The transcription of human α1(I) procollagen gene (COL1A1) is suppressed by tumour necrosis factor-α through proximal short promoter elements: evidence for suppression mechanisms mediated by two nuclear-factor-binding sites

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Recent studies have demonstrated that tumour necrosis factor-α (TNF-α) decreases α1(I) procollagen gene (COL1A1) expression in cultured human dermal fibroblasts. The purpose of this study was to analyse the transcriptional control of COL1A1 by TNF-α. Cultured human dermal fibroblasts were transiently transfected with plasmids containing 5′ flanking sequences of COL1A1 fused to the chloramphenicol acetyltransferase (CAT) gene, and were incubated for 48 h in medium with or without TNF-α. TNF-α inhibited the CAT activity of fibroblasts transfected with plasmids containing 2.3 kb of 5′ flanking sequences of COL1A1, whereas the activity of control plasmids containing the herpes simplex thymidine kinase promoter gene (pBLCAT) was unaltered. A series of deletion constructs or various small substitution mutations of the COL1A1 5′ flanking region fused to the CAT gene were also transfected, and CAT activity was measured after incubation with TNF-α. TNF-α suppressed COL1A1 promoter activity through proximal short promoter elements containing only 107 bp. Short substitution mutations between -101 and -97 bp or between -46 and -38 bp abolished TNF-α suppression of COL1A1 promoter activity. DNA–protein complex formation was observed involving both sites in gel retardation assays. These results suggest that TNF-α suppressed COL1A1 promoter activity through elements located between -101 and -97 bp and between -46 and -38 bp of the COL1A1 promoter, and that the suppression involved DNA–protein interactions.

INTRODUCTION

Type I collagen, a major protein product synthesized by fibroblasts, is the most abundant protein in bones, tendons and dermis and the most abundant fibrillar collagen in a number of other connective tissues, such as cornea and blood-vessel walls. It is composed of two α1 chains and one α2 chain, which interact with each other to form a characteristic triple helix. Regulation of type I collagen biosynthesis plays an important role in many physiological and pathological events. Although some studies showed translational control of type I collagen gene expression [1], most evidence indicates that the expression of type I collagen genes is regulated at the level of transcription. Changes in the synthesis of type I collagen occur during embryonic development, wound healing and in various forms of fibrosis [2,3]. Recently, many cytokines have been shown to cause specific effects on the expression of genes encoding connective tissue components, especially collagen. Several studies have demonstrated that the synthesis of type I collagen can be modulated *in vitro* by numerous cytokines, including transforming growth factor-β (TGF-β) [4,5], interleukin 1 [6], γ-interferon [7,8] and tumour necrosis factor-α (TNF-α) [9,10].

To understand how collagen transcription is selectively controlled in physiological and pathological conditions, a number of studies have recently focused on the identification and characterization of *cis*-acting DNA regulatory elements in mammalian collagen genes [11–15]. More recently, progress has been accomplished in characterizing some of the *trans*-acting nuclear factors that bind to collagen gene regulatory sequences [16–18]. Induction of type I collagen gene expression by TGF-β has been investigated in more detail [19–22].

TNF-α is a cytokine mainly produced by monocytes/macrophages that is able to modulate the biological functions of a large variety of cells and to cause pleiotropic effects, including a potent inhibition of collagen gene expression in dermal fibroblasts [9,10,23]. Levels of α1(I) procollagen mRNA have been shown to be down-regulated by incubation of dermal fibroblasts with TNF-α [9,10], and reduced transcription levels of the gene in nuclear run-off assays have been reported [24]. However, the *cis*-acting DNA regulatory elements of the human α1(I) procollagen (COL1A1) gene promoter responsible for the down-regulation of transcription by TNF-α have not been elucidated. In the present study, we analysed the regulation of the COL1A1 promoter by TNF-α, employing DNA-mediated transfection experiments with chimaeric gene constructs containing the promoter of COL1A1 and various deletions and substitution mutations of the regulatory elements of the gene. We found that TNF-α suppresses the transcription of COL1A1 and that the suppression was mediated by two *cis*-acting elements contained within the proximal short promoter region of the gene.

Abbreviations used: COL1A1, the gene coding for the α1(I) collagen chain; CAT, chloramphenicol acetyltransferase; TNF-α, tumour necrosis factor-α; TGF-β, transforming growth factor-β; DTT, dithiothreitol; NF, nuclear factor; CBF, CCAAT-binding factor.

