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Human Collagen Krox Up-regulates Type I Collagen Expression in Normal and Scleroderma Fibroblasts through Interaction with Sp1 and Sp3 Transcription Factors*⒮

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Despite several investigations, the transcriptional mechanisms that regulate the expression of both type I collagen genes (*COL1A1* and *COL1A2*) in either physiological or pathological situations, such as scleroderma, are not completely known. We have investigated the role of hc-Krox transcription factor on type I collagen expression by human dermal fibroblasts. hc-Krox exerted a stimulating effect on type I collagen protein synthesis and enhanced the corresponding mRNA steady-state levels of *COL1A1* and *COL1A2* in foreskin fibroblasts (FF), adult normal fibroblasts (ANF), and scleroderma fibroblasts (SF). Forced hc-Krox expression was found to up-regulate *COL1A1* transcription through a −112/−61-bp sequence in FF, ANF, and SF. Knockdown of hc-Krox by short interfering RNA and decoy strategies confirmed the transactivating effect of hc-Krox and decreased substantially *COL1A1* transcription levels in all fibroblast types. The −112/−61-bp sequence bound specifically hc-Krox but also Sp1 and CBF. Attempts to elucidate the potential interactions between hc-Krox, Sp1, and Sp3 revealed that all of them co-immunoprecipitate from FF cellular extracts when a c-Krox antibody was used and bind to the *COL1A1* promoter in chromatin immunoprecipitation assays. Moreover, hc-Krox DNA binding activity to its *COL1A1*-responsive element is increased in SF, cells producing higher amounts of type I collagen compared with ANF and FF. These data suggest that the regulation of *COL1A1* gene transcription in human dermal fibroblasts involves a complex machinery that implicates at least three transcription proteins, hc-Krox, Sp1, and Sp3, which could act in concert to up-regulate *COL1A1* transcriptional activity and provide evidence for a pro-fibrotic role of hc-Krox.

Fibroblasts represent the main cellular population of dermis. Their principal function is to maintain the extracellular matrix (ECM)[5] homeostasis. A complex network of cytokines/growth factors, acting by autocrine and paracrine mechanisms, controls the fibroblast metabolism and ensures the tissue integrity. In a normal situation, there is a balance between synthesis and degradation of the matrix components, including elastic fibers, proteoglycans, and collagens (1, 2). This equilibrium is disrupted in pathological processes, such as fibrosis, and characterized by excessive production of matrix proteins, particularly of type I collagen, which is the major structural component of dermal ECM (2).

Type I collagen is composed of two α1 chains and one α2 chain, encoded by two distinct genes, *COL1A1* and *COL1A2*, respectively (3). Coordinated transcription of these genes involves similar *cis* elements interacting with the same transcription factors, such as Sp1, Sp3, CBF (CCAAT-binding factor), and c-Krox (collagen-Krüppel box) (4–15). The relative proportions and/or interactions between these and other transcription factors are responsible for the modulation of type I collagen expression under physiopathological conditions.

Human c-Krox (hc-Krox) is a zinc finger transcription factor belonging to the Krox family proteins and binds to GC-rich sequences. This protein harbors a BTB-POZ (for "broad complex, tramtrack, and bric à brac/pox virus and zinc finger") domain in its N-terminal extremity, which is responsible for protein homo- and heterodimerization and for the inhibition of

[5] The abbreviations used are: ECM, extracellular matrix; siRNA, short interfering RNA; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; FF, foreskin fibroblast; ANF, adult normal fibroblast; SF, scleroderma fibroblast; CBF, CCAAT-binding factor; c-Krox, collagen-Krüppel box; hc, human collagen; mc, mouse collagen.

transcriptional activity of ECM-targeted genes (16). hc-Krox has already been found to inhibit type I collagen (*COL1A1* and *COL1A2*), fibronectin, and elastin genes in fibroblastic cells and uridine diphosphoglucose dehydrogenase (*UGDH*) in rabbit articular chondrocytes (17, 18).

