CHARACTERIZATION OF THE c-MYB-RESPONSIVE REGION AND
REGULATION OF THE HUMAN TYPE I COLLAGEN α2 CHAIN GENE BY c-MYB

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SUMMARY

We have further characterized the role of c-Myb and B-Myb in the regulation of human type I collagen α2 chain gene expression in fibroblastic cells. We have identified four potential Myb-binding sites (MBS) in the promoter. Transactivation assays performed in several human fibroblast cell lines on wild type and mutant promoter-reporter constructs demonstrated that c-Myb, but not B-Myb, is able to transactivate the human type I collagen α2 chain gene promoter via the MBS-containing region. EMSA experiments showed that c-Myb specifically binds to each of the four MBS, however the mutagenesis of site MBS-4 completely inhibited transactivation by c-Myb, at least in the context of the full-length promoter construct. In agreement with these transactivation results we found that c-myb−/− mouse embryo fibroblasts (MEFs) showed a selective lack of expression of type I collagen α2 chain gene, but maintained the expression of fibronectin and type III collagen. Furthermore, TGF-β was able to induce type I collagen α2 chain gene expression in c-myb−/− MEFs, implying that the TGF-β-signaling pathway is maintained in these cells and that the absence of COL1A2 gene expression in c-myb−/− MEFs is a direct consequence of the lack of c-Myb. The demonstration of the importance of c-Myb in the regulation of the type I collagen α2 chain gene suggests that uncontrolled expression of c-Myb could be an underlying mechanism in the pathogenesis of fibrotic disorders characterized by excessive deposition of type I collagen.
KEY WORDS

c-myb/myb-genes/transcription factor/collagen/scleroderma/fibrosis
INTRODUCTION

The myb oncogene family is composed of c-myb, A-myb and B-myb genes, each encoding a distinct nuclear protein that displays transcription factor activity (1). The myb genes are structurally related and initially they were believed to be expressed only in hematopoietic cells, where they were found to play a pivotal role in the regulation of growth and development (2,3,4,5). However, the different myb family members have been reported to be expressed and to function in some other cell types. Hence, although little information is available relating to A-Myb, it has been demonstrated that c-Myb and B-Myb are expressed in epithelial cells (6), and fibroblasts (7). In the latter cells, the role of the Myb proteins has not been completely elucidated and, despite their structural homology and similar patterns of expression, B-myb and c-myb may exert opposite effects through the repression or activation of the same gene(s) (9,10,11,12,13). With regard to c-Myb, it has been demonstrated that it can induce insulin-like-growth factor-1 (IGF-1) independent growth (14) and that it can control fibroblast proliferation through the regulation of the intracellular Ca++ concentration (15) as well as the expression of cell-cycle related genes, such as PCNA (16).

c-Myb protein can activate or repress gene transcription by binding directly to the promoters of genes as in the case of mim-1 (17), cdc2 (18), c-myc (19,20), CD4 (21), the HTLV-1 LTR (22) and c-myb itself (23). However, despite intensive investigations, not many genes regulated by c-Myb have been identified so far.
Following the finding of abnormal expression of *c-myb* genes in quiescent scleroderma fibroblasts, a disease characterized by an augmented production of extracellular matrix protein, we have speculated that c-Myb but not B-Myb could be involved in the up-regulation of the type I collagen promoter (8). However, the employment of animal and not human type I collagen promoters, the use of the 3T3 fibroblast cell line rather than human fibroblasts, and the lack of identification and characterization of the region of the promoter involved in c-Myb binding has left open the issue of whether c-Myb can modulate collagen gene expression (24).

In view of the potential relevance of this finding for the understanding of the pathogenesis of scleroderma and of fibrotic disease in general we have, therefore, decided to elucidate more exhaustively the role of c-Myb and B-Myb in the expression of the human type I collagen gene. We have cloned the type I collagen α2 chain (COL1A2) promoter and screened it for the presence of potential Myb-binding sites (MBSs). Transactivation of the COL1A2 promoter by c-Myb and B-Myb has then been investigated in human fibroblasts and human fibroblast cell lines, and the regions of the promoter which are critical for Myb-mediated transactivation have been determined. Finally, the requirement for c-Myb in the expression of the type I collagen α2 chain gene has been demonstrated by comparison of embryonic fibroblasts derived from wild type and *c-myb* -/- mice and of normal human skin fibroblasts over expressing c-Myb or a dominant negative c-Myb derivative.
EXPERIMENTAL PROCEDURES

Computer analysis of the putative myb-binding-sites contained in the COL1A2 promoter

The whole DNA sequence of the COL1A1 and COL1A2 promoters (accession numbers J03559, U06669, and AF 004877, respectively) was subjected to computer analysis and screened for putative myb-binding-sites (MBSs) using the software MatInspector v2.2 (25). The computer analysis utilised matrices derived from the published MBSs consensus sequence (26,27,28) and results were expressed in matrix similarity, where a value of 1 corresponds to complete homology.

Cloning of the human COL1A2 promoter

The 5’ flanking region of human COL1A2 gene, spanning from -2430 to +5 bp (according to the Gene Bank Sequence AF 004877) containing a CAAT-binding site (-220) and a TATA -box (-170), was cloned from genomic DNA of human fibroblasts using a PCR approach. Briefly, fibroblast genomic DNA was extracted from monolayer cultures of normal human fibroblasts using a commercial kit (Qiagen) according to the manufacturer’s instructions. Genomic DNA (250 ng) was amplified in a final volume of 50 µl containing 50 mM KCl/10 mM Tris-HCl/1.5 mM MgCl₂/0.2 mM each dNTP and 15 pmol of forward and reverse primers (COL-F: TTACCACCGGTCCCTTTGAGTTCATTTC and COL-B: GCACCTAGACATGCAGACTCCT), and 2 units of LA-Taq polymerase (TaKaRa). PCR (20 cycles of 1 min 95°C, 1 min
55°C, 3 min 70°C) was carried out in a PTC-200 thermal cycler (MJ Research). The resulting DNA fragment of 2430 bp was extracted from a 1% agarose gel, purified using Gene-Clean kit (Qiagen) and cloned within the multiple cloning site region (mcsr) of the pT-Adv vector (Clontech) using standard ligation procedure. Both strands of the COL1A2 promoter inserted in the recombinant plasmid pTCOL1A2-P were extensively sequenced to exclude the possibility of random mutations inserted by PCR reaction. The COL1A2 promoter was further digested from pTCOL1A2-P using SacI and EcoRV, gel purified and subcloned between the SacI and SmaI sites upstream of the firefly luciferase gene of the Promega vectors pGL3-Basic (pGbC1A2-P) and pGL3-Enhancer (pGECA2-P) in which the SV40 enhancer sequences are located downstream of the mcsr. Briefly, 100 ng of COL1A2 promoter DNA was mixed with 30 ng of vector in 66 mM Tris-HCl pH7.6/6.6 mM MgCl\(_2\)/10 mM DTT/0.1 mM ATP/2 µM Hexamino-CoCl and 450U T4 DNA ligase. The ligation mix was incubated at 14°C overnight and 3 µl of the mix were used to transform DH5-α competent bacteria.