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We also observed that the binding activities of two factors that recognize sequences within these elements were altered by incubation of cultured fibroblasts with TNF-α.

MATERIALS AND METHODS

Human recombinant TNF-α was donated by Dainippon Pharmaceutical Co. Ltd. (Osaka, Japan). The concentrations of TNF-α used in this study were 0.1–10 ng/ml, and a concentration of 1 ng/ml TNF-α was employed in most of the experiments.

Fibroblast cultures

Fibroblast strains were established from skin biopsies obtained from three healthy volunteer subjects. Primary cultures of dermal fibroblasts were established by an out-growth method, as described previously [25]. The fibroblast cultures were maintained in Dulbecco's modified Eagle's medium with 10 % inactivated fetal calf serum in a CO_2 incubator at 37 °C. The cells were used in early passage (third to ninth).

DNA constructs

The construction of plasmid p2.3Kα1(I)CAT has been described previously [20]. The plasmid contains the human COL1A1 promoter region encompassing −2.3 kb to +42 bp fused to the CAT gene. Plasmid p804α1(I)CAT was constructed by digestion of plasmid p2.3Kα1(I)CAT with *Not*I. Plasmid p332α1(I)CAT was produced by digestion of p804α1(I)CAT with *Xba*I. A deletion from the *Xba*I site at −332 bp in p332α1(I)CAT towards the transcription-initiation site was made employing the exonuclease III digestion procedure of Henikoff [26]. The exact deletion end-point was determined by sequencing, and a clone with a 5′ end-point at −194 bp was selected for subsequent studies. Plasmid p107α1(I)CAT was constructed by digestion of p332α1(I)CAT with *Alu*I (Figure 1). All deletion mutations were selected in a way that preserved the DNA sequences around the CCAAT box (−101 to −97 bp) and the TATA box (−28 to −22bp). Four different short substitution mutations (M1, M3, M4 and M5) were constructed in the wild-type COL1A1 promoter region spanning nucleotides −332 to +42, using synthetic double-stranded oligonucleotides located between two appropriate restriction sites, and were linked to the CAT gene (see Figure 4). The mutant M2 construct was obtained by digestion of p332α1(I)CAT with *Eag*I, followed by digestion with exonuclease III and mung bean nuclease. Exact deletion points were verified by DNA sequencing. The plasmid pBLCAT [27], containing the herpes herpes simplex thymidine kinase promoter fused to the CAT gene, was used as a positive control in each experiment. The plasmid pLTRAP, containing the Rous sarcoma virus long-terminal-repeat gene fused to the human placental alkaline phosphatase gene, was constructed from the plasmid RCASBP/AP [28] by deleting *Xho*I–*Xho*I fragments.

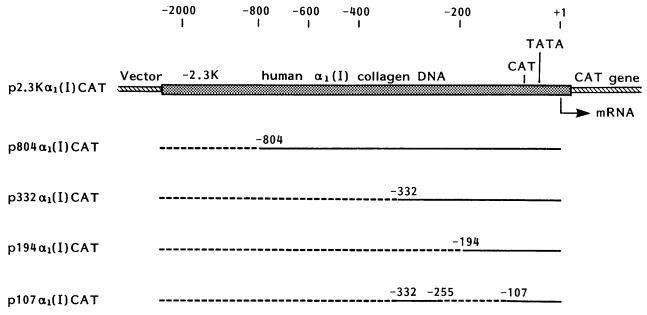

Figure 1 Diagram showing the recombinant plasmids containing various deletions in the promoter region of the human COL1A1 gene fused to the CAT gene

The details of preparation of each construct are described in the Materials and methods section. The following restriction sites were utilized in the preparation of these constructs: *Not*I at −2292 bp, *Hin*dIII at −804 bp, *Xba*I at −332 bp and *Alu*I at −107 bp. The sequence of the construct with a 5′ end point at −194 bp was verified by DNA sequencing.

Transfections and TNF-α treatment of cultured fibroblasts

A total of 4.5×10^5 cells was plated in 100-mm-diam. dishes and cultured as described above. Transfections were performed 24 h later, employing the calcium phosphate co-precipitation method, as described previously [29]. Cells were co-transfected with 20 μg of COL1A1-CAT plasmid DNA or with pBLCAT plasmid DNA and 1 μg of the pLTRAP plasmid DNA as an internal standard to monitor for tranfection efficiency. At a time of 4 h after addition of the DNA, the cells were shocked with 5 ml of 15 % glycerol in Hepes-buffered sodium phosphate solutions for 1 min. Then, the glycerol shock solutions were removed and fresh medium containing 10 % fetal calf serum and human recombinant TNF-α was added. Control cells incubated in parallel received culture medium without TNF-α. The control and TNF-α-treated cells were incubated for an additional 48 h and then were harvested for assays of CAT and alkaline phosphatase activities as described below.