On the other hand, the murine homologue of hc-Krox, mouse c-Krox (mc-Krox), was first cloned as a partial cDNA lacking the BTB-POZ domain and was demonstrated to be an activator of transcription of mouse *Col1a1* and *Col1a2* genes in fibroblastic cells (14). This transcription factor is expressed in a quite specific manner in the dermis, although it is absent in bone (14, 15). Its expression can be detected at 9.5 days post-coitum in the myodermatomes and it precedes type I collagen expression. These data, reporting the co-localization of type I collagen and c-Krox expression in skin fibroblasts, suggest the possible involvement of the *trans* factor in the fibrotic process, particularly in scleroderma. Further support to this hypothesis comes from the fact that the full-length mc-Krox (containing the BTB-POZ domain) was recently found to promote thymocyte differentiation into CD4+ T cells rather than into CD8+ T cells (19, 20). Knowing that during the early stages of scleroderma disease there is an activation of CD4+ T cells, rather than of CD8+ T cells (21), we may suppose that c-Krox plays an important role in the early manifestation of this disease that leads to fibrotic reaction in later stages.

Sp1 and Sp3 are ubiquitous proteins belonging to the family of Sp transcription factors recognizing GC and TC boxes, like c-Krox (22). Sp1 is a well characterized activator of type I collagen genes (6, 12, 23–25); however, its function seems to depend on the cellular and/or molecular context. It has been shown to inhibit mouse *Col1a1* gene in NIH-3T3 fibroblasts (26) and to function as a co-inhibitor of human *COL1A2* gene transcription when it interacts with the transcription factor Fli-1 in human skin fibroblasts (27). Additionally, Sp1 may play a role in fibrotic diseases, because it is one of the mediators of transforming growth factor-β1, a profibrogenic factor that activates human *COL1A1* gene transcription (10, 22, 28). In this regard, its binding activity is increased in human scleroderma fibroblasts and in hepatic stellate cells, compared with normal cells (23, 29).

Sp3 shares identical binding sites and affinities with Sp1. Consequently, it often behaves like an Sp1 antagonist in many systems, competing with the latter for the same DNA sites, reducing Sp1 binding activity, and inducing inhibition of gene transcription (30–33). Moreover, Sp3 on its own exerts bifunctional effects on gene transcription. These effects can be cell- and/or promoter-specific, but it can also depend on the number of binding sites available for Sp3 on a given gene (34–35). Recent findings show that there exist four Sp3 isoforms, resulting from alternative translational start sites. Thus, Sp3 does not appear as a simple Sp1 homologue but is rather controlled by complex mechanisms of expression (36).

In this study, we investigated the functions of hc-Krox in human dermal fibroblasts. We found that hc-Krox increases type I collagen synthesis in human foreskin fibroblasts (FF) obtained from young children, adult normal fibroblasts (ANF), and scleroderma fibroblasts (SF). Moreover, hc-Krox enhances human *COL1A1* transcriptional activity in FF, ANF, and SF

through a −112/−61-bp promoter sequence. The factor can bind to this promoter region *in vitro* and *in vivo*, as well as Sp1 and CBF.

Our results reveal for the first time the transactivating role of hc-Krox on human *COL1A1* gene in normal and pathological human dermal fibroblasts and provide evidence for interactions between hc-Krox, Sp1, and Sp3. Moreover, the data suggest that hc-Krox is a pro-fibrogenic factor, because its DNA binding is increased in SF compared with ANF, and this is correlated with enhanced type I collagen expression, and this offers new perspectives for therapeutic strategies that could be applied to fibrotic diseases and aging-related skin disorders.

EXPERIMENTAL PROCEDURES

Cell Cultures—Children foreskin samples were provided by Dr. P. Ravasse (Dept. of Children Surgery, Caen Hospital, Caen, France). Human ANF and SF were obtained from forearm biopsies of either healthy donors or patients suffering from localized scleroderma (Prof. F.-X. Maquart, Laboratory of Medical Biochemistry and Molecular Biology, Reims, France). Fibroblasts were obtained after explant cultures and seeded at 2.5×10^6 cells/cm^2 in 175-cm^2 flasks, in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (100 IU/ml), streptomycin (100 μg/ml), and fungizone (0.25 μg/ml) in a 5% CO$_2$ environment. They were passaged with a trypsin (0.05%), EDTA (0.25 mM) solution (Invitrogen) after reaching confluency. All the experiments were performed on cells between 5 and 9 passages.