**Plasmids**

The pGbC1A2-P and p-GECA-P recombinant plasmids were derived as described above. The pGL-KHK plasmid was created by digesting KHK-CAT (24) with BamHI and HindIII. The resulting DNA fragment containing the KHK synthetic promoter was purified by gel electrophoresis and end-blunted in a mix containing 100 µM of each dNTP/0.01% BSA/33 mM Tris-Ac pH 7.9/66 mM Kac/10 mM MgAc/0.5
mM DTT and 4U of T4 DNA polymerase. The blunted KHK promoter was then subcloned into the Sma I site of the pGL3-Basic mcsr, upstream the firefly luciferase gene.

The pQCM plasmid, used for the production of the c-Myb recombinant protein, was obtained by digesting pSGC-myb, carrying the full-length coding region of the human c-myb gene, with NcoI and BglII and subcloning the resulting c-myb gene fragment (+1 to +1200 bp) upstream and in frame with a six-histidine tag into the NcoI/BglII sites of the pQE-60 plasmid mcsr (Qiagen, Hilden, Germany). This c-myb DNA fragment contained the sequences encoding the R1-R3 domains that are necessary for c-Myb DNA binding activity. The pSGC and pSGB plasmids, containing the entire coding region of the c-myb and B-myb genes, respectively, and the pCys 130 plasmid, carrying the entire coding region of c-myb gene mutated at codon 130, have already been described (24). The pGREMyb plasmid, containing the full coding region of the c-myb gene, and the pGREMen plasmid, in which sequences encoding the R2 and R3 domains of c-Myb and the Drosophila engrailed gene encoding the alanine-rich repressor domain (15) are linked (a kind gift of Prof. M. Simons, Angiogenesis Research Center, Cardiovascular Division, Boston, USA).

All recombinant plasmids were grown and further purified using the Endofree maxiprep method (Qiagen, Hilden, Germany).

Nested-deletions of the human COL1A2 promoter

The pTCOL1A2-P plasmid was used in PCR reactions to obtain the promoter
deletions. Nested primers starting from different regions of the COL1A2 promoter (del1F-1500: AGCCTTTCAACCTAGGGCCTG, del 2F-1269: GCCTCAGCAAAGGCAAGCTAG, del3F-950: TGGAGCCCTCCACCCTACAA, del4F-575: GGACAGCTCC TGCT TTATCG, del5F-290: TTCGCTCCCTCTCTGCGCCC) and a backward primer (GCCCATCTGCAGAATTCGGCTT) were used in PCR reactions to generate five different DNA fragments spanning from -1500 to -290. Since the first two MBSs are closely located at position -1025 and -1045, it was not possible to separate them efficiently, and therefore they were both deleted in the pGLD-1269 plasmid. Template DNA (250 ng) was amplified in a final volume of 50 µl containing 50 mM KCl/10 mM Tris-HCl/2 mM MgCl2/0.2 mM each dNTP/15 pmol of each primer and 2 units of LA-Taq polimerase (TaKaRa, Otsu, Shiga, Japan). PCR reactions (1 min 95°C, 1 min 55°C, 3 min 70°C) were carried out in an MJ Research PTC-200 thermal cycler for 20 cycles. Each fragment was gel purified and subcloned into the mcsr of pT-Adv vector (Clontech, Palo Alto, California) and thereafter into the SacI-Smal sites of the pGL3Basic plasmid mcsr to obtain the pGLD-1500, pGLD-1269, pGLD-950, pGLD-575, and pGLD-290 recombinant plasmids, respectively. The pGLD1095 plasmid was obtained by the digestion of pGbC1A2-P with NcoI and the resulting COL1A2 promoter fragment, spanning from position -1095 to the NcoI site of the pGL3-Basic vector, was subcloned within the NcoI site of the pGL3-Basic vector mcsr. All plasmids were extensively sequenced to check for mutations introduced by PCR.
Site-directed mutagenesis

The pGbC1A2-P and pGL-D1095 plasmids, containing only one MBS at position -1000, were used as templates for site-directed mutagenesis. The sequences of the primers used were: MBM-1: CGGTCTCCAGGTGATATCAGTCGTGTCGGAGTGCCAG, MBM-2: CGGTCTCCAGGTCACTAGTAGTCGTGTCGGAGTGCCAG, MBM-3: CGGTCCCAGGTCCGCGGTAGTCGTGTCGGAGTGCCAG, both in the sense and antisense orientations, each incorporating a restriction enzyme (EcoRV, SpeI, and SacII, respectively) for the screening of recombinant mutated plasmids. Only the MBM-3 primers were used to mutagenize the MBS-4 of the pGbC1A2-P plasmid (containing the full-length COL1A2 promoter). The site-directed mutagenesis was achieved using the “Ex-Site PCR-Based Site-Directed Mutagenesis” kit (Stratagene, La Jolla, USA). Each primer was phosphorylated at 37°C for 30 min in 1X kinase buffer (TaKaRa, Otsu, Shiga, Japan) containing 10 U of T4 polynucleotide kinase (Takara, Otsu, Shiga, Japan). 30 pmol of phosphorylated sense and antisense primers were then used in a mix containing 400 ng of template DNA, 5U of Exsite DNA polymerase blend/0.2 mM dNTPs/20 mM Tris-HCl pH 8.8/10 mM KCl/10 mM (NH₄)₂SO₄/2 mM Mg SO₄/0.1 % Triton X-100/0.1 mg/ml BSA. The PCR reaction was carried out in an MJ Research PTC-200 Thermal Cycler. At the end of the reaction, 10U of DpnI restriction enzyme and 2.5U of Pfu polymerase were added and the mix was incubated at 37°C for 2 hrs and 72°C for 1 hr. An aliquot of PCR products was further ligated at 14°C overnight with 5 mM rATP and 4U T4 DNA ligase in 10 mM Tris-HCl pH 8.8/5 mM KCl/5 mM (NH₄)₂SO₄/0.1 % Triton X-100/0.1 mg/ml BSA. Ten microliters of
the reaction were used to transform XL-1-Blue competent bacteria. All recombinant plasmids were screened for the inserted mutations using the above mentioned restriction enzymes and thereafter both strands were extensively sequenced using a CEQ-2000 DNA sequencing instrument (Beckman, Palo Alto, California).