Assays of CAT and alkaline phosphatase activities

Cell extracts were prepared by mechanically detaching the cells in 2.5 ml of 10 mM Tris/HCl (pH 7.8)/2.7 mM NaCl/1 mM EDTA and by centrifugation at 5000 ***g*** at 4 °C for 5 min. The cell pellets were resuspended in 150 μl of 0.25 M Tris/HCl (pH 7.5)/0.1 % Triton X-100 and were lysed by three cycles of freeze–thawing. CAT activity in the cell extracts was determined employing [^{14}C]chloramphenicol according to the method described by Gorman et al. [29]. The conversion of chloramphenicol into acetylated forms was quantified by scraping the corresponding areas from the TLC plates and measuring radioactivity by liquid scintillation spectroscopy. CAT activities were expressed as d.p.m. of acetylated chloramphenicol/h per mg of protein. Approx. 2.7×10^5 d.p.m. was used for each assay. Alkaline phosphatase activity was assayed with 10 μl of the extracts, as described by Yoon et al. [30].

Nuclear-extract preparation

For the preparation of nuclear extracts, confluent cells cultured in 150 mm × 25 mm dishes were washed twice with PBS and harvested. The nuclei were isolated according to the method of Groudine et al. [31]. The nuclei were then suspended in 4 vol. of 20 mM Hepes, pH 7.9, containing 1 mM EGTA, 0.5 mM dithiothreitol (DTT), 400 mM NaCl, 5 % (v/v) glycerol, 0.01 % Nonidet P-40, 0.5 mM PMSF, 10 μg/ml leupeptin and 10 μg/ml pepstatin A. The mixture was stirred for 30 min at 4 °C and was centrifuged for 5 min at 4 °C in an Eppendorf centrifuge. The supernatants were dialysed for 4 h against a buffer consisting of 20 mM Hepes, pH 7.9, 50 mM NaCl, 0.1 mM Na_2EDTA, 0.5 mM DTT, 0.5 mM PMSF and 20 % (v/v) glycerol, and were centrifuged for 5 min at 4 °C in an Eppendorf centrifuge. The supernatants were used as nuclear extracts. Protein concentrations were assayed with a protein assay (Bio-Rad Laboratories, Richmond, CA, U.S.A.) [32]. The protein concentration of the extract was about 2 mg/ml, and the extracts of each fibroblast

strain were adjusted to a 1 mg/ml protein concentration with the same buffer that was used for dialysis.

Gel retardation assay

The assay was performed as previously described [12]. Briefly, 1–2 μl of nuclear extracts containing between 1 and 4 μg of total protein were incubated for 20 min at 26 °C in 10 μl of a mixture containing 20 mM Hepes, pH 7.5, 0.5 mg/ml BSA, 150 mM NaCl, 1 mM Na_2EDTA, 0.5 mM DTT, 2.5 μg of poly(dI–dC) and 104 c.p.m. (approx. 0.2 ng) of [^{32}P]-labelled double-stranded oligonucleotides labelled at the 5′ end by T4 polynucleotide kinase. Following the binding reaction, 2 μl of a sample buffer consisting of 30 % (v/v) glycerol, 0.2 % (w/v) Bromophenol Blue and 0.2 % (w/v) Xylene Cyanol was added, and the DNA–protein complexes were separated by electrophoresis in a 6 % polyacrylamide gel in TBE buffer (89 mM Tris, 89 mM boric acid and 8 mM Na_2EDTA). Then the gel was submitted to fluorography, and the fluorograph was scanned and densitometrically analysed with a Fujix Bas 2000 bioimaging analyser (Fuji, Tokyo Japan).