Collagen Labeling and Assay—Fibroblasts were seeded at 0.17×10^5 cells/9.6-cm^2 wells, in 10% fetal calf serum-containing Dulbecco's modified Eagle's medium, and transiently transfected, or not, by the calcium phosphate precipitation method with the hc-Krox expression vector and/or the corresponding insertless plasmid (pSG5/hc-Krox). After 15 h, the culture medium was replaced by the same fresh medium supplemented with β-aminopropionitrile (50 μg/ml) and ascorbic acid (50 μg/ml) for a further 24-h period. The same medium was then renewed, and [^{3}H]proline (2 μCi/ml) (PerkinElmer Life Sciences) was added. After 24 h, the culture medium was collected, and the labeled collagen was assayed, using the bacterial collagenase method (37). The cell layer was scraped, and the extracts were sonicated. They were used to assay cell protein levels, using the Bradford colorimetric assay. Levels of collagen and noncollagenous proteins were corrected for total protein amount.

An alternative method of transfection was also used to estimate collagen neosynthesis in the siRNA knockdown experiments. Fibroblasts were trypsinized at confluency and then distributed in Eppendorf tubes (1×10^6 cells per 1.5-ml tube). Cells were centrifuged for 10 min at $200 \times g$ and transfected using the Nucleofector apparatus (AMAXA, Köln, Germany), according to the manufacturer's instructions. After the transfection of hc-Krox siRNA (1 μg), the cells were plated and incubated in the same media, and the bacterial collagenase assay was performed as described above.

Site-directed Mutagenesis—Mutant collagen promoters were made using the −804hCOL-LUC as a template for PCR together with mutated oligonucleotides as follows. A 30-mer

TABLE 1

Primers used in real time RT-PCR experiments (Eurogentec)

Gene of interest	Forward primer (orientation 5′ → 3′)	Reverse primer (orientation 5′ → 3′)
COL1A1	CACCAATCACCTGCGGTACAGAA	CAGATCACGTCATCGCACAAC
COL1A2	AAAACATCCCAGCCAAGAACTG	AAACTGGCTGCCAGCATTG
hc-Krox	AGGGTTCTGGAAGATGAAATGAGT	AAGAGGACCAACTTTAGCCAATACTT
COL3A1	TCTTGGTCAGTCCTATGCGGATA	CATCGCAGAGAACGGATCCT
ARNr 18 S	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

spanning the collagen promoter sequence from −84 to −55 was synthesized containing either G to T mutations at positions −61 and −62 or C to A mutations at positions −73 and −74. PCR was performed with a single oligonucleotide primer and the −804hCOL-LUC template for 18 cycles using *Pfu* Turbo polymerase (Stratagene) as directed by the manufacturer. The PCR product was digested with DpnI restriction endonuclease to eliminate the input template, followed by transformation of *Escherichia coli*. Plasmid DNA extracted from bacterial colonies was sequenced using the pGL2 primer (Promega) to confirm the presence of the desired mutations. This gave rise to the −804mut−73/−74 and −804mut−61/−62 constructs. The −112 mutant constructs were created by using the −804 mutant constructs as PCR templates with an upstream 18-mer beginning at −112, and the pGL2 as downstream primer. All plasmids were sequenced prior to transfection to confirm the presence of the correct mutations and the promoter length.

Transient Transfections with COL1A1 Constructs—Fibroblasts were passaged after reaching confluency and then distributed in Eppendorf tubes (1×10^6 cells per 1.5-ml tube). Cells were centrifuged for 10 min at $200 \times g$ and transfected using the Nucleofector apparatus and an NHDF kit (AMAXA, Köln, Germany), according to the manufacturer's instructions. For each sample, 2 μg of pSV40/β-gal expression vector, 2 μg of reporter plasmids, and 1 μg of hc-Krox expression vector were used. After transfection, cells were seeded in 9.6-cm² wells and incubated 12 h at 37 °C. The medium was then replaced, and cells were harvested 6 h later to determine luciferase and β-galactosidase activities, as well as the protein amount (38).

