B-Myb acts as a repressor of human COL1A1 collagen gene expression by interacting with Sp1 and CBF factors in scleroderma fibroblasts

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We investigated the role of B-Myb, a cell-cycle-regulated transcription factor, in the expression of the α1 (I) pro-collagen gene (COL1A1) in scleroderma fibroblasts. Scleroderma or SSc (systemic sclerosis) is a fibrotic disease characterized by excessive production of extracellular matrix components, especially type I collagen. Northern-blot analysis showed an inverse relationship between COL1A1 mRNA expression and that of B-Myb during exponential cell growth and during quiescence in human SSc fibroblasts. Overexpression of B-Myb in SSc fibroblasts was correlated with decreased COL1A1 mRNA expression. Transient transfections localized the down-regulatory effect of B-Myb to a region containing the proximal 174 bp of the COL1A1 promoter that does not contain B-Myb consensus binding sites. Gel-shift analysis, using nuclear extracts from normal and SSc fibroblasts transfected with B-Myb, showed no differences in DNA–protein complex formation when compared with the nuclear extracts from mock-transfected cells. However, we found that B-Myb decreases Sp1 (specificity protein 1) and CBF (CCAAT-binding factor) binding for their specific sites localized in the 174 bp COL1A1 proximal promoter. These results were also confirmed using B-Myb-immunodepleted nuclear extracts. Furthermore, immunoprecipitation assays using SSc nuclear extracts demonstrated a physical interaction of B-Myb with Sp1 and CBF transcription factors, and also an interaction between Sp1 and CBF. In addition, by employing full-length or deleted B-Myb cDNA construct, we found that B-Myb down-regulates the COL1A1 proximal promoter through its C-terminal domain. Thus these results suggest that B-Myb may be an important factor in the pathway(s) regulating collagen production in SSc fibroblasts.

Key words: collagen, Myb, promoter, regulation, scleroderma, transcription factor.

INTRODUCTION

B-Myb belongs to a family of transcription factors that includes two other members, A-Myb and c-Myb, which are important in controlling cell proliferation and differentiation [1]. Unlike other members of the family, B-Myb is expressed ubiquitously and its biological activity is regulated at multiple levels in a cell-cycle-dependent manner. Expression of B-Myb is significantly induced during the G1–S transition and S-phase of the cell cycle and down-modulated in resting and differentiated cells [2,3]. The fact that B-Myb is highly expressed during S-phase suggests that it modulates transcription at this stage of the cell cycle. Several studies have reported that B-Myb expression promotes cell proliferation, whereas extrinsic expression of B-Myb results in the suppression of cell differentiation induced by different agents [4–6]. B-Myb is regulated at the protein level by phosphorylation [7,8] and acetylation [9] in a cell-type-specific fashion. It has been shown that a sequence-specific MBS (Myb-binding site) is not always necessary for B-Myb function [8]. In fact, an increasing body of evidence has indicated the dependence of B-Myb activity on its interaction with other nuclear factors such as cyclin D1 [10] and poly(ADP-ribose) polymerase [11]. The effects of B-Myb on gene promoter activity are complex. B-Myb transactivates the promoter of several genes, many of which are involved in cell proliferation and survival, such as DNA topoisomerase IIα [12], c-*myc* [13], Hsp70 [14], B-Myb itself [15], apolipoprotein J [16], Bcl2 [17] and murine surfactant Protein A [18]. In contrast, B-Myb can repress the promoter activity of the genes encoding type I [19], type V [20] and type VI collagens [21].

Type I collagen is a heterotrimeric molecule consisting of two α1 (I) chains and one α2 (I) chain and is produced in tissues such as skin and bone by fibroblasts and osteoblasts [22–25]. Scleroderma or SSc (systemic sclerosis) is an auto-immune disease of unknown origin characterized by skin thickening, fibrosis and microvascular changes often associated with progressive internal organ involvement [26,27]. Excessive accumulation of extracellular matrix components in affected tissues is a central event in the pathogenesis of SSc [22]. Fibroblasts cultured from SSc skin biopsies exhibit higher than normal expression levels of matrix components, such as type I collagen, a phenotype that is maintained during several passages [23,24]. Previous work using bovine aortic SMCs (smooth-muscle cells) demonstrated that type I collagen is down-regulated by the overexpression of the transcription factor B-Myb [19,20]. Moreover, whereas B-Myb mRNA levels have been found to increase during cellular proliferation and to decrease in quiescent SMC cells, type I collagen expression, instead, varies inversely with the cellular growth state [19,20]. The ability of B-Myb to down-regulate collagen gene expression led us to investigate the potential role of B-Myb in the control of type I collagen (COL1A1 gene) expression in SSc fibroblasts. We have previously found that a short region of -174 bp of the COL1A1 proximal promoter was responsible for most of the transcriptional activity of the COL1A1 gene *in vitro* in normal and in SSc fibroblasts [28]. We

Abbreviations used: CAT, chloramphenicol acetyltransferase; CBF, CCAAT-binding factor; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility-shift assay; HA, haemagglutinin; MBS, Myb-binding site; SMC, smooth-muscle cell; Sp1, specificity protein 1; SSc, systemic sclerosis.

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further demonstrated that Sp1 (specificity protein 1) and CBF (CCAAT binding factor), also called NF-Y or CP1, probably play a central role in the increased expression of COL1A1 seen in dermal fibroblasts from patients with SSc. Specifically, we have shown that these transcription factors interact with the proximal promoter region of COL1A1 and that there is increased DNA binding activity to the Sp1 [28] and CBF *cis* elements [25] in SSc nuclear extracts when compared with normal fibroblasts.