**Cells and cell cultures**

Normal human skin fibroblasts (NSF) were obtained from punch biopsies taken from the forearms of healthy subjects. Primary explant cultures were established in 25 cm² culture flasks in MEM medium containing 10% FCS, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 ug/ml) and amphotericin-B (0,25 mg/ml). MEM medium with these supplements is hereafter referred to as ‘culture medium’. Normal fibroblast human cell lines WS-1, HUDE, and HFL-1 were purchased from ATTC and grown in DMEM medium containing 10% FCS, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 ug/ml) and amphotericin-B, (0.25 mg/ml). Normal human fibroblastic cell lines transfected with pGreMyb and pGreMen plasmids were grown in culture medium containing 0.2 μM dexamethasone (15). Monolayer cultures were maintained at 37°C in 5% CO₂. Fibroblasts at the fifth passage were used for all experiments. *c-myb* knock-out (*c-myb*⁻⁻)(29) embryonic fibroblasts (MEFs) were cultured in DMEM containing 20% FCS, 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 ug/ml) and amphotericin-B, (0.25 mg/ml). Fibroblasts at the fourth passage were used for Northern blot analysis. For the stimulation experiments
of wt and c-myb/- MEFs, recombinant TGF-β was used at a final concentration of 2 ng/ml.

**Cell transfection**

For transfection experiments, confluent fibroblasts were harvested with trypsin and plated in 60 mm dishes in culture medium. After 24 hrs, the medium was discarded, replaced with fresh culture medium and the cells transfected. Transfection experiments were carried out in triplicate using a liposomal method (Effectene, Qiagen) in the presence of an Enhancer and a DNA condensation buffer (according to the manufacturer’s instructions). Plasmid DNA was purified with Endofree maxi-kit (Qiagen) to remove bacterial endotoxins. Cells were transfected with a mix containing 0.7 µg of reporter plasmid ± 1.5 µg of effector plasmid, 8 µl Enhancer, 25 µl/µg DNA of Effectene, and culture medium containing 10% FCS. To control transfection efficiency, the pRLSV reporter plasmid (Promega), coding for Renilla luciferase, was used in all experiments at a concentration of 0.05 µg/60 mm dish. After 12 hrs, the medium was changed and 24 hrs later the cells were harvested and lysed in 100 µl of PBL buffer (Promega). Following the manufacturers instructions, 20 µl of the supernatant were used in the Dual Reporter Luciferase Assay in which the activities of Firefly and Renilla luciferases are sequentially measured from a single sample using two different enzymatic substrates. The luciferase activities of the samples were measured with a TD-20/20 luminometer (Turner design) and the ratio of Renilla to Firefly luciferase values was used to normalize all of the transfection experiments.
Western blot analysis

Cleared cell lysates, obtained from NSF cells that had been cultured and transfected as described above, were precipitated with 20% trichloroacetic acid and aliquots containing the same amount of protein were subjected to SDS/PAGE on 10% gels, according to standard procedures. The transfer from the gels to nitrocellulose membranes (Bio-Rad, Milan, Italy) was achieved using a Trans Blot Cell apparatus (Bio-Rad) and blotting was performed at 4°C, 30 V overnight in 25 mM Tris/192 mM glycine/20% (v/v) methanol/3.5 mM SDS. To ensure that comparable amounts of proteins had been transferred to the nitrocellulose membranes, proteins were revealed on by staining with 0.05% (v/v) Ponceau S (Sigma) for 1 min. The blots were then rinsed in TBS (50 mM Tris/170 mM NaCl/0.2% (v/v) Tween 20, pH 7.5) and incubated for 4 hrs in Blotto solution (TBS containing 5% non-fat dried milk), before incubating overnight at 4°C with rabbit anti-c-Myb (Geneka) diluted 1:1000 in Blotto solution. The blots were then rinsed in TBS with several changes and then incubated for 60 min at room temperature in a horseradish peroxidase-labelled donkey anti-rabbit IgG (Amersham, Little Chalfont, UK) diluted 1:1000 in TBS. After further washing, bound antibodies were detected using enhanced chemiluminescence detection reagents (Amersham). Images were analysed using a Molecular Images FX (Bio-Rad).

Production of the recombinant Myb-HIS fusion protein

pQCM and the control plasmid pQE40 (Qiagen) were used to transform pREP-MC5 chemicompetent bacteria (Qiagen, Hilden, Germany). Single bacterial colonies
were picked from LB plates, grown in an orbital shaker at 37°C in NZYC broth and induced with 0.5 mM isopropyl-thiogalactoside (IPTG) for 5 hrs. An aliquot of the culture was lysed in 8M urea and subjected to Western blot analysis to check the expression of recombinant proteins using the Penta-HIS antibody (a mouse monoclonal IgG1 antibody raised against the six-histidine tag coupled to horseradish peroxidase, Qiagen, Hilden, Germany). Western blot analyses were carried out as described above and following the manufacturer’s experimental conditions with minor variations. The batch purification of Myb-HIS recombinant protein was achieved using an extraction method under native conditions. Briefly, bacterial pellets were resuspended in 5 ml/g of B-PER II reagent (Pierce, Rockford, USA) containing 20 mM imidazole and 0.1 mM PMSF, briefly vortexed and then incubated at room temperature for 30 min. Thereafter, the solution was centrifuged at 4°C for 30 min and the cleared lysate was recovered. 2 ml of 50% nickel-nitrilotriacetic acid (Ni-NTA) slurry matrix (Qiagen, Hilden, Germany) were added to the lysate and gently mixed by shaking at 4°C for 1 hr. The lysate-Ni-NTA mixture was loaded onto a column, washed twice with 50 mM NaH$_2$PO$_4$/300 mM NaCl, and Myb-HIS recombinant protein was eluted with 50 mM NaH$_2$PO$_4$/300 mM NaCl/250 mM imidazole. The purity of Myb-HIS recombinant protein in the eluate was assessed by Western blot analyses using the Penta-HIS antibody described above.