RESULTS

TNF-α suppresses COL1A1 promoter activity in dermal fibroblasts

The human COL1A1 promoter–reporter gene construct p2.3Kα1(I)CAT, which contains 2.3 kb of 5′ flanking sequences of COL1A1 fused to the CAT gene, was used in this study for transient cell transfections to determine the effects of TNF-α on its promoter activity. After transfection and incubation with TNF-α, the fibroblasts were harvested and the CAT activity was measured as an index of promoter activity. The effects of TNF-α on COL1A1 promoter activity were first tested by adding various concentrations of this cytokine to the culture medium. In previous studies it was shown that treatment of human dermal fibroblasts with various non-cytotoxic doses (0.1–1000 ng/ml) of TNF-α significantly decreased collagen production [9]. Therefore in this study we tested the effects of increasing concentrations of TNF-α (0.1, 1 and 10 ng/ml) on the promoter activity of COL1A1. Quantification of the results revealed that CAT activity was decreased in a dose-dependent manner at the concentrations of 0.1 and 1 ng/ml TNF-α (Figure 2). Increasing the concentration of TNF-α to 10 ng/ml did not further decrease COL1A1 promoter activity (results not shown). On the other hand, the CAT activity driven by pBLCAT, a positive-control plasmid containing the herpes simplex thymidine kinase promoter gene, was unchanged in transfected fibroblasts incubated with either 0.1, 1 or 10 ng/ml TNF-α. Thus, 1 ng/ml of TNF-α was used in all subsequent experiments.

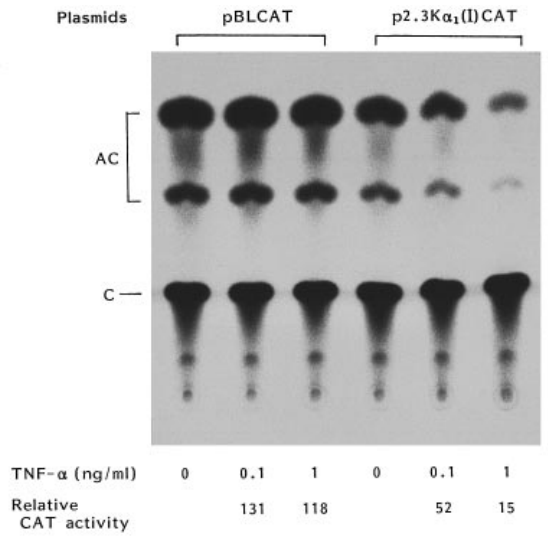

Figure 2 Effects of TNF-α on the activity of the transcriptional regulatory elements of the human COL1A1 gene in transfected cultured fibroblasts

Cultured human dermal fibroblasts were transiently co-transfected with either p2.3Kα1(I)CAT or pBLCAT (as a positive-control plasmid) and with the Rous sarcoma virus long-terminal-repeat–alkaline phosphatase construct pLTRAP (as an internal standard) and were incubated in Dulbecco's modified Eagle's medium containing 10% serum, with or without TNF-α, for 48 h. The CAT and alkaline phosphatase activities were determined as described in the Materials and methods section. CAT activity in TNF-α-treated cells is expressed relative to the CAT activity in untreated cells (100%). The acetylated (AC) and non-acetylated (C) chloramphenicol were separated by TLC.

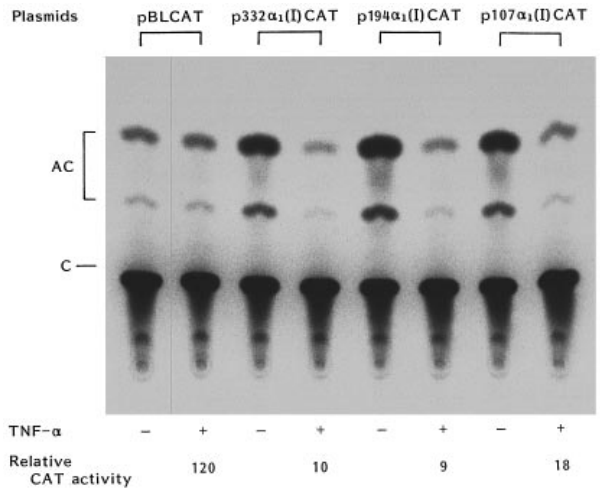

Figure 3 Regulation of the human COL1A1 promoter activity by TNF-α

A representative CAT assay demonstrating the effect of TNF-α on three deletion constructs of plasmid 332α1(I)CAT is shown. Human dermal fibroblasts were transfected with plasmids p332α1(I)CAT, p194α1(I)CAT, p107α1(I)CAT or pBLCAT (as a positive control). Following a 48-h incubation with or without TNF-α, CAT activity in the cell extracts was determined as described in the Materials and methods section. Values are expressed relative to the CAT activity in untreated cells. The acetylated (AC) and non-acetylated (C) chloramphenicol were separated by TLC.