Here we show that B-Myb decreases the expression of the human COL1A1 gene in normal and SSc fibroblasts. We localize the down-regulatory effect of B-Myb to the -174 bp of the COL1A1 proximal promoter and show that B-Myb partially inhibits Sp1 and CBF binding for their consensus sites in the proximal promoter region of the gene. We also found that a physical interaction of B-Myb with Sp1 and CBF factors occurs, which may be responsible for the down-regulatory effects on COL1A1 expression.

EXPERIMENTAL

Cell culture and time-course conditions

Dermal fibroblast cell lines were established from patients with diffuse SSc of recent onset and rapid progression, as previously reported [25,28]. All patients studied were observed at the Scleroderma Center of Thomas Jefferson University and fulfilled the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for the classification of SSc [29]. All studies were approved by the IRB. Only untreated cases were studied to avoid spurious results caused by the various therapeutic agents. In all cases, the cell lines were obtained from full-thickness skin biopsy samples that were surgically excised from the leading edge of clinically apparent SSc lesions [28]. Control fibroblasts were obtained from age- and sex-matched individuals undergoing surgical procedures for unrelated purposes. For all studies, only early-passage fibroblasts (<8) were examined to avoid the possibility of phenotypic changes during extended subculture. Cells were routinely maintained in DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % (v/v) FBS (foetal bovine serum; Invitrogen, Carlsbad, CA, U.S.A.), 1 % vitamins, 2 mM L-glutamine and antibiotics, and incubated at 37 °C in a 5 % CO_2 humidified atmosphere. For serum stimulation studies, cells were plated at an initial density of 5×10^5 cells in 100 mm dishes and grown in DMEM supplemented with 10 % FBS for 1 day (subconfluent cultures). Cultures were then serum-depleted for 48 h by culture in 0.1 % FBS–DMEM and then stimulated with 10 % FBS for 0, 24 and 48 h. Total RNA was isolated at the indicated times and analysed as described below.

Transient transfections of human dermal fibroblasts

Cells were transfected by using the lipid-based transfection reagents supplied in the FuGene 6 kit (Roche, Indianapolis, IN, U.S.A.). For CAT (chloramphenicol acetyltransferase) assays, cells were plated at 70 % confluency in 60 mm dishes and co-transfected with 1 μg of CAT-containing constructs (-5.3, -2.3, -675, -369, -174 and -84 bp) of the human COL1A1 promoter [30] and 0.5 μg of several CMV (cytomegalovirus) constructs containing either the full-length B-Myb cDNA or mutated forms (ΔN/B-Myb, Eco/B-Myb or Sca/B-Myb). Empty CMV vector was used as a negative control. The cells were harvested 48 h after transfection. The protein concentration of the extracts was determined using the Bio-Rad reagent (Bio-Rad, Hercules, CA, U.S.A.). For studies of the effect of B-Myb overexpression on COL1A1 mRNA levels, cells at 70 % confluency in 100 mm dishes were transfected with either 2 μg/plate of human CMV B-Myb expression vector for 48 h or empty CMV vector as control. Additionally, in the same studies in normal and SSc fibroblasts, we also co-transfected 2 μg/plate of the Capture-Tec™ pHook-1 plasmid (Invitrogen) to isolate transfected cells. Transfection of the pHook plasmid into cells results in the expression of a single-chain antibody (sFv) that recognizes a specific hapten, which is displayed on the surface of transfected cells. These were then isolated from the untransfected cells by binding to hapten-coated magnetic beads. The selection procedure was carried out following the manufacturer's instructions (Invitrogen).

β-Galactosidase and CAT assays

For all experiments, transfection efficiency was corrected by co-transfecting with 0.2 μg of CMV vector containing the *Escherichia coli* β-galactosidase cDNA (ClonTech, Palo Alto, CA, U.S.A.), assaying for β-galactosidase activity according to the manufacturer's instructions (ClonTech). After each sample was normalized for β-galactosidase activity, 2 μl of chloramphenicol (55.0 mCi/mmol 1,2-^{14}C; Amersham Biosciences, Piscataway, NJ, U.S.A.) and 15 μl (3.5 mg/ml) of acetyl-CoA (Roche) were used in a final volume of 120 μl of incubation reaction. CAT activity was determined in cell extracts by TLC (Baker-flex; provided by J. T. Baker, Div. of Mallinckrodt Baker, Phillipsburg, NJ, U.S.A.). The CAT activities were quantified by densitometry (Image-Quant version 5.1; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Northern-blot analysis

Total RNA was prepared using the RNeasy kit (Qiagen, Valencia, CA, U.S.A.). Total RNA (8 μg) was electrophoresed on formaldehyde agarose gels, and transferred to nylon membrane (Hybond-N; Amersham Biosciences) as described previously [31]. The probes were labelled with [^{32}P]dCTP (3000 Ci/mmol; Amersham Biosciences) using the Rediprimer II kit (Amersham) and hybridized in QuikHyb solution (Stratagene, La Jolla, CA, U.S.A.) following the conditions described by the manufacturer. The filters were exposed to X-ray film (Eastman Kodak, Rochester, NY, U.S.A.) in cassettes with intensifying screens (Kodak) at -70 °C.