Electrophoretic mobility shift assay (EMSA)

EMSA experiments were carried out using the Myb-HIS recombinant protein and
four double-stranded oligonucleotides (MBS-1: CCTCTCCCTAGTAGGGAGTGGAGG GTTGGATGGAGGCGGC, MBS-2: TGGAGGCGGCCAGAGAAGAGGGAAGTTGGGT GCTGGGGAGAGAGTTAACA, MBS-3: GGACCGGGGGCTACAGGGAGGGTGA AGGGTCCAGCTC, MBS-4: GTTCTCGGTCTCCAGGTCGGTTGGAGTCGTGTCGGAC TGC) the sequences of which (listed from 5’ to 3’) are complementary to COL1A2 promoter regions that contain each of the four MBS (see Figure 1A). The gel purified KHK promoter, obtained by the digestion of the pGL-KHK plasmid with HindIII and BamHI was used as a cold competitor. Anti-c-Myb polyclonal antibody (Geneka, Montreal, Quebec, Canada), raised against a peptide derived from amino acid residues 2-16 of human c-Myb, was used to block the binding activity of Myb-HIS recombinant protein to its MBS. The Penta-HIS antibody described above was used to supershift the DNA-Myb-HIS complex. The complementary single strand oligonucleotides were annealed at 95°C for 3 min, at 55°C for 2 min and at 37°C for 15 min in 10 mM Tris-HCl pH 7.5/10 mM MgCl₂/50 mM NaCl. Three hundred nanograms of double stranded oligonucleotides were then labelled with α-³²P-dCTP (3000 Ci/mmole) using 2.5 U of Klenow DNA enzyme and purified on a silica-column (Qiagen). The binding reaction was carried out at room temperature for 30 min using 25000 cpm of labelled DNA, 1 µg of poly dI-dC, and 10 µg of Myb-HIS in 50 mM NaCl/20 mM Hepes pH8/1 mM EDTA/10 mM DTT/0.5 % non-fat dried milk/5 % glycerol in a final volume of 12 µl. For supershift and blocking experiments the binding reaction was 2 µl of Penta-HIS or anti-c-Myb polyclonal antibody, respectively, were
preincubated at 4 °C for 15 min in the absence of the target DNA. After the incubation period, 1.5 µl of loading buffer (250 mM Tris-HCl pH 7.5, 40 % glycerol) were added and the samples were immediately loaded onto a 6 % acrylamide Retardation Gel (Novex). Gel electrophoresis was carried out in a cold room at 100 V for 2 hrs. The gel was then fixed with 10 % acetic acid/10% Methanol, dried and exposed using the FX-Molecular Imager system (BIORAD).

**Northern analysis**

The plasmid used for the detection of the type I collagen α2 chain mRNA (Hf32) and type III collagen were a kind gift of Dr. C.M. Lapiere (Laboratoire de Biologie des Tissue Conjonctifs, University of Liege, Belgium). The fibronectin and TGF-βcDNAs were purchased from ATCC. The probe was labelled with 32P-dCTP by standard random priming procedures to specific activities of 5 x 10^8 cpm/µg DNA. Total cellular RNA was extracted according to the guanidine isothiocyantate-cesium chloride method. Eight micrograms of total RNA were loaded in each lane of a 1% agarose-formaldehyde gel and transferred to nylon membranes by standard blotting procedures. RNA was fixed to the membranes by baking or UV-crosslinking. Prehybridization was performed for 7 hrs at 60°C in 1M NaCl/1% SDS/10% dextran sulfate and 100 mg/ml denatured salmon sperm DNA. Hybridization was performed overnight at 60°C in the same buffer by adding 1.2 x 10^6 cpm/ml of labelled probe. The filters were washed twice for 10 min at 60°C with 2 x SSC/1 % SDS, 15 min with 0.5 x SSC/0.5 % SDS and finally twice at room temperature with a large volume of 0.5
The blots were briefly dried, exposed in screen cassette BIORAD and hybridization signals were obtained using a Molecular Images FX (Bio-Rad Laboratories, Hercules, California).

Semiquantitative analysis of c-myb RNA by PCR in mouse embryonic fibroblasts (MEFs)

Total cellular RNA was extracted from MEFs as described above. Two micrograms of total RNA were directly reverse-transcribed in 5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl pH 8.3, 2.5 µM random hexamers, 1 mM dNTP, 1 U/ml RNase inhibitor and 2.5 U/ml M-MLV reverse transcriptase. The samples were incubated for 10 min at room temperature and then for 45 min at 42°C. Three microlitres of the reverse transcription reactions were amplified by PCR in 2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, 0.2 mM each dNTP, 2.5 U/ml Taq DNA polymerase and 5 ng/ml of each primer: c-myb 1, ACTCAACTGCCCAATGAAGTCG; c-myb 2, TTCCTGTTCCACCTTGCG; actin 1, ATCGTGGGCGCCCTAGGCACCA; actin 2, TTGGCCTTAGGATTCAGAGGG. PCR was performed in an MJ-PT200 thermal-cycler using the following cycle: 30” 95 °C, 30” 58 °C, 30” 72 °C, for 18 cycles. The predicted sizes of the RT-PCR products were 445 bp and 244 bp, respectively. An aliquot of each reaction was run in a 1.5 % ultrapure agarose gel (BRL) and transferred to nylon membranes by standard Southern blotting procedures.
RESULTS

The promoter of the human type I collagen α2 chain gene contains four putative Myb-binding sites

In a previous report, we have shown that c-Myb can up-regulate rat α1(I) collagen and mouse α2(I) collagen promoters by 6-10 fold whereas B-Myb was inactive (8). Here we have tried to ascertain whether c-Myb could also up-regulate human type I collagen gene expression. Using the computerized software MatInspector v2.2, we have analysed the DNA sequence of the promoter of the human type I collagen α2 chain (COL1A2-P), and found four putative MBSs. These are located between positions −1400 to −1000 (henceforth defined as a "Myb-Responsive Region" (M-RR)) and display a high degree of homology with the published consensus sequence, the degree of matrix similarity ranging from 0.87 to 0.93, where 1 corresponds to complete homology (Figure 1A).

When we extended the software analysis to the DNA sequence of the human COL1A1 gene promoter, from the transcription start site to −2000 bp, we did not find myb-binding sites. Thus, we have focused our investigation on the regulation of COL1A2 gene expression by c-myb.

c-Myb, but not B-Myb, up-regulates the promoter of the gene encoding the α2 chain of human type I collagen in human fibroblasts

To demonstrate that c-Myb is able to transactivate the human COL1A2 promoter,
we co-transfected the reporter plasmid pGbC1A2-P (containing the COL1A2 promoter inserted upstream the firefly luciferase gene - Figure 1B) and either the pSGC or pSGB CMV promoter-driven plasmids which express human c-Myb or B-Myb, respectively. Western blot analysis confirmed that c-Myb was efficiently expressed from the pSGC vector (Figure 2B). In a different set of experiments, the pGECA-2 plasmid, in which the COL1A2 promoter is inserted upstream an SV40 enhancer cassette, was used as the reporter vector (Figure 1C). The transfection assays demonstrated that c-Myb, but not B-Myb, up-regulates expression driven by the COL1A2 promoter by 6-8 fold in the WS-1 and HUDE cell lines, and in normal skin fibroblasts (NSF). A stronger degree of transactivation (10-15 fold) was obtained in the HFL-1 cell line (Figure 2A).