Effects of TNF-α on the transcriptional activity of deletion derivatives of COL1A1 promoter

In order to determine the regions of COL1A1 promoter that contain elements responsive to TNF-α, the effects of TNF-α on the CAT activity of fibroblasts transfected with COL1A1 promoter and various deletion constructs were investigated. The promoter activity of the 2300 bp COL1A1 construct was decreased by approx. 80 % in fibroblasts treated with 1 ng/ml TNF-α. Similar results were obtained when fibroblasts transfected with either 804 or 332 bp of the COL1A1 promoter fused to the CAT gene were treated with TNF-α (results not shown). TNF-α also decreased the CAT levels of fibroblasts transfected with plasmids containing COL1A1 promoter deletions with 5′ end-points at −332 bp, −194 bp or −107 bp with TNF-α (Figure 3).

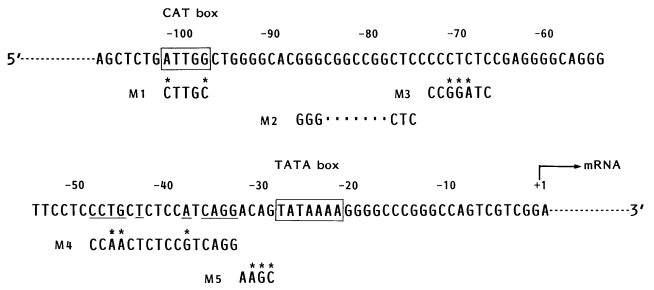

Figure 4 Mapping of the TNF-α-responsive region in the human COL1A1 promoter sequence

Map of the human COL1A1 promoter (from nucleotides −107 to +1) with indicated positions of the CCAAT (CAT) and TATA boxes. The positions of each substitution mutation, indicated by asterisks (M1, M3, M4 and M5), and the deletion, indicated by dots (M2), are shown under the relative positions of the wild-type gene. The underlined nucleotides represent an inverted-repeat element.

Localization of COL1A1 promoter regions responsive to TNF-α

Because the results described above indicated that TNF-α reduced COL1A1 transcription through a region encompassing only −107 bp of COL1A1 promoter, we determined the effects of TNF-α on CAT activity in fibroblasts transfected with COL1A1 promoter constructs with small substitution mutations in the short proximal promoter region (Figure 4). Treatment with TNF-α did not decrease the CAT activities of fibroblasts transfected with plasmids containing either a −101 to −97 bp (CCAAT box) substitution (pM1) or a −46 to −38 bp substitution (pM4). In contrast, the CAT activities of cells transfected with plasmids containing a −84 to −78 bp deletion (pM2), a −71 to −69 bp substitution (pM3) or a −31 to −29 bp substitution (pM5) were decreased by treatment with TNF-α (Figure 5). Quantification of the data revealed that the ratio of CAT activity in TNF-α-treated fibroblasts to CAT activity in control fibroblasts (without TNF-α), each with plasmids containing mutations pM1 and pM4, was significantly higher than the ratio of CAT activities in cells transfected with a wild-type plasmid compared with control fibroblasts (Table 1).

Nuclear factor binding to COL1A1 regions responsive to TNF-α

In order to elucidate the occurrence of *trans*-acting protein–DNA interactions that may participate in the suppressive effects of TNF-α on COL1A1 promoter activity, gel retardation assays with promoter constructs encompassing the TNF-α-responsive regions were performed. The assays were performed employing a 48 bp double-stranded oligonucleotide containing sequences encompassing −91 to −110 bp and nuclear extracts from untreated dermal fibroblasts. A major retarded band indicating the formation of DNA–protein complex was observed (Figure 6A). The complex formation was competed by addition of excess amounts of wild-type unlabelled oligonucleotide encompassing −91 to −110 bp but was not competed by addition of the same fragment containing the 2 bp substitution mutation (M1) shown in Figure 4 (Figure 6A). No qualitative or quantitative differences in the formation of this DNA–protein complex were noted when the binding of nuclear extracts prepared from untreated or TNF-α-treated cells to the oligonucleotide encompassing sequences −91 to −110bp was examined (Figure 6B). Excess amounts of unlabelled oligonucleotide containing sequences encompassing −74 to −94 bp of the mouse α2(I) procollagen gene promoter also competed for binding (results not shown).