EMSA (electrophoretic mobility-shift assay)

Confluent cells were washed with PBS and nuclear extracts were prepared [25]. EMSA using nuclear extracts from human dermal fibroblasts was performed as described previously [32]. Protein concentration of the extracts was determined using the Bio-Rad reagent. An 85 and a 46 bp DNA fragment (Figure 3A) were used as probes in all EMSA experiments. The fragments were obtained by PCR amplification employing the following primers: S9 at -134 bp (5′-CTCCCAAATTGGGGGCCGGGC-3′), S14 at -109 bp (5′-TGCCCCAGCCAATCAGAGCT-3′) and S2 at -50 bp (5′-AGGAACCCTGCCCCTCGGAGA-3′) of the human COL1A1 promoter. Oligonucleotides containing consensus sequences for Sp1 and for CBF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Oligonucleotides for Myb-binding site 'tcgacacattaTAACGGtttttttagc' were obtained from the Nucleic Acid Facility at Thomas Jefferson University (Philadelphia, PA, U.S.A.). Radioactive probes were generated by phosphorylating the 5′-ends with polynucleotide kinase (Roche) and [γ-^{32}P]ATP (Amersham Biosciences). Binding reactions containing 5–10 μg of nuclear extracts, 2 μg of poly (dI/dC) and approx. 50 000 c.p.m. of (0.2 ng) radiolabelled probes were incubated for 20 min at room temperature in a buffer containing

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40 mM KCl, 15 mM Hepes (pH 7.9), 1 mM EDTA, 0.2 mM dithiothreitol, 1 mM $MgCl_2$ and 5 % (v/v) glycerol in a total volume of 20 μl. Sp1 and CBF antibodies for supershift assays were obtained from Santa Cruz Biotechnology.

Immunodepletion

Nuclear extracts (100 μg) were incubated at 4 °C for 1 h with IgG, anti-HA (haemagglutinin)-B-Myb-antibody, Sp1, CBF-A or CBF-B polyclonal antibodies and then for 1 h with Protein G-Plus agarose conjugate. After centrifugation, supernatants were used for EMSA. Immunoprecipitated proteins were washed four times in a lysis buffer [20 mM Tris/HCl, pH 7.2/150 mM NaCl/0.1 % Nonidet P40/10 % (v/v) glycerol/1 mM dithiothreitol/100 mM sodium fluoride/10 μg/ml aprotinin, leupeptin and PMSF], resolved by SDS/PAGE and blotted with the anti-HA-probe, anti-B-Myb, anti-Sp1, anti-CBF-A and anti-CBF-B antibodies.

Immunoprecipitation/Western-blot analysis

Normal and SSc fibroblasts were lysed in the lysis buffer. Nuclear extracts were precleared with 30 μl of Protein G-Plus agarose conjugate and immunoprecipitated with IgG, HA tag, Sp1, CBF-A or CBF-B polyclonal antibodies (Santa Cruz Biotechnology) overnight at 4 °C. Protein G-Plus (20 μl) agarose conjugate was added and the samples were rocked for 1 h at 4 °C. After three washes, samples were electrophoresed on SDS/polyacrylamide gel and transferred to a PVDF-Plus membrane (MSI, Westboro, MA, U.S.A.). Western-blot analysis was performed with B-Myb N-19, B-Myb C-20 and Sp1 polyclonal antibodies (Santa Cruz Biotechnology) in Blotto (5 % dry milk in PBS) plus 0.1 % Nonidet P40. After washings and incubation with secondary antibody coupled with peroxidase, the filter was developed using a chemiluminescent substrate according to the manufacturer's instructions (ECL®-plus, Amersham Biosciences).

RESULTS

B-Myb down-regulates the expression of COL1A1 in human dermal fibroblasts

To analyse the correlation between B-Myb and COL1A1 mRNA expression in normal and SSc fibroblasts, Northern-blot analysis was performed (Figure 1A). Serum-deprived (0.1 % FBS) quiescent cells were stimulated with 10 % FBS, and mRNA levels of B-Myb and COL1A1 were analysed after 0, 24 and 48 h. B-Myb mRNA reached its highest level of expression in both fibroblasts after 24 h (lanes 2N and 2S), and then started decreasing at 48 h when the cells were confluent. In contrast, COL1A1 mRNA levels were higher (lanes 3N and 3S) with respect to the exponentially growing cells (lanes 2N and 2S). The fact that no differences in levels of COL1A1 expression between normal and SSc fibroblasts were observed could be explained by the effects of serum depletion on the growth of the cells used in these studies. In the 48 h sample 3S, when compared with the 3N sample, we did observe a slight increase in mRNA levels.

To assess the effect of B-Myb on COL1A1 mRNA levels, the full-length B-Myb cDNA was transfected into subconfluent SSc fibroblasts for 48 h. Total RNA was isolated and Northern-blot analysis was performed (Figure 1B). Decreased COL1A1 mRNA expression was observed in B-Myb-transfected fibroblasts (lane 2) compared with mock-transfected cells (lanes 1), without affecting glyceraldehyde-3-phosphate dehydrogenase and ribosomal (28 and 18 S) RNA levels (results not shown). Similar results, showing a decrease in COL1A1 expression using total RNA from normal fibroblasts transfected and overexpressing the ectopic B-Myb, were previously obtained [36]. These results, taken together, suggest that endogenous B-Myb expression (Figure 1A) and ectopic B-Myb overexpression (Figure 1B) in dermal fibroblasts down-regulate COL1A1 mRNA levels.

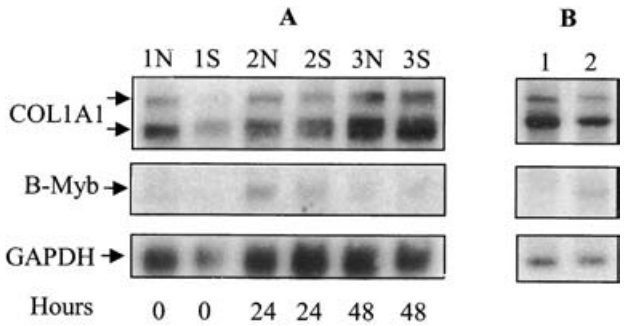

Figure 1 B-Myb down-regulates COL1A1 steady-state mRNA levels in human dermal fibroblasts

(**A**) Northern-blot analysis demonstrating the inverse relationship of α1 (I) pro-collagen mRNA (COL1A1) and that of B-Myb during cell proliferation. Normal and SSc fibroblasts were serum-starved with 0.1 % FBS for 48 h and stimulated with 10 % FBS for 0 h (lanes 1N and 1S), 24 h (lanes 2N and 2S) and 48 h (lanes 3N and 3S). (**B**) Ectopic expression of the full-length B-Myb cDNA by transient transfection in SSc fibroblasts correlated with decreased COL1A1 mRNA levels. Northern-blot analysis was performed using total RNA (8 μg) from mock-transfected (lane 1) and B-Myb transfected SSc fibroblasts (lane 2). In both experiments, a glyceraldehyde-3-phosphate dehydrogenase cDNA probe was used as a loading control.