Furthermore, in the same cell types we performed co-transfection assays using the expression plasmids pSGC and pSGB and the control reporter plasmid pGL-KHK, which carries the KHK synthetic promoter inserted upstream the luciferase gene. In this case, both c-Myb and B-myb were able to stimulate luciferase expression 8-fold (Figure 2C). Moreover, co-transfection assays using pSGC and pSGB together with the reporter plasmid pGbC1A2-P demonstrated that B-Myb could partially inhibit transactivation of the COL1A2 promoter driven by c-Myb (Figure 2D).

The strength of the Myb-induced transactivation was also evaluated by transfecting the pSGC or pSGB expression vectors together with the reporter pGECA2-P in which the COL1A2 promoter was linked to SV40 enhancer sequences. Stimulation of this promoter/enhancer combination by c-Myb was equivalent to that
seen with the pGbC1A2-P reporter (data not shown), thus demonstrating that enhancer region(s) are not required for the effect of c-Myb on the COL1A2 promoter.

These results clearly prove that the human COL1A2 promoter is strongly and specifically transactivated by c-Myb in several human fibroblastic cell types, whereas B-myb can potentially modulate this transactivation through partial inhibition of the positive effect of c-Myb.

Transactivation of the human type I collagen α2 chain gene promoter requires the presence of the 400 bp Myb-responsive region (M-RR)

Next we investigated the role that the four MBSs of the 400 bp COL1A2 promoter Myb-responsive region (M-RR) might play in c-Myb-mediated transactivation. We created a set of pGL-plasmids that carry different deletions of COL1A2 promoter from -1500 to -290 in order to remove progressively each MBS (Figure 3A). Transfection assays utilising the pSGC expression vector together with promoter constructs containing deletions to -950, -575 and -250 demonstrated that one or more MBSs were sufficient to allow c-Myb-dependent activation, but that the loss of all four MBSs completely ablated the response to the transactivator (Figure 3B).

These results prove that the presence of the 400 bp M-RR in the COL1A2 promoter is necessary for c-Myb-dependent transactivation and suggest that each MBS could be important in mediating the effect.

Mutagenesis of MBS-4 or mutation of c-Myb abrogates Myb-dependent
transactivation of the human type I collagen α2 chain gene promoter

To show further that c-Myb acts upon the MBS in the COL1A2 promoter, mutagenesis experiments were performed on MBS-4 at position 1000. MBS-4 (GGTTGG) was mutated in the context of i) plasmid pGbC1A2-P, which contains all four MBS, to CCGCGG (MBM-3), and ii) plasmid pGLD’1095, which contains only MBS-4, to sequences GATATC, ACTAGT, or CCGCGG (MBM-1, MBM-2, or MBM-3, respectively). In transfection assays, c-Myb expressed from pSGC was unable to stimulate luciferase expression from the mutated pGLD’1095-based plasmids, demonstrating the requirement for the integrity of MBS-4 (Figure 4A). Moreover, mutation of MBS-4 alone in the context of the full length COL1A2 promoter strongly decreased up-regulation by c-Myb (2.3 times less than on the wt-promoter - Figure 4A).

To demonstrate that c-Myb is directly involved in transactivation of the COL1A2 promoter we used an expression vector, pCys130, which encodes a mutated c-Myb protein containing a Cys to Ser mutation at position 130 that abrogates the binding ability of c-Myb to DNA (30,31). The mutated c-Myb protein was unable to activate luciferase expression from either the wild-type pGLD-1095 or the full length COL1A2 promoter (Figure 4B).

These data demonstrate that both MBS integrity and fully functional c-Myb protein are necessary for the c-Myb-mediated transactivation of the COL1A2 promoter. Furthermore, of the four MBS, MBS-4 seems to play the pivotal role in COL1A2 promoter transactivation by c-Myb.
c-Myb protein binds to the MBSs DNA sequences in the promoter of the human type I collagen α2 chain gene promoter

In view of results described above, we have investigated the DNA binding capacity of c-Myb to the MBSs DNA sequences that are present in the M-RR of the COL1A2 promoter. For this purpose we produced a c-Myb recombinant protein, truncated at position +1200, conjugated to a six-hystidine tag. This protein, Myb-HIS, contains the three repeat domains (R1, R2, and R3) that are necessary for DNA binding ability (32,33,34). Gel retardation experiments (EMSA) demonstrated that Myb-HIS is able to bind to the KHK synthetic promoter (Figure 5A) and to a labelled double-stranded oligonucleotide containing the sequence of MBS-4 (Figure 5B). The Myb-HIS/DNA complexes were similar to those obtained using nuclear extracts from the K562 cell line (Figure 5A and B, lane 2). The binding activity was abrogated by incubating Myb-HIS with a 100-fold molar excess of cold competitor (Figure 5A and B, lane 4) and with an anti-c-Myb polyclonal antibody raised against the region of the protein that contains the R1-R3 domains (Figure 5A and B, lane 5). The same results were obtained using three different double-strand oligonucleotides, each one containing one of the three other MBS (MBS1-3) from the COL1A2 promoter (data not shown).

To confirm the binding specificity of Myb-HIS to the MBS, we carried out binding reactions using the Penta-HIS antibody, raised against the six-hystidine tag of the recombinant protein, to super-shift the Myb-HIS/DNA complex. The reason for using this antibody was that the available anti-c-Myb antibodies were directed
against the COOH-terminus of the protein, which is deleted in our recombinant Myb-HIS, and because the hystidine tag does not usually participate in the function of recombinant proteins (35-40). Moreover, the Penta-HIS antibody easily detected the recombinant protein in Western blot analysis (data not shown). Gel retardation experiments demonstrated that the Penta-HIS antibody was able to supershift the Myb-HIS/DNA complex (Figure 5C, lane 4). Binding reactions between Myb-HIS protein and double-stranded oligonucleotides containing the three mutated MBS described above (MBM 1-3) demonstrated that the mutated sites could no longer specifically bind Myb-HIS (Figure 5C, lanes 5-7). These data demonstrate that the Myb-HIS recombinant protein, containing the R1-R3 DNA binding domains conjugated to a six-hystidine tag, can specifically bind to both the KHK synthetic promoter and to several double-stranded oligonucleotides that contain the COL1A2 promoter MBSs and that the Myb-HIS/DNA complex can be super-shifted by the Penta-HIS antibody. Specificity of binding is indicated by the fact that binding can be blocked by: a) a molar excess of cold competitor; b) an anti-c-Myb antibody directed against the R1-R3 DNA binding domains; and c) mutation of the MBSs.