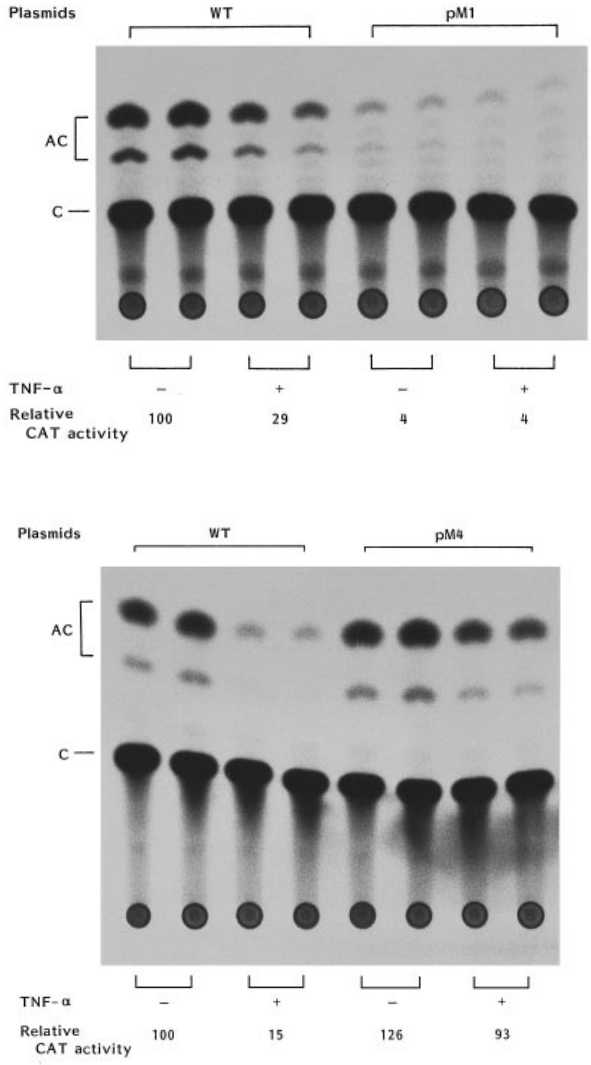

Figure 5 Effect of promoter mutations on TNF-α-induced reduction of human COL1A1 transcription

The chimaeric constructs used contain the following sequences: −332–+42 bp (wild type; WT); −101–−97 bp substitution (M1); −84–−78 bp deletion (M2); −71–−69 bp substitution (M3); −46–−38 bp substitution (M4); and −31–−29 bp substitution (M5). Only results obtained with M1 and M4 are shown. Human dermal fibroblasts were transiently transfected with WT, M1 (top) or M4 (bottom). Values are the means of duplicate cultures and are expressed relative to those obtained with the WT plasmid [p332α1(I)CAT] without TNF-α treatment. C and AC are defined in Figure 3.

Gel retardation assays were also performed using a 54-bp double-stranded oligonucleotide containing sequences encompassing −27 to −54 bp and crude nuclear extracts prepared from untreated fibroblasts. A major retarded band was found that was competed by addition of excess amounts of wild-type unlabelled oligonucleotide encompassing −27 to −54 bp, but was not competed by addition of either the same fragments containing the 3 bp substitution mutation (M4) shown in Figure 4 or the fragment containing the 3 bp substitution mutation of CCTGCTCTCCATGATC (M4′) (Figure 6C). The binding activity of nuclear extracts prepared from TNF-α-treated fibroblasts to this oligonucleotide was higher than that from untreated fibroblasts (Figure 6D).

Table 1 Effects of various COL1A1 promoter mutations on the inhibition of its transcriptional activity by TNF-α

The chimaeric constructs employed are described in the Materials and methods section and in the legends to Figure 5 and Figure 6. Values are expressed relative to the values obtained with p332α1(I)CAT (WT) without TNF-α treatment. The values shown are the ratio of treated over untreated cells ±S.D. The number of independent tests performed is indicated in parentheses.

Plasmid	Relative CAT activity (%)		TNF-α-treated cells/control cells (without TNF-α)
	Without TNF-α	With TNF-α	
WT	100 (10)	35±12 (10)	0.35±0.12
pM1	7±4 (4)	8±4 (4)	1.14±0.14*
pM2	73±7 (4)	30±3 (4)	0.14±0.04
pM3	70±6 (4)	18±3 (4)	0.26±0.04
pM4	121±16 (4)	97±28 (4)	0.80±0.23*
pM5	45±2 (4)	14±3 (4)	0.31±0.10

* The ratio value is statistically higher than that of plasmid WT (Mann–Whitney test, $P < 0.01$).