B-Myb down-regulates the activity of the proximal region of the COL1A1 promoter

To determine the COL1A1 promoter region affected by B-Myb, we co-transfected SSc fibroblasts with several CAT-containing deletion constructs (−2300, −675, −369, −174 and −84 bp) of the human COL1A1 promoter region (Figure 2A) together with either an expression vector containing the B-*myb* cDNA driven by the CMV promoter or the CMV (no insert) control plasmid. Figure 2(B) shows a representative duplicate experiment. We identified a region between −174 and −84 bp of the proximal COL1A1 promoter that was strongly down-regulated (up to 60 %) by B-Myb overexpression (Figure 2C). Moreover, we also transfected COL1A1 deletion constructs containing more distal regions of the promoter (−5.3, −4.0 and −3.4 kb) and confirmed that the down-regulation was restricted to the proximal promoter region (results not shown). These results suggest that B-Myb acts as a repressor of COL1A1 expression in human dermal SSc fibroblasts at the transcriptional level by interacting with the proximal promoter region of the gene.

Overexpression of B-Myb decreases the binding activity of Sp1 and CBF in the COL1A1 proximal promoter

Sequence analysis of the COL1A1 promoter region affected by B-Myb overexpression showed that it does not contain MBS. This region, however, has been demonstrated to interact with some other transcription factors (Figures 3A and 3B) such as CBF, Sp1, Sp3 and NF-1 [25,33–35]. To examine whether B-Myb could bind directly to this region, gel-shift assays were performed using different fragments of the COL1A1 promoter (S9-S2 and S9-S14, see Figure 3A) and a glutathione-S-transferase B-Myb DNA-binding domain (GST-B-Myb-DBD) fusion protein [11], but no retarded complexes were detected (results not shown).

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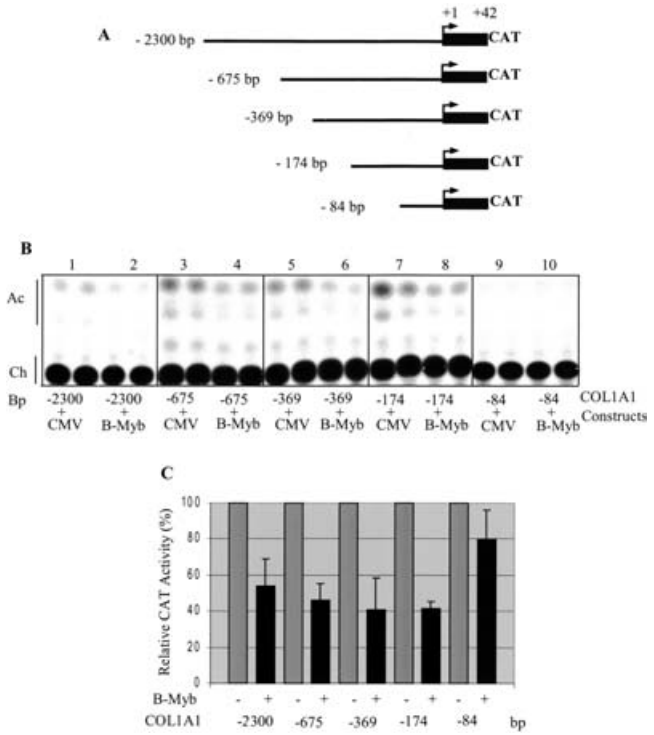

Figure 2 CAT assay demonstrating that overexpression of B-Myb is correlated with a down-regulation of the COL1A1 proximal promoter activity

(**A**) Schematic representation of the COL1A1 promoter CAT constructs. (**B**) SSc fibroblasts were co-transfected in duplicate with several CAT-containing deletion constructs of the human COL1A1 promoter (−2300, −675, −369, −174 and −84 bp) and the empty CMV vector (−) or the CMV vector expressing B-Myb (+). Ch, [^{14}C]chloramphenicol; Ac, acetylated form of [^{14}C]chloramphenicol. (**C**) Histograms represent the results of three different experiments, and the error bars represent S.D. CAT activities, corrected for differences in transfection efficiencies, are compared with the activity observed with the insertless plasmid (CMV), which was arbitrarily set at 100 %.

