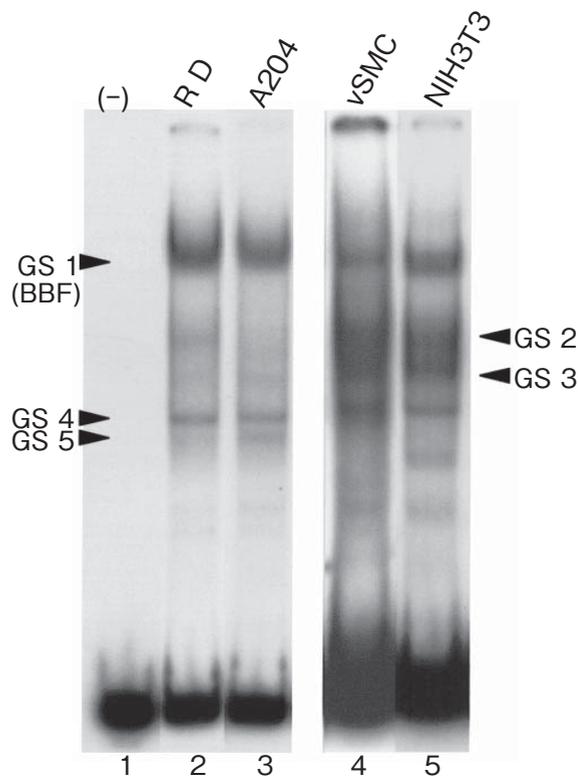
Fig. 4   EMSA analysis of the *COL3A1* promoter. The [³²P]-labeled −97 to −31 probe was incubated with nuclear extracts from RD, A204, vSMC and NIH3T3 cells. The DNA-protein complexes are the GS1(BBF), GS2, GS3, GS4 and GS5 bands, which are indicated by arrowheads. Lane 1 contains no nuclear extract. Extra bands were sometimes seen depending on the preparation of the nuclear extracts.
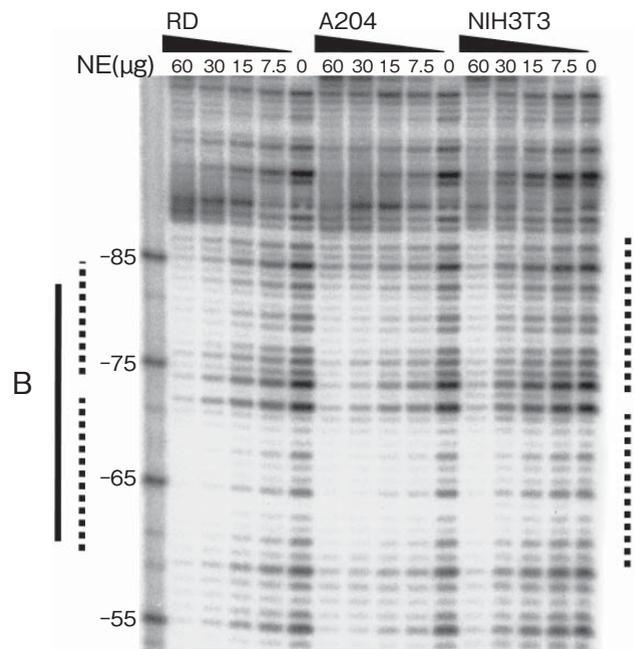
Fig. 5   DNase I footprinting analysis of the region around the B element of the *COL3A1* promoter. The [³²P]-labeled −97 to −31 probe was incubated with RD, A204 and NIH3T3 nuclear extracts. B: B element. Dotted lines indicate protected regions.

resistance gene. The constructs were transfected into A204 and RD cells, and stably transformed cells were selected with G418 or blasticidin.

Microscopy. Cultured cells were observed using an inverted microscope (TE2000-U, Nikon, Tokyo, Japan). Stably transfected cells were identified and photographed using a microscope-attached camera (Olympus BX-50, with the Keyence VB-6000/6010 microscope).

Results

COL3A1 gene expression. We examined the expression of the type III collagen gene in several cells using Northern blot analysis (Fig. 1). The expression was abundant in RD and vSMC cells. Low-level expression was noted in NIH3T3 cells, and no expression took place in A204 cells, although a high level of transcriptional activity was seen (Fig. 1 and Fig. 2). Gene expression was never detected in HT1080 cells.