All the above evidence clearly demonstrates that Myb-HIS binds to the human type I collagen α2 chain gene promoter, requiring specific interaction between the DNA-binding domain of c-Myb and the MBS.

The lack of transcription of type I collagen α2 chain mRNA in c-myb<sup>−/−</sup> embryonic fibroblasts can be restored by ectopic expression of c-Myb.
To confirm the relationship between c-Myb and type I collagen α2 chain gene expression, we studied mouse embryonic fibroblasts derived from embryos homozygous for an inactivating mutation in the c-myb gene (c-myb−/− MEFs). Although c-myb−/− embryos do not survive beyond day 16 of gestation because of a severe impairment of fetal hematopoiesis, MEFs can be grown in cultures for several weeks (15) and are suitable for the study of extracellular matrix gene expression. The results of a representative Northern blot analysis of mRNA from MEFs c-myb−/− cells (out of a total of five experiments) are shown in Figure 6A. Cells lacking c-Myb expressed very low levels of α2(I) collagen mRNA when compared to wild type cells, a faint band being visible only after long exposures of the autoradiograms. This defect is specific since the same cells maintained the expression of other important extracellular matrix proteins, such as type III collagen and fibronectin (Figure 6A). The transfection of the c-myb−/− MEFs with a plasmid encoding c-Myb restored the expression of the α2(I) collagen mRNA to a level comparable to that of the wt MEFs (Figure 6B) implying that the lack of the constitutive expression of α2(I) collagen gene is caused by the lack of c-Myb expression and not by a global impairment of other signalling pathways. This latter conclusion is further supported by the normal expression of the α2(I) collagen gene, but not c-myb, when the c-myb−/− MEFs are stimulated by TGF-β (Figure 6C). Wild type MEFs exhibited increases in both c-myb and COL1A2 gene expression after TGF-β stimulation (Figure 6C), thus mimicking the response seen in human dermal fibroblasts (24).
Normal human skin fibroblasts expressing a dominant negative version of c-Myb lose the ability to express the type I collagen gene

Finally, to validate and confirm in human cells the data obtained in c-myb−/− MEFs, we transfected normal human skin fibroblasts (NSFs) with a plasmid constitutively expressing wild type c-Myb (pGREMyb) or a dominant negative form of c-Myb (pGREMen). NSFs transfected with pGREMyb displayed an augmented expression of the type I collagen gene whereas they lost the ability to express the gene when transfected with pGREMen (Figure 7).

This demonstrates that c-Myb also plays a crucial role in the regulation of the type I collagen gene in human cells.
DISCUSSION

Type I collagen is a heterotrimeric molecule consisting of two \( \alpha_1(I) \) chains and one \( \alpha_2(I) \) chain. Fibroblasts and osteoblasts are the major collagen-producing cells in tissues such as a skin and bone that present large amounts of type I collagen (41). Fibroblasts also appear to be the collagen-releasing cells in those tissues that contain smaller amounts of type I collagen. Hence, since the genes encoding the \( \alpha_1(I) \) and \( \alpha_2(I) \) chains are expressed in several cell types, at distinct stages of development and under various physiologic conditions and their regulation is consequently complex (42-44). Interactions between a number of \textit{cis}-regulatory elements and sequence specific trans-acting factors are involved in the stage-specific and tissue-specific regulation of type I collagen gene transcription. Common \textit{cis}-acting elements present in the genes encoding both the \( \alpha_1 \) and \( \alpha_2 \) polypeptides have been demonstrated to be responsible for their co-regulation (46).

Recent studies have examined collagen gene transcriptional regulation using the human and mouse \( \alpha_1(I) \) and \( \alpha_2(I) \) gene promoter regions (COL1A1 and COL1A2) and have led to the identification of several \textit{cis}-elements and transcription factors that control constitutive expression. Thus, Ets factors (45), CCAAT binding factor (46,47), K-ROX (48,49), NFkB (50), Sp-1 (51,52) and SMADs (53,54) have all been found to regulate type I collagen gene expression. These transcription factors may act alone or in concert, as is seen for example in the case of regulation elicited by the of TGF-\( \beta \) signalling pathway which has a positive effect on COL1A2 promoter activity in
cooperation with Sp-1 (55,56).

At present the mechanisms that regulate the expression of collagen genes in pathologic fibroblasts are not known. Cells isolated from fibrotic tissues have an activated phenotype. Both the COL1A1 and COL1A2 promoters exhibit several fold higher activity when studied in scleroderma fibroblasts as compared to fibroblasts derived from healthy controls (57,58). In activated Ito cells derived from cirrhotic liver increased COL1A1 mRNA levels correlate with increased Sp-1 binding to the promoter (59), however, the specific cis-elements and trans-acting factors responsible for abnormal regulation of promoter activity in fibrotic disease have not been conclusively elucidated.

Previous work by our group have shown that c-Myb, a transcription factor involved in differentiation and proliferation of hematopoietic cells, is expressed by scleroderma fibroblasts cultured in serum-deprived medium and is able to transactivate mouse and rat type I collagen gene promoters (8,24). Since a detailed study of the transcriptional regulation of the COL1A2 promoter by c-Myb may shed light on the pathogenesis of scleroderma and could lead to possible therapeutic strategies against this incurable disease, we felt it important to investigate more thoroughly the relationship between c-Myb and COL1A2 promoter regulation.

Previous studies have identified several functional cis-acting elements in the 350 bp proximal region of the human COL1A2 gene promoter (44,52), whereas the data presented here demonstrate that c-Myb stimulates transcription by binding to a Myb-Responsive Region (M-RR) containing four Myb binding sites (MBS) located between
-1400 and -1000 bp upstream of the initiation site. Multiple MBS are often present in promoters regulated by c-Myb (33), so that the clustering of the COL1A2 promoter MBS implies that the M-RR could play a key role in the regulation of COL1A2 gene expression by c-Myb. Consistent with this hypothesis, we have found that deletion of the M-RR abrogates the ability of c-Myb to regulate the COL1A2 promoter in transactivation assays.