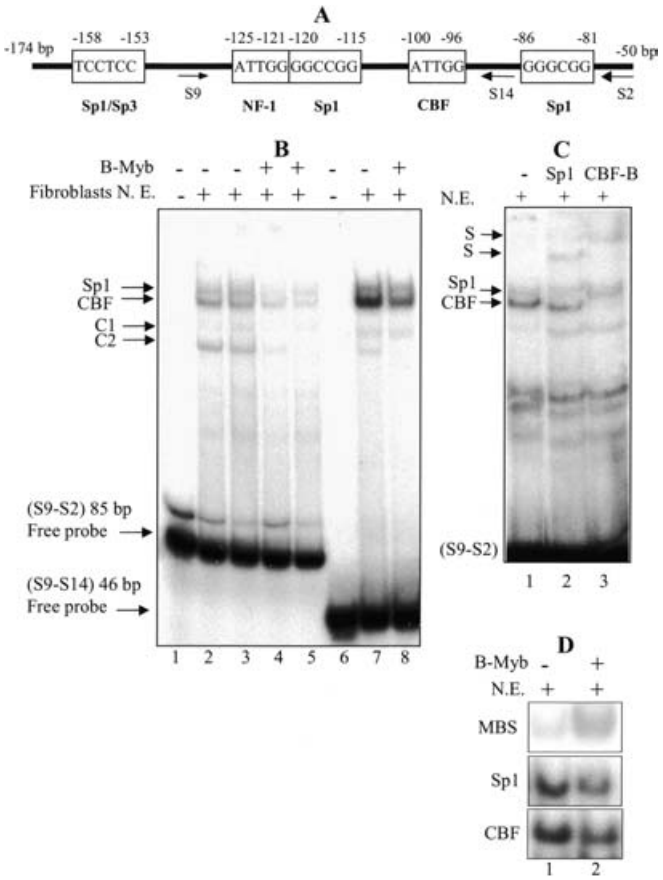

Figure 3 B-Myb decreases the binding activity of Sp1 and CBF to COL1A1 proximal promoter region

EMSAs were performed using the 85 bp or the 46 bp promoter fragments generated by PCR using the S9-S2 or S9-S14 primers respectively. (**A**) Schematic diagram of the potential transcription binding sites in the proximal COL1A1 promoter. (**B**) Nuclear extracts (NE) from SSc fibroblasts transfected with the empty CMV vector (lanes 2, 3 and 7) and with the CMV vector expressing the full-length B-Myb (lanes 4, 5 and 8) were incubated with the (S9-S2) 85 bp (lanes 2–5) and (S9-S14) 46 bp (lanes 7 and 8) probes. Two different concentrations of NE (6 μg lanes 2, 4 and 12 μg lanes 3 and 5) were used with the (S9-S2) 85 bp probe. Note the marked decrease (lanes 4 and 5) in Sp1 and CBF DNA-binding activity in NE from B-Myb-transfected SSc fibroblasts compared with the mock-transfected cells. Lanes 1 and 6 contain probe alone, (S9-S2) 85 bp and (S9-S14) 46 bp probes respectively. (**C**) EMSA using the 85 bp probe and nuclear extracts from SSc fibroblasts (lane 1) and supershift analysis by using Sp1 (lane 2) and CBF antibodies (lane 3) showing the protein–DNA complexes formed by the transcription factors Sp1 and CBF in the proximal promoter region of COL1A1. Supershifted complexes are denoted by the letter S. (**D**) B-MBS, Sp1 or CBF consensus oligonucleotides were used in EMSA to test the binding efficiency of nuclear extracts from mock-transfected cells (−) and from B-Myb-transfected cells (+).

Furthermore, gel shift using nuclear extracts from B-Myb or mock-transfected SSc fibroblasts were performed. The results, shown in Figure 3(B), suggest that B-Myb does not directly interact with the COL1A1 proximal promoter since no additional band(s) was observed when using nuclear extracts from cells overexpressing B-Myb (lanes 4, 5 and 8) compared with the mock-transfected fibroblasts (lanes 2, 3 and 7). In contrast, a significant decrease in DNA-binding activity was observed for Sp1 and CBF complexes (lanes 4, 5 and 8). In addition, a faster migrating C2 complex, not yet identified, seems also to be modulated by B-Myb overexpression, whereas a fainter C1 complex, also not yet identified, appears to be unaffected (lane 8). Supershifted complexes using Sp1 and CBF (Figure 3C, lanes 2 and 3) antibodies in the proximal promoter region of COL1A1 are also shown. We have also noted a partial supershift for Sp1 (lane 2) showing a remaining band that could be due to a partial shift of Sp1 or to another complex, most probably Sp3 for which we have previously demonstrated binding in the same promoter region [25]. Furthermore, we performed gel-shift assays using B-Myb, Sp1 and CBF-specific consensus oligonucleotides and nuclear extracts from B-*myb* or mock-transfected SSc fibroblasts (Figure 3D). The DNA-binding activity of CBF and Sp1 to their consensus sequences was decreased (approx. 40–60 %) when the cells were transfected with B-Myb. The nuclear extracts from B-Myb-transfected fibroblasts showed a much stronger binding activity compared with the mock-transfected cells when the specific B-MBS was used as a probe, confirming that transfected B-Myb is functional (Figure 3D, top panel). The results suggest that B-Myb does not bind directly to the DNA, but is capable of inhibiting partially the binding of Sp1 and CBF to their consensus sites in the COL1A1 proximal promoter region.

B-Myb interacts with the Sp1–CBF complex in immunodepleted SSc fibroblast nuclear extracts

To assess whether B-Myb binds specifically to the DNA–protein complex formed by Sp1 and CBF of the COL1A1 proximal promoter region, we transfected the CMV-WT/B-Myb construct encoding the full-length B-Myb fused to the HA tag into SSc fibroblasts. Nuclear extracts from SSc fibroblasts overexpressing

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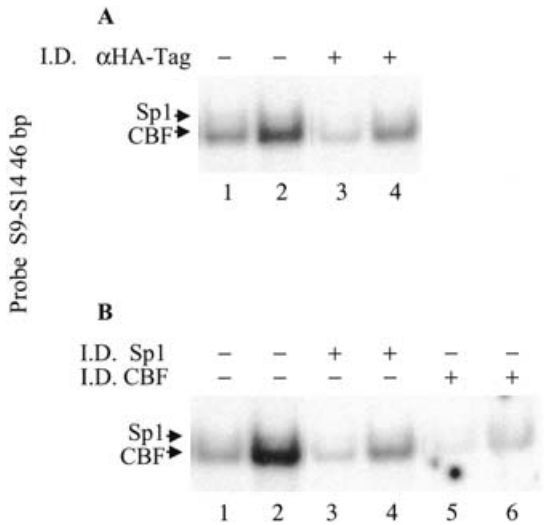

Figure 4 B-Myb interacts with the Sp1/CBF complex in immunodepleted SSc fibroblast nuclear extracts

(**A**) EMSA using the 46 bp promoter fragment and nuclear extracts from HA-B-Myb-transfected SSc fibroblasts pretreated with antibody to rabbit IgG (lanes 1 and 2) and HA-tagged antibody (lanes 3 and 4). Two different concentrations of nuclear extracts (6 and 12 μg) were used. (**B**) EMSA using the 46 bp promoter fragment and Sp1 (lanes 3 and 4) or CBF-B (lanes 5 and 6) immunodepleted (ID) fractions. Lanes 1 and 2 represent nuclear fractions pretreated with αIgG as control.