DISCUSSION

Recent studies have shown that many cytokines cause potent and specific effects on the expression of genes encoding connective-tissue components, especially collagen. TGF-β has been shown to be a potent stimulator of collagen synthesis in fibroblasts in various experimental conditions both *in vivo* and *in vitro*. Previous studies using chimaeric type I collagen promoter–reporter gene constructs have shown that enhancement of COL1A1 transcriptional activity by TGF-β is mediated by an Sp1-binding site [20,21] or a nuclear factor (NF)-1 binding site [19]. TNF-α is a macrophage-derived multifunctional cytokine with pleiotropic effects, which include pro-inflammatory and immunomodulatory properties. TNF-α has also been shown to be involved in the remodelling of the extracellular matrix that occurs in a variety of physiologic and pathologic events. The profound effects of the cytokine on the extracellular matrix include inhibiton of collagen production as well as stimulation of the synthesis of various metalloproteases [9,10,22–24]. The mechanisms involved in the multiple effects of TNF-α have not been completely elucidated, although recent studies have identified a complex cascade of events that includes interactions of the cytokine with specific cell-membrane receptors and the transduction of its effects leading to the activation of a variety of nuclear binding factors, including those of the NFκB and activator protein-1 families of proteins [23]. Although TNF-α has been shown to inhibit collagen-gene transcription in dermal fibroblasts, the *cis*-acting DNA regulatory elements of the COL1A1 promoter gene responsible for the down-regulation of transcription induced by this cytokine have not been completely identified. A recent study examined the *cis*- and *trans*-regulatory factors that are involved in the inhibition of the transcriptional activity of the human α2(I) collagen gene(COL1A2) [22]. These studies identified a TNF-α-responsive element and indicated the convergence of TNF-α-dependent signals upon the same sequence that mediates the responsiveness of the gene promoter to TGF-β. Furthermore, these studies showed that TNF-α-treated cells displayed higher levels of DNA-binding proteins that specifically interacted with three distinct regions of the proximal COL1A2 promoter and excluded the participation of NFκB and activator protein-1 in the TNF-α-induced inhibition of COL1A2 transcriptional activity. The identified COL1A2 promoter regions are localized in close proximity to and overlap with a previously identified TGF-β-response element that includes a Sp-1-binding site. However, the sequences surrounding the CCAAT sequence did not appear to be involved in TNF-α modulation of COL1A2 transcriptional activity in this study. In contrast, in the present study, we found that TNF-α suppressed COL1A1 promoter activity through DNA sequences corresponding to the CCAAT-binding factor (CBF)-binding site [16] as well as by elements

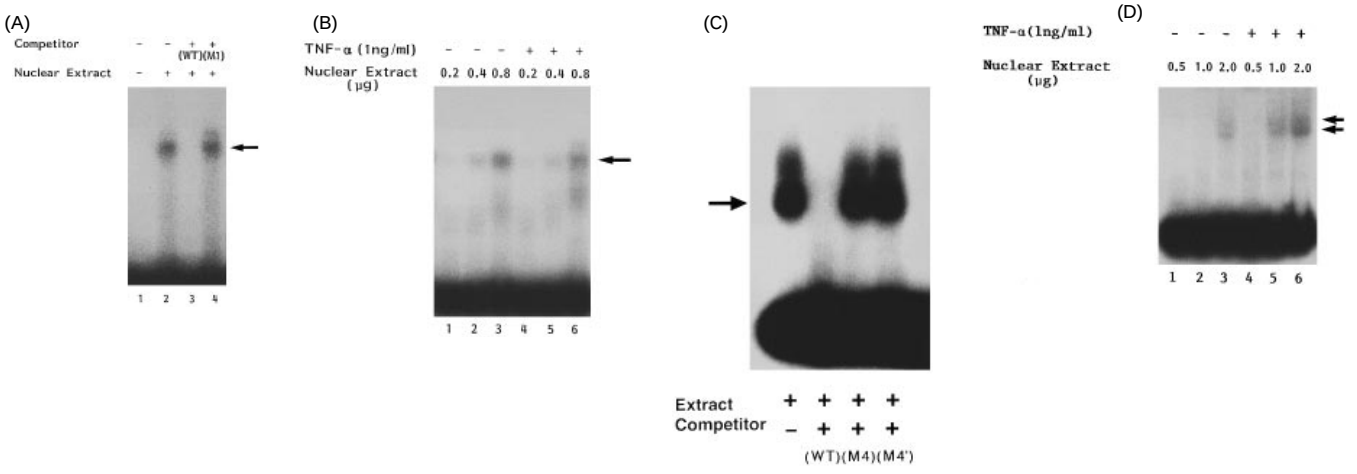

Figure 6 Gel retardation assay of nuclear proteins that bind to human COL1A1 TNF-α-responsive elements