Characterization of the COL3A1 proximal promoter. Previously, we reported that the region from -96 to -34 was essential to transcriptional activity in A204 and NIH3T3 cells, which we determined by using 6 constructs harboring progressive
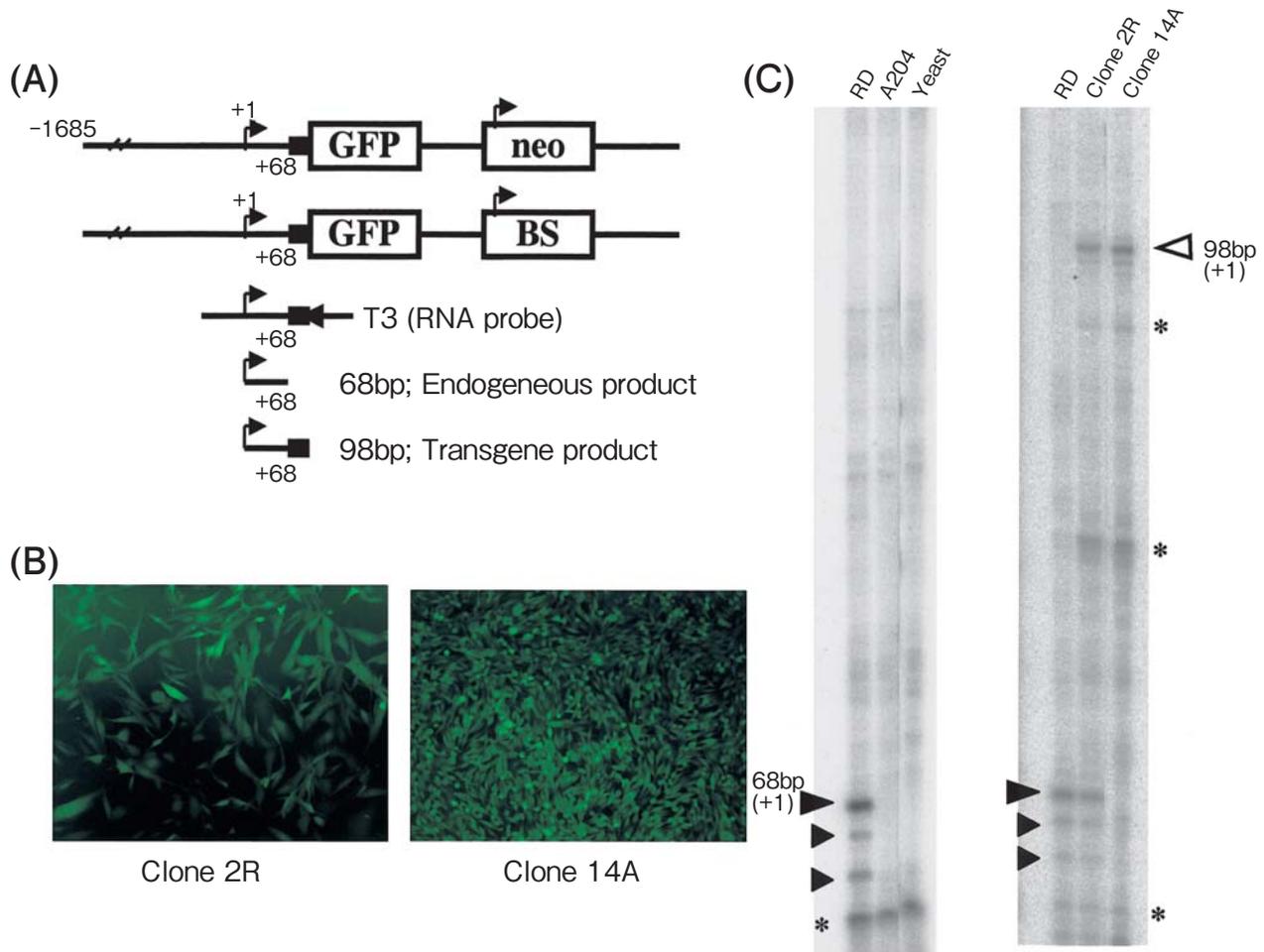
Fig. 6 Stable transfection with a GFP-linked *COL3A1* promoter. (**A**) Schematic illustration of constructs of the GFP-linked *COL3A1* promoter, the RNA probe, and gene products. (**B**) Stably transfected cells. Note that clone 2R and clone 14A derived from RD and A204 cell lines, respectively, show expression of type III collagen. (**C**) RNase protection assay using stably transfected cells. Large and small closed triangles, an open triangle, and asterisks indicate major and minor endogenous gene products, the transgenic products, and nonspecific bands. Note that 2 minor transcripts from the endogenous gene were seen in the RD cells and in clone 2R.