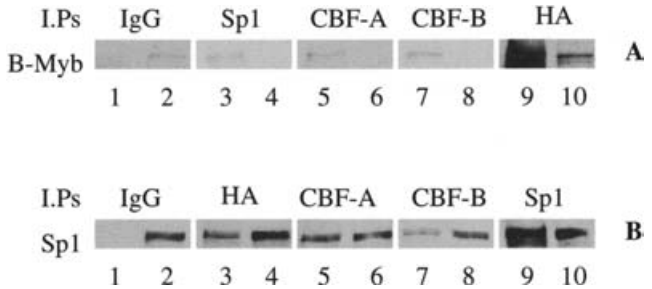

Figure 5 B-Myb physically interacts with Sp1 and CBF in SSc fibroblasts

(**A**) Nuclear extracts were prepared from SSc fibroblasts and 100 μg were used for each immunoprecipitated with IgG (lane 1) Sp1 (lane 3), CBF-A (lane 5) and CBF-B (lane 7) antibodies. In lane 9, cells were transfected with HA-B-Myb and immunoprecipitated with HA tag, as positive control. The membrane was probed with B-Myb antibody, and both endogenous (lanes 2, 3, 5 and 7) and exogenous B-*myb* (lanes 9 and 10) proteins were detected. (**B**) SSc fibroblast nuclear extracts were immunoprecipitated with IgG (lane 1), HA (lane 3), CBF-A (lane 5), CBF-B (lane 7) and Sp1 (lane 9) antibodies. The membrane was probed with Sp1 antibody. In lane 2, cells were transfected with HA-B-Myb and immunoprecipitated with HA antibody. As indicated in (**A**, **B**), lanes 2, 4, 6, 8 and 10 represent the unbound fractions of the respective immunoprecipitated antibody reactions.

HA-B-Myb were depleted of B-Myb using HA antibody and tested in EMSA with the S9–S14 ^{32}P-labelled 46 bp probe (Figure 4A). The results show that pretreatment with the HA antibody decreased the formation of the retarded DNA–protein complex formed by Sp1 and CBF (lanes 3 and 4) compared with the fraction incubated with an antibody to IgG (lanes 1 and 2), suggesting that the B-Myb-immunodepleted nuclear extracts were also partially deprived of Sp1 and CBF. The specificity of the decrease in Sp1- and CBF-binding efficiency was confirmed using the same probe with the Sp1- or CBF-immunodepleted fractions (Figure 4B, lanes 3–6). The supernatant and pellet fractions were then tested for B-Myb, Sp1 and CBF by Western blot to confirm effective depletion from the fractions used for EMSA (results not shown). As demonstrated in Figure 4(B), the Sp1-immunodepleted fraction also shows a decrease in CBF-binding (lanes 3 and 4) and the CBF-immunodepleted fraction shows a decrease in Sp1 binding (lanes 5 and 6), suggesting the occurrence of a physical interaction between Sp1 and CBF, already demonstrated in rat fatty acid synthase, the human metalloproteinase-2 and natriuretic receptor genes [37–39].

B-Myb, Sp1 and CBF co-immunoprecipitate in SSc fibroblasts

To investigate further, whether B-Myb interacts *in vivo* with Sp1 and/or CBF transcription factors, immunoprecipitation assays were performed using nuclear extracts from SSc fibroblasts with or without transfection of the CMV-B-Myb construct expressing the full-length B-Myb fused to the HA tag. The nuclear extracts (100 μg) were incubated with either IgG, HA, Sp1, CBF-A or CBF-B antibodies (Figures 5A and 5B) and the samples were electrophoresed on an SDS/polyacrylamide gel. Western blotting using antibodies against B-Myb demonstrated that the Sp1, CBF-A, CBF-B and HA (as control) were positive for B-Myb (Figure 5A, lanes 3, 5, 7 and 9) and negative for IgG immunoprecipitate (lane 1). In fact, B-Myb was positive only in the unbound IgG fraction (lane 2) and negative in all the other fractions (lanes 4, 6 and 8), except lane 10, where B-Myb was overexpressed using the HA-B-Myb construct. We have also found interaction between B-Myb and Sp1 when the HA-B-Myb immunoprecipitate was probed with the Sp1 antibody (Figure 5B, lane 3). In addition, we detected interaction of the CBF-A (lane 5) and CBF-B (lane 7) immunoprecipitates with Sp1, confirming a physical interaction between Sp1 and CBF as previously demonstrated for other genes [37–39]. Also, as described in Figure 5(A), the interaction for the IgG fraction was only in the unbound immunoprecipitate (Figure 5B, lane 2). We also observed that Sp1 was recognized in the unbound immunoprecipitate fractions of HA, CBF-A, CBF-B and Sp1 (lanes 4, 6, 8 and 10). This result could be explained by the excess of SSc nuclear extracts (100 μg) used in each reaction and/or to the high amount of Sp1 protein present in SSc fibroblasts [28].