The sequences of the four potential MBS suggest that each could be involved in the specific binding of c-Myb to the COL1A2 promoter. Indeed, using gel shift experiments we have shown that c-Myb binds to each MBS, however, mutagenesis experiments demonstrated that MBS-4 plays a pivotal role in up-regulation of the COL1A2 promoter by c-Myb. In fact, the presence of the MBS-4 in the COL1A2 promoter appears to be sufficient to allow strong transactivation by c-Myb. This finding could be explained by the fact that the different means by which c-Myb activates its target gene promoters via MBS do not necessarily require cooperative interactions between multiple c-Myb molecules (60). However, it is noteworthy that the COL1A2 promoter with a mutation only in MBS-4 is still transactivated to some extent by c-Myb suggesting that MBS-1, MBS-2 and MBS-3 are perhaps important in cooperation with MBS-4.

We have shown that the conclusion that B-Myb cannot transactivate the COL1A2 promoter in mouse fibroblasts (24) holds true in human cells. Indeed, while c-Myb activates both the COL1A2 and synthetic KHK promoters, B-Myb, which should be capable of binding the same MBS, only activates the KHK construct and can partially
inhibit the transactivation of the COL1A2 promoter that is driven by c-Myb. Two possible explanations for this distinction are; i) that B-Myb works as a repressor of type I collagen gene expression, as demonstrated by our experiments and by others (12), and ii) that c-Myb may regulate human type I collagen expression in cooperation with an as yet unknown protein which does not operate in conjunction with B-Myb.

Our most direct evidence for a close link between c-Myb and COL1A2 gene expression has been provided by showing the lack of type I collagen expression in MEFs derived from c-Myb−/− embryos. That the absence of c-Myb has a specific effect on the COL1A2 gene was apparent since the expression of type III collagen and fibronectin was normal in c-Myb−/− MEFs.

Since the constitutive, but not the TGF-β induced expression of the COL1A2 gene is abrogated in c-Myb−/− MEFs, our data imply that TGF-β responsive cellular pathways are not affected in these cells. Furthermore, as already demonstrated in human dermal fibroblasts (24), wild type MEFs stimulated by TGF-β showed an increase in c-Myb gene expression, implying that c-Myb may regulate both constitutive and cytokine-induced expression of the type I collagen gene. However, although TGF-β induces c-Myb expression, c-Myb is not obligatory for COL1A2 induction by this cytokine since TGF-β induces COL1A2 in the absence of c-Myb in c-Myb−/− MEFs.

Thus, in summary, c-Myb is not the only transcription factor in the pathway that leads to collagen following TGF-β stimulation and c-Myb, as shown in unstimulated

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cells (Fig. 8C), may be under the control of factors distinct from TGF-β. These findings raise intriguing questions about the role of c-myb in the pathogenesis of SSc where it is over-expressed in quiescent SSc fibroblasts and TGF-β is considered a key pathogenetic factor, and suggest that the scenario is more complex than so far known, with distinct intracellular (collagen transcription factors) and extracellular factors (cytokines, growth factors, etc.) involved.

It is noteworthy that we have confirmed the results obtained in mouse embryonic fibroblasts lacking the c-myb gene by expressing a dominant negative c-Myb protein in normal human skin fibroblasts (NSF). As already demonstrated in 3T3 fibroblasts, such a dominant negative c-Myb protein suppresses transcription normally stimulated by endogenous c-Myb and blocks cell cycle progression in these cells (15).

Induction of the dominant negative c-Myb in NSF led to the abrogation of COL1A2 gene expression, confirming that c-Myb plays a crucial role in the regulation of this gene transcription in both mouse (24) and, as shown here, human fibroblasts. In this regard, it is noteworthy that, whereas the regions recognized by c-Myb in the mouse and human COL1A2 gene promoter have a different location, they display a high degree of homology. Furthermore, c-Myb modulates the expression of type I collagen genes in a species-specific manner, as shown in the case of other transcription factors (61). In fact, whereas multiple MBSs are present in the rat promoter of COL1A1 (unpublished data) and in the promoter of mouse and human COL1A2 genes, no MBSs have been found in the human COL1A1 promoter. It is unlikely that this finding is to be ascribed to the limited extension of the published sequences of the human
COL1A1 promoter (62,63), since we have analyzed a region spanning from the transcription start site to ~2000 bp which has the size of the promoter region of COL1A2 and rat COL1A1 genes where MBSs are present.

Thus, in summary, it can be speculated that in humans c-myb modulates the expression of type I collagen gene expression acting preferentially on the COL1A2 gene and that the precise mechanisms employed by c-Myb have evolved in a species-specific way (64).

In conclusion, in linking c-Myb to the expression of type I collagen in human fibroblasts, the present work emphasizes the physiological role of c-Myb in human fibroblasts and its potential importance in fibrotic conditions such as a scleroderma (SSc), in which the augmented production of collagen could be maintained by the deregulated expression of this gene (8). Further studies will be necessary to more clearly elucidate the role by c-Myb in fibrotic disorders.
ACKNOWLEDGEMENTS

This work has been supported by Telethon grant n. 0822. JF is a Wellcome Trust Senior Basic Biomedical Research Fellow.
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FIGURE LEGENDS

Figure 1. Schematic representation of the human type I collagen α2 chain gene (COL1A2) promoter and of plasmids used in transactivation assays

(A) The COL1A2 promoter and the position of c-Myb-binding sites (MBSs) - NcoI: position of the restriction enzyme site NcoI, used to obtain the pGL-D1095 plasmid (see Materials and Methods for more details). The table shows from left to right; the nucleotide sequences of the four MBSs (the core sequence regions are in bold and underlined); the nucleotide position within the COL1A2 promoter; and the values of matrix similarities to the MBS consensus sequences, where a value of 1 corresponds to complete homology. (B) The recombinant plasmid pGbC1A2-P obtained by cloning the COL1A2 promoter between the SacI-SmaI sites of the pGL3-Basic mcsr, upstream the firefly luciferase gene (LUC+). (C) The recombinant plasmid pGECA2-P obtained by cloning the COL1A2 promoter between the SacI-SmaI sites of the pGL3-Enhancer mcsr, upstream the firefly luciferase gene (LUC+) and an SV-40 enhancer cassette (SV40-Enh).