5'-deletions of the 1.7 kb promoter fragment [12]. The data from RD and vSMC cells were consistent with those previously reported. No promoter activity, from any of the 6 constructs, was detected in HT1080 cells (Fig. 2).

Previously, we noted that the promoter activity was located at the region from −80 to −60 in A204 cells, and from −90 to −70 in NIH3T3 cells, and suggested that a repressor was bound downstream of the B element in these cells [12]. In the present study, we examined the promoter activities in the same regions of RD and vSMC cells, both of which show high expression of type Ⅲ collagen (Fig. 3). Using the promoter constructs bearing the 10 bp internal deletion, we found that promoter activity decreased when the −80 to −60 region was removed

from RD cells, and when the −90 to −70 region was excised from vSMC cells. Promoter activity increased when the −60 to −50 region was eliminated from RD cells, and when the −70 to −50 segment was excised from vSMC cells. The promoter activity patterns shown by RD and vSMC cells were similar to those exhibited by A204 and NIH3T3 cells, respectively [12]. EMSA analysis using a [^{32}P]-labeled −97 to −31 probe showed 3 bands in A204 cells and 5 bands in NIH3T3 cells [12]. The pattern shown by RD cells was similar to that demonstrated by A204 cells, although band GS5 was absent in RD cells (Fig. 4, lanes 2 and 3). On the other hand, vSMC cells showed a pattern similar to that of NIH3T3 cells (Fig. 4, lanes 4 and 5). In a DNase footprinting experiment, protected areas
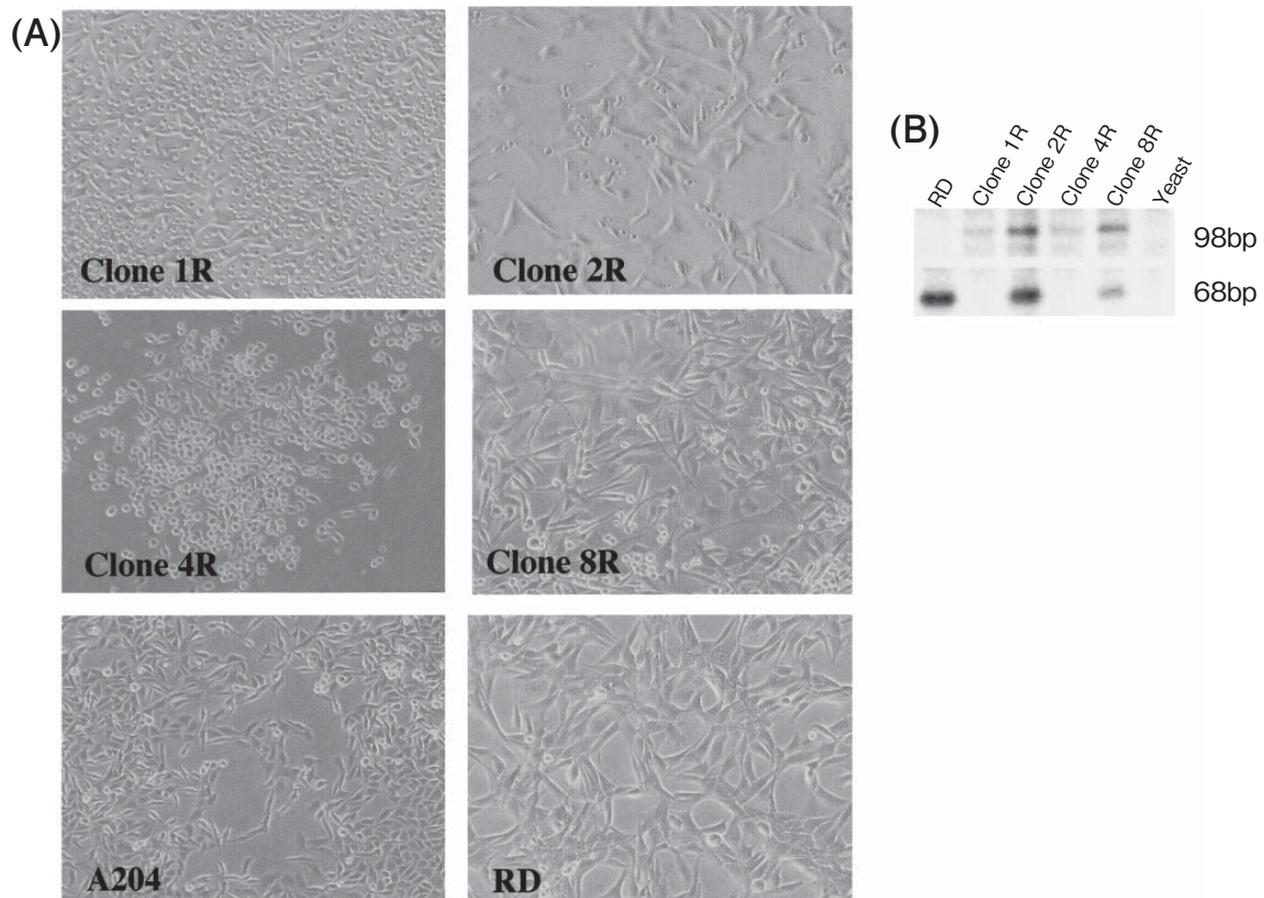
Fig. 7   Stable transfection with a GFP-linked *COL3A1* promoter. (A) Stably transfected cells. Clones 1R, 2R, 4R and 8R were derived from RD cells. Note that the cell shapes of clones 1R and 4R are similar to that of A204 cells, but not to that of RD cells. (B) RNase protection assay using stably transfected cells. Data from clones 1R, 2R, 4R and 8R are shown.