B-Myb down-regulates COL1A1 expression through its C-terminal domain in SSc fibroblasts

To determine which region(s) of B-Myb (Figure 6A) was required for the modulation of COL1A1, the transactivation domains of two C-terminal truncated forms encoding amino acids 1–508 (Sca/B-Myb) or amino acids 1–350 (Eco/B-Myb) and an N-terminal truncated cDNA (ΔN/B-Myb) that lacks the DNA-binding domain repeats were analysed for their effects on the COL1A1 proximal promoter. The wild-type and mutated forms of B-*myb* were tested for their expression in SSc fibroblasts (Figure 6B) and co-transfected into SSc fibroblasts with the -174 COL1A1 promoter construct and CAT assays performed (Figure 6C). Our results showed $>50\%$ decrease in COL1A1 promoter activity compared with mock-transfected cells (CMV), when the full-length B-Myb (B-Myb) or the N-terminal-deleted form of B-Myb (ΔN/B-Myb) was co-transfected with the -174 bp deleted construct promoter (Figures 6C and 6D). In contrast, no change in COL1A1 promoter activity was observed when the B-Myb mutants (Eco/B-Myb or Sca-B-Myb) lacking the C-terminal domain were overexpressed (Figures 6C and 6D). It is possible that the C-terminal B-Myb mutants are expressed at lower levels compared with the wild-type protein (Figure 6B, lanes 2–4). However, the N-terminal mutant (lane 5) is expressed

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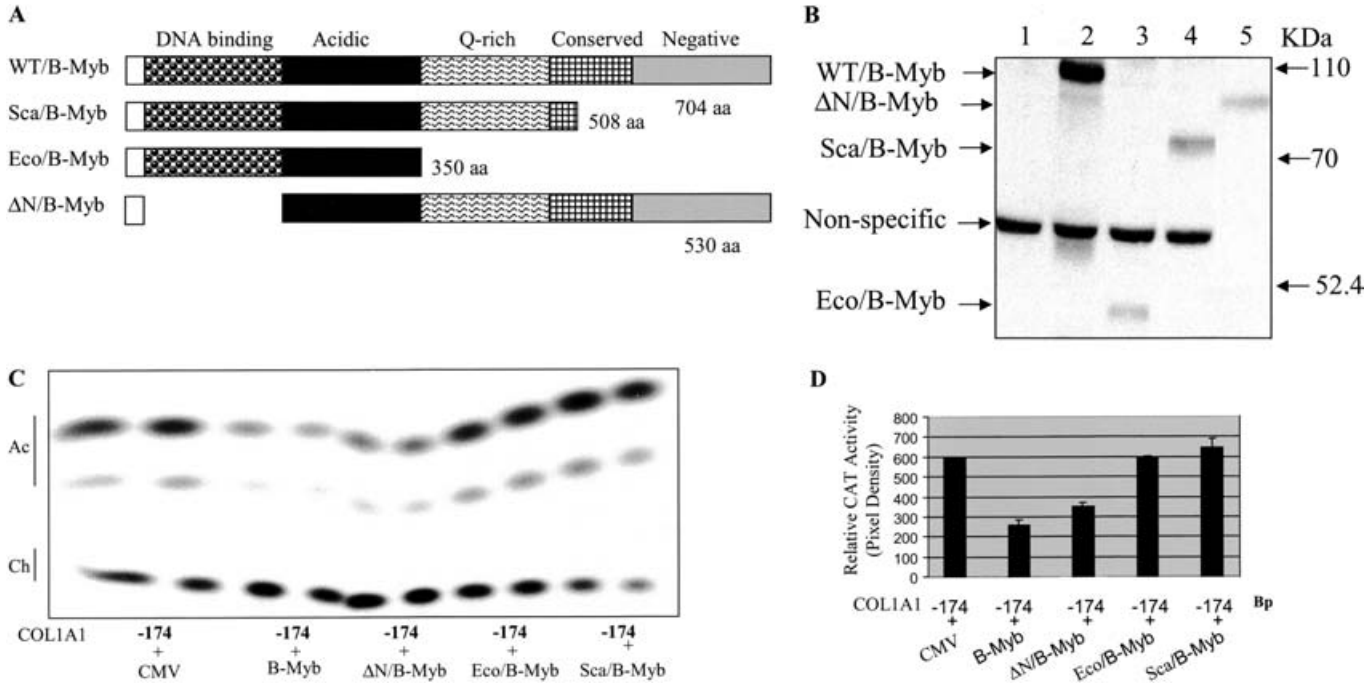

Figure 6 B-Myb down-regulates COL1A1 promoter activity through its C-terminal domain

(**A**) Schematic representation of the full-length B-Myb and the deletion mutants of B-Myb. (**B**) Western blot showing cellular extracts from mock-transfected cells (lane 1), full-length B-Myb (lane 2) and mutated forms, Eco/B-Myb (lane 3) and Sca/B-Myb (lane 4) and ΔN/B-Myb (lane 5) overexpressed in SSc fibroblasts. (**C**) The −174 deletion CAT construct of the human COL1A1 promoter was co-transfected in duplicate with empty vector (CMV) or with CMV vector containing the cDNA for WT/B-Myb, ΔN/B-Myb, Eco/DNA or Sca/DNA. The COL1A1 promoter activity decreased when the cDNA of WT/B-Myb or of ΔN/B-Myb was employed. No effects were observed when B-Myb cDNAs deleted for the C-terminal domain Sca/B-Myb and Eco/B-Myb were used (+). Ch, [^{14}C]chloramphenicol; Ac, acetylated form of [^{14}C]chloramphenicol. (**D**) Histograms represent the results of three different experiments. Error bars represent S.D.

at lower or similar levels than the C-terminal mutants, yet its activity is similar to that of wild-type B-Myb (Figures 6C and 6D). Thus expression levels of the B-Myb C-terminal mutants do not explain their loss of function. These results indicate that the region of B-Myb involved in the down-regulation of COL1A1 could be localized, only within the C-terminal of the protein.