Figure 2. Transient transactivation of the COL1A2 promoter by c-Myb or B-Myb in human fibroblasts

(A) Co-transfection of human fetal lung (HFL), human dermal embryonic cells (HUDE
and WS1) and normal human skin fibroblast cells (NSF) with the COL1A2 promoter-reporter (COL1A2-P) and c-Myb or B-Myb expression vectors. The results represent the mean of three separate experiments. All transfections also included the pRL-SV vector that encodes renilla luciferase. The ratios of renilla to firefly luciferase activities obtained with the "Dual Reporter Luciferase Assay" were used to normalize all the transfection experiments. (B) Western-blot analysis of NSF cells transfected with COL1A2 promoter-reporter (COL1A2-P) ± the c-Myb expression vector pSGC. Affinity-purified rabbit anti-c-Myb polyclonal antibody was used as the primary antibody and western blot detection was carried out following the manufacturer's instructions. K562: nuclear extract prepared from K562 human chronic myelogenous leukaemia cell line, used as positive control for c-Myb. (C) Co-transfection of normal human skin fibroblast cells (NSF) with pGL-KHK plasmid (carrying the KHK synthetic promoter), and c-Myb or B-Myb expression vectors. The results represent the mean of three distinct experiments. Normalisation for transfection efficiency was performed as described in (A). (D) Co-transfection of normal human skin fibroblast cells (NSF) with COL1A2 promoter-reporter (COL1A2-P) together with c-Myb (pSGC) and B-Myb (pSGB) expression vectors, at different molar ratios (eg 1/1 = 1µg/1µg). The results represent the mean of three distinct experiments. Normalisation for transfection efficiency was performed as described in (A).

Figure 3. Transient transactivation of full length and deleted COL1A2 promoter-reporter constructs by c-Myb in NSF cells
The six nested deletions (D1500 to D290) of the full length COL1A2 promoter (FL) are schematically represented on the left. The position of the c-Myb binding sites (mbs) are represented by the black boxes. Results of transfections +/- the c-Myb expression vector pSGC are indicated. The fold transactivation values are indicated on the right of the figure. Results represent the mean of three separate experiments. Normalisation for transfection efficiency was performed as described in the legend to Figure 2A.

Figure 4. Transient transactivation of the wild type and mutated MBS COL1A2 promoter-reporters by c-Myb and c-Myb(Cys130) in NSF cells

(A) Co-transfection of normal human skin fibroblast cells (NSF) with or without the pSGC c-Myb expression vector together with either the pGbC1A2-P and pGLD1095 promoter-reporters or with equivalent constructs containing either the full length promoter with only the MBS-4 mutated (pGbC1A2-P-MBS4-mut) or the shorter promoter containing one of three different mutations in the MBS-4 sequence (MBM-1, -2 and -3). The MBS core sequence present in each construct is indicated and the mutated residues are shown in lower case type. (B) Co-transfection of normal human skin fibroblast cells (NSF) with the full length COL1A2 promoter-reporter construct (COL1A2-P) and the expression vector pSGC encoding either wild type c-Myb or the version mutated at cysteine 130 (Cys130). Results represent the mean of three distinct experiments. Normalisation for transfection efficiency was performed as described in the legend to Figure 2A.
Figure 5. Results of gel retardation experiments (EMSA)

(A) EMSA of K562 nuclear extract (ne) and Myb-HIS recombinant protein incubated with labelled KHK synthetic promoter, containing eight Myb binding sites, and resolved on a non-denaturing polyacrylamide gel. Comp: 100-fold molar excess of KHK synthetic promoter unlabelled competitor. Anti-myb: supershift using anti-c-Myb polyclonal antibody raised against a peptide derived from amino acid residues 2-16 of human c-Myb. (B) EMSA performed as in (A) except that the labelled probe oligonucleotide contained MBS-4 from the COL1A2 promoter. (C) Emst performed using the Myb-HIS recombinant with labelled double-stranded oligonucleotide containing the sequence of wild type MBS-4 (MBS-4 wt; lanes 1-4) or one of the three mutated MBS-4 sequences elsewhere described (lanes 5-7, MBM-1, -2, -3). Comp (lane 2): 100-fold molar excess of KHK synthetic promoter unlabelled competitor. Anti-myb (lane 3): supershift using anti-c-Myb polyclonal antibody. Anti-HIS (lane 4): supershift using the mouse monoclonal Penta-HIS antibody. A band is clearly visible in a higher position than the control MYB-HIS/DNA complex (lane 1).

Figure 6. Northern blot and RT-PCR analysis of the expression of extracellular matrix proteins and c-myb in wild type and c-myb--/- mouse embryonic fibroblasts (MEFs)

(A) Northern blot analysis of RNA prepared from wild type (wt) and c-myb--/- mouse embryonic fibroblasts (MEFs). Eight micrograms of total RNA were loaded in
each lane of a 1\% agarose-formaldehyde gel. The membrane was hybridized successively with labeled cDNA probes for the $\alpha(2)$ type collagen gene (COL1A2), fibronectin, the type III collagen gene (COL3) and $\beta$-actin. Autoradiographic exposure was for 24 hrs, except for COL1A2 gene (72 hrs). (B) Northern blot analysis of RNA prepared from wild type (wt) and $c-myb^{-/-}$ mouse embryonic fibroblasts (MEFs) ± transfected with the pGREMyb plasmid, expressing inducible full length c-Myb ($c-myb^{+/+}$). Cells were cultured in the presence of 0.2 $\mu$M dexamethasone. (C) The upper part shows northern blot analysis of RNA prepared from wild type (wt) and $c-myb^{-/-}$ MEFs ± TGF-β (2 ng/ml). The membrane was hybridized with radiolabeled cDNA probes for the $\alpha(2)$ type collagen gene (COL1A2) and $\beta$-actin. The lower part is an RT-PCR analysis of $c-myb$ RNA and a corresponding control reaction using primers specific for $\beta$-actin.

Figure 7. Northern blot analysis of type I collagen mRNA from normal human skin fibroblasts (NSFs) overexpressing c-Myb or a dominant negative c-Myb variant.

Northern blot analysis of RNA prepared from normal human skin fibroblasts (NSFs) transfected with the pGREMyb plasmid, expressing inducible full length c-Myb ($c-myb^{+/+}$) or with pGREMen, expressing a dominant negative variant of c-Myb ($c-myb^{-/-}$) ± 0.2 $\mu$M dexamethasone (+Dexa). Eight micrograms of total RNA were loaded in each lane of a 1\% agarose-formaldehyde gel. The membrane was
hybridized with labeled cDNA probes for the α(2) type collagen gene (COL1A2) and β-actin. Autoradiographic exposure was for 24 hrs.
Characterization of the c-MYB-responsive region of the human type I collagen \(\text{alfa}_2\) chain gene

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J. Biol. Chem. published online November 6, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204392200

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