gradually appeared from -83 to -75, and from -72 to -59, both in A204 and RD cells, whereas the protected areas were located from -90 to -75 and from -72 to -59 in NIH3T3 cells (Fig. 5).

Analysis using cells stably transfected with a GFP-linked COL3A1 promoter.   To study a chromosomally-inserted type Ⅲ collagen promoter, we stably transfected GFP-linked promoter fragments (Fig. 6A) into A204 and RD cells. Clone 2R and clone 14A that were derived from RD and A204 cells, respectively, resembled the original cells morphologically (Fig. 6B). Both the endogenous and transgenic type Ⅲ collagen promoters were active in clone 2R, while only the transgenic promoter functioned in clone 14A (Fig. 6C). However, several clones such as 1R and 4R derived from RD cells showed morphological changes (Fig. 7A). The endogenous gene was nonfunctional in these clones (Fig. 7B). We also examined DNA-protein complexes in the nuclear extract of clone 1R using [32P]-labeled -97 to -31 oligonucleotides. Complexes of GS1, GS4 and GS5 were seen in clone 1R (Fig. 8). The pattern was similar to that shown by A204 cells, and differed from that of the clone parent, the RD cell line.

Discussion

Previously, we characterized the proximal promoter of the *COL3A1* gene [12]. The DNA fragment from -96 to -34 is necessary to activate transcription. In the present study, we confirmed that this region is important in RD and vSMC cells, both of which show high expression of type Ⅲ collagen. Using a luciferase assay, an electrophoretic mobility shift assay, and a DNase footprinting assay, we showed that 2 types of multifactor complex bind to the DNA region in the vicinity of the B element. Taken together with previous data [12], these findings show that the complexes in A204 and RD cells bind to the -80 to -60 region, while those of NIH3T3 and vSMC cells attach to DNA in the -90 to -70 area [12] (Fig. 3).

To explain the discrepancy between Northern blot information and luciferase assay data using A204 cells (Fig. 1 and Fig. 2), we determined the sequences of the proximal promoters in A204 and RD cells. No differences were found (data not