DISCUSSION

In the present study, we have demonstrated that the transcription factor B-Myb inhibits the expression of the COL1A1 gene in human dermal fibroblasts. We have localized the inhibitory activity within 174 bp of the transcription start site of the COL1A1 promoter region. Furthermore, we have shown that B-Myb decreases Sp1 and CBF binding to their specific binding sites situated in the same region. Using SSc nuclear extracts, we found a physical interaction between B-Myb and Sp1 and CBF transcription factors that may be responsible for the down-regulatory effects on COL1A1 expression. We have also determined that the region of B-Myb involved in this modulation is contained within the C-terminal domain of the protein.

Genes encoding the α1 (COL1A1) and α2 (COL1A2) chains of type I collagen are expressed in different cell types at distinct stages of development and under several physiological conditions [40,41]. Excessive production of type I collagen is a key feature of fibrotic disorders of the lung, liver, kidney, skin and heart, and diseases such as SSc and hypertension, often leading to severe organ dysfunction [26,42,43]. Increased collagen deposition is a central event in the pathogenesis of SSc, but the mechanisms responsible for the pathological increase in the expression of collagen genes in SSc have not been clearly defined [22,26]. Previous studies suggested that, in addition to enhanced extracellular production and deposition, an abnormality of fibroblast growth might contribute to the fibrotic lesions [42]. The levels of COL1A1 mRNA are low in cultures of exponentially growing SMCs and increase as the cells reach confluence, whereas the levels of B-Myb mRNA expression are high in proliferating cells [19,20]. Piccinini et al. [44] have shown that B-Myb is expressed in proliferating SSc fibroblasts and its expression peaks 24 h after serum starvation, as has been found in the present study (Figure 1). The same authors have observed that c-Myb, but not B-Myb, up-regulates type I collagen expression [45], and that B-Myb may partially inhibit transactivation of the COL1A2 promoter driven by c-Myb [46]. These results suggest that B-Myb and c-Myb may exert opposite effects through the repression or activation of the same gene(s).

In the present study, we focused on the assessment of regulation of B-Myb of COL1A1 in human SSc fibroblasts. It has already been shown that B-Myb overexpression induces a strong down-regulation (90 %) of the COL1A1 and COL1A2 collagen gene promoters in bovine aortic SMCs [19]. Here, we demonstrated that B-Myb expression is inversely correlated with that of COL1A1 in normal and SSc fibroblasts (Figure 1A). The fact that no apparent increase in COL1A1 levels was seen in SSc versus normal fibroblasts could be due to the conditions of serum starvation or to a random variation of COL1A1 RNA levels among different SSc fibroblasts as we have previously observed [47]. The 174 bp COL1A1 promoter sequence that contains binding sites for CBF, Sp1 and other transcription factors, mediates maximal promoter activity of the COL1A1 promoter in human dermal fibroblasts [25,28]. Absence of MBSs in the 174 bp promoter region and up

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to −2000 bp (present study and [46]) suggests that the effect of B-Myb on COL1A1 expression does not occur by means of a direct protein–DNA interaction (Figure 3), but, instead, through a possible interaction of B-Myb with the DNA–protein complex formed by Sp1 and CBF (Figures 4 and 5). In addition, we found that Sp1 and CBF interact with each other, as previously shown [37–39], and B-*myb* may cause destabilization of these two positive factors positioned only 15 bp apart in the COL1A1 promoter (Figure 3).

Therefore two distinct mechanisms may be responsible for down-modulation of COL1A1 and COL1A2 genes and probably work in concert to decrease collagen I production in dermal fibroblasts. The first mechanism has been described by other groups [45,46], and may involve direct B-Myb binding to its consensus sequence in the COL1A2 promoter, resulting in squelching of other active Myb family members, such as c-Myb [46]. The other mechanism, described in this study, proposes a direct physical interaction between B-Myb and the CBF–Sp1 complex (Figures 4 and 5), which could be responsible for B-Myb-mediated reduction of Sp1 and CBF binding to their DNA-consensus sequences (Figure 3), and decreased COL1A1 transcription (Figures 2 and 6). So far, no direct binding of B-Myb to the COL1A2 promoter has been reported, despite the presence of MBSs [46]. The C-terminal domain of B-Myb has been shown to contain a conserved region with significant homology to other members of the *myb* gene family. The conserved region has been shown to be critical for transactivation by B-Myb, presumably via the binding of cofactors involved in the modification and activation of B-Myb [48]. In agreement with these observations, the analysis of the region of B-Myb involved in the down-modulation of COL1A1 indicates that the C-terminal region of B-Myb, but not the DNA-binding domain, is essential for this activity (Figure 6). Previously, we reported that B-Myb co-operates with the transcription factor Sp1, resulting in the synergistic activation of the B-Myb promoter in SAOS 2 osteosarcoma cells [32]. In the light of the results described here, we hypothesize that B-Myb and the Sp1–CBF complex are involved in either co-operative or antagonistic interactions, depending on the promoter and/or cellular context.

In conclusion, B-Myb probably contributes to the regulation of COL1A1 expression in SSc as well as normal dermal fibroblasts and may exert its effect by decreasing Sp1 and CBF binding to their specific sites in the proximal promoter region. Analysis of the role of post-transcriptional modification of B-Myb, such as phosphorylation and acetylation, and their role in the mechanistic interaction occurring among B-Myb, Sp1 and CBF are worth further investigation. Elucidation of the modes of action of regulatory factors of extracellular matrix proteins is essential towards understanding pathological fibrotic processes, such as SSc.

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