(**A**) Crude nuclear extract (1 μg) from fibroblasts incubated without TNF-α was examined for binding activity to a DNA segment encompassing −91 to −110 bp of COL1A1, using a 200-fold excess of unlabelled double-stranded oligonucleotide as a competitor. −, absence; WT, wild type; M1, see the legend to Figure 4. (**B**) Crude nuclear extracts (0.2–0.8 μg) from fibroblasts incubated with (+) or without (−) TNF-α (1 ng/ml) were examined for binding activity to a DNA segment encompassing −91 to −111 bp of COL1A1. (**C**) Crude nuclear extract (2 μg) from dermal fibroblasts incubated without TNF-α was examined for binding activity to a DNA segment encompassing −46 to −38 bp of COL1A1 by gel retardation assay, using a 54 bp double-stranded oligonucleotide encompassing −54 to −27 bp of COL1A1. A 200-fold excess of unlabelled double-stranded oligonucleotide was used as a competitor. −, absence; WT, wild type; M4, see the legend to Figure 5; M4′, CCTGCTCTCCATGATC. (**D**) Crude nuclear extracts (0.5 to 2.0 μg) from fibroblasts incubated with (+) or without (−) TNF-α (1 ng/ml) were assayed for binding activity to a DNA segment encompassing −46 to −38 bp of COL1A1.

localized in a sequence encompassing −46 to −38 bp of the COL1A1 promoter. We also found that the suppression of transcription of COL1A1 was not mediated by an Sp-1 binding site, as shown by the failure of a substitution mutation of this binding site (M2) to alter the formation of DNA–protein complexes. The discrepancies between these two studies suggest that TNF-α may modulate differentially the transcriptional activities of COL1A1 and COL1A2, and that this differential regulation involves different regulatory elements in the promoters of these two genes.

We also identified binding activity that recognizes the segment spanning −46 to −38 bp of the COL1A1 promoter gene, using an oligonucleotide spanning −54 to −27 bp of the gene. This segment displays an incomplete inverted repeat (5′-CCTGCT/ATCAGG-3′) that could possibly represent a binding site for two subunits of a homodimer. The binding of nuclear extracts prepared from TNF-α-treated fibroblasts to this segment was greater than that from untreated fibroblasts. Therefore the binding factor seems to be a repressor of COL1A2 gene transcription; however, transient transfection assays using plasmids containing two different 3 bp substitution mutations of this segment (M4 and M4′) did not show increased activity of the reporter gene.

CBF, a factor composed of three different polypeptides [33], binds to the CCAAT sequence in both mouse COL1A1 and COL1A2 and activates their transcription [16]. There is high similarity between the mouse and human genes in the sequences surrounding the CCAAT sequence [15,34]. In this study, we have shown that there is binding activity to an oligonucleotide containing the CCAAT sequence (−101 to −97 bp) of human COL1A1. This activity is competed by addition of excess amounts of an oligonucleotide containing the CCAAT sequence of mouse COL1A2 (results not shown). Although the transient transfection experiments using a construct with a substitution in the CCAAT sequence (pM1) indicated suppression of COL1A1 transcription through at least the CBF-binding site, the CBF-binding activity of nuclear extracts prepared from TNF-α-treated fibroblasts was the same as that from untreated fibroblasts. There are three possible explanations for these discrepant results. First, it is possible that treatment with TNF-α does not affect the DNA-binding properties of CBF but causes a modification that renders it inactive. Secondly, treatment with TNF-α may remove or inactivate a second factor that does not bind directly to the DNA but that is required for optimal transcriptional activity of the COL1A1 promoter. Thirdly, TNF-α treatment may activate a factor that inhibits the activity of CBF. Regardless of the mechanisms involved, it is apparent from our studies that TNF-α exerts a potent inhibitory effect on the transcriptional activity of the COL1A1 promoter, which appears to be mediated by DNA-binding proteins that interact with two regulatory elements localized in the proximal region of the promoter. Identification and characterization of these proteins may provide further insights into the complex mechanisms that regulate the transcriptional activity of collagen genes under normal and pathologic conditions and may provide novel therapeutic avenues for the treatment of various fibrotic diseases.

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