shown). Next, we examined the possible influence of DNA methylation on promoter expression. We treated A204 cells with the DNA methyltransferase inhibitor, 5-azacytidine [19]. The expression of the type Ⅲ collagen gene was not affected by this drug (data not shown). We considered other influences on promoter expression. Using transfection with a GFP-linked type Ⅲ collagen promoter fragment, we obtained stably transfected cells that retained the character of the original cells in terms of cell shape and gene expression (Fig. 6B and 6C). However, in
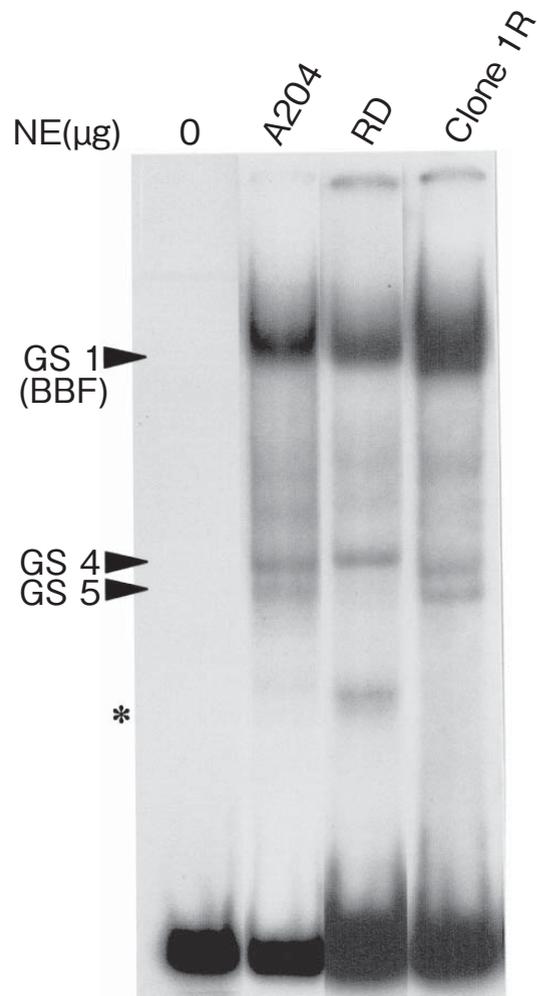
Fig. 8   EMSA analysis using nuclear extracts from A204 cells, RD cells and clone 1R. The [32P]-labeled -97 to -31 probe was incubated with nuclear extracts from A204 cells, RD cells, or clone 1R cells. The DNA-protein complexes are the GS1 (BBF), GS4 and GS5 bands indicated by arrowheads. An asterisk indicates a nonspecific band. Lane 1 contains no nuclear extract.
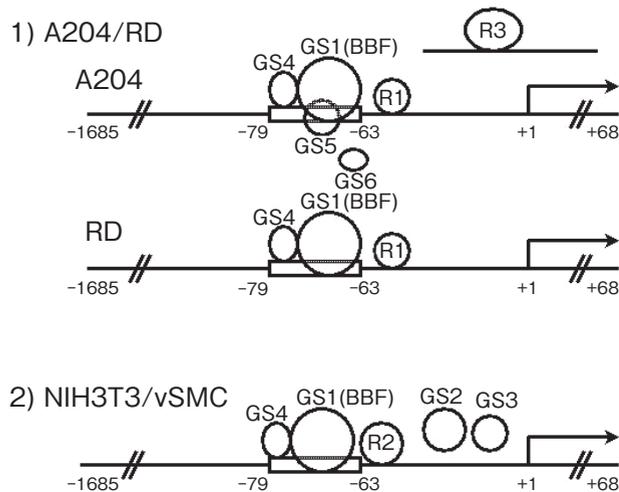
Fig. 9   Schematic summary of proteins binding to DNA in the proximal promoter region of the *COL3A* gene. The overview includes data from a previous study [12] and from the present study. R1, R2 and R3 are hypothetical repressors.

several clones derived from RD cells, the cell character seemed to alter to become that of A204 cells (Fig. 7 and Fig. 8). Morphologically, the cells changed from spindle-shaped to round. The endogenous type III collagen gene was not expressed and the GS5 band was noted in the DNA-protein complex bound to the B element. These data suggest that A204 cells express a specific repressor that binds to some region upstream of -1685 or downstream of $+68$ in the endogenous gene. We do not know if this unknown repressor functions alone or interacts with other factors such as GS5, which is also specifically expressed in A204 cells.

   In the collagen family, the regulation of type I collagen expression has received much attention. 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 have been identified, including ubiquitous factors such as Sp1/Sp3 and CBF/NF-Y for basal activity [20, 21]. Other factors, such as c-Krox, BFCOL1 and IF-1, are also involved in the transcriptional regulation of type I collagen genes [22–24]. In the *COL3A1* gene, repressors seem to bind downstream of the B element (Fig. 3), and to the distal region from the proximal promoter in A204 cells, as mentioned above. Tissue expression of type III collagen is similar to that of type I collagen.

However, transcriptional control of the type III collagen gene appears to be distinct. A unique range of complexes consisting of both positive and negative transcription factors may be involved in *COL3A1* gene regulation (Fig. 9).

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