The other human *COL1A1* plasmid constructs were described previously (39). pK1A4 Luc and pK1A4mut Luc plasmids were described previously (14).

siRNA Experiments—The following siRNA sequences have been designed by Qiagen (Courtaboeuf, France): hc-Krox siRNA forward, r(UCGCUGCUUGCAUGGAGAU)dTdT, and reverse, r(AUCUCCAUGCAAGCAGCGA)dTdG; control siRNA forward, r(UUCUCCGAACGUGUCACGU)dTdT, and reverse, r(UUCUCCGAACGUGUCACGU)dTdT.

FF, ANF, and SF were transfected as described earlier, with the following modifications. For each sample of 1×10^6 cells, 1 or 1.5 μg of hc-Krox siRNA or control siRNA were added to the transfection mixture. 12 h after transfection, the medium was replaced with fresh medium, and 6 h later, cells were harvested to measure the transcriptional activity or to extract total RNAs.

RNA Extraction and Real Time RT-PCR Analysis—Fibroblasts were seeded in 9.6- or 55-cm² wells and transiently transfected by the AMAXA Nucleofector apparatus or the calcium phosphate precipitation method, respectively, in the presence of the hc-Krox expression vector (pSG5/hc-Krox) and/or the corresponding insertless plasmid (pSG5). 12 h

TABLE 2

Oligonucleotides used in this study (Eurogentec)

Oligonucleotide name	Sequence (potential binding sites for CBF, Sp1/Sp3, and c-Krox transcription factors are in boldface)
SOX9 ("decoy" control)	5′–AGCCCCATTCATGAGAGACGAGGT–3′
−112/−61 wild type	5′–AGGCAGCTCTG**ATTGG**CTGGGGCAC**GGGCGG** CCGG**CTCCCCC**TCTCCGAGGG–3′
Aα_1(I) wild type	5′–CTTGC**GGGAGGGG**GGGCGCTGGGTGGACA–3′

after Nucleofector transfection, and 17 h after transfection by the calcium phosphate precipitation procedure, the medium was replaced by fresh medium. 6 h (nucleofection procedure) or 24 h (calcium phosphate precipitation) later, cells were harvested. Total RNA were extracted (40), and 2 μg were reverse-transcribed and analyzed (18). Real time RT-PCRs were performed (18), using sequence-specific primers (Table 1) (Eurogentec, Angers, France) defined with the "Primer Express" software (Applied Biosystems, Courtabœuf, France). Analysis of relative gene expression was done by using the $2^{-\Delta Ct}$ method (41).

Nuclear Extracts and Gel Retardation Assays—Nuclear extracts were prepared using a mini-preparation procedure (42), and gel retardation assays were performed with the oligonucleotides shown in Table 2 or in Fig. 5A. The probes were end-labeled with [γ-^{32}P]dATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase (Promega, Charbonnières, France). FF, ANF, and SF nuclear extracts (5 or 7.5 μg) were incubated for 10 min at room temperature with the probe (5 fmol) in 20 μl of a specific binding buffer (43) and in the presence of 1 μg of poly(dI-dC)·poly(dI-dC) (Amersham Biosciences, Orsay, France), used as a DNA nonspecific competitor. For antibody interference reactions, nuclear extracts were incubated with specific antibodies directed against c-Krox (antibody developed in collaboration between our laboratory and Novotec (Lyon, France)), Sp1, Sp3, and CBFb (Tebu-Bio SA, Le Perray en Yvelines, France) for 10 min at room temperature and then 10 min at 4 °C. Finally, the probe was added, and a 10-min incubation at room temperature was performed. Samples were then fractionated by electrophoresis for 1.5 h at 150 V on a 5% polyacrylamide gel (acrylamide/bisacrylamide at 30:1) in 0.5× TBE (45 mM Tris borate, 1 mM Na$_2$EDTA) and visualized by autoradiography.

Western Blot Analysis—Fibroblasts were seeded in 55-cm² wells and at 90% confluency, transiently transfected by the calcium phosphate precipitation method in the presence of an expression vector containing or not the hc-Krox cDNA. 17 h after transfection, the medium was replaced by fresh medium and 24 h later, cells were harvested, and cellular extracts were prepared as described previously (18). The cellular proteins were fractionated on an 8% polyacrylamide gel

A

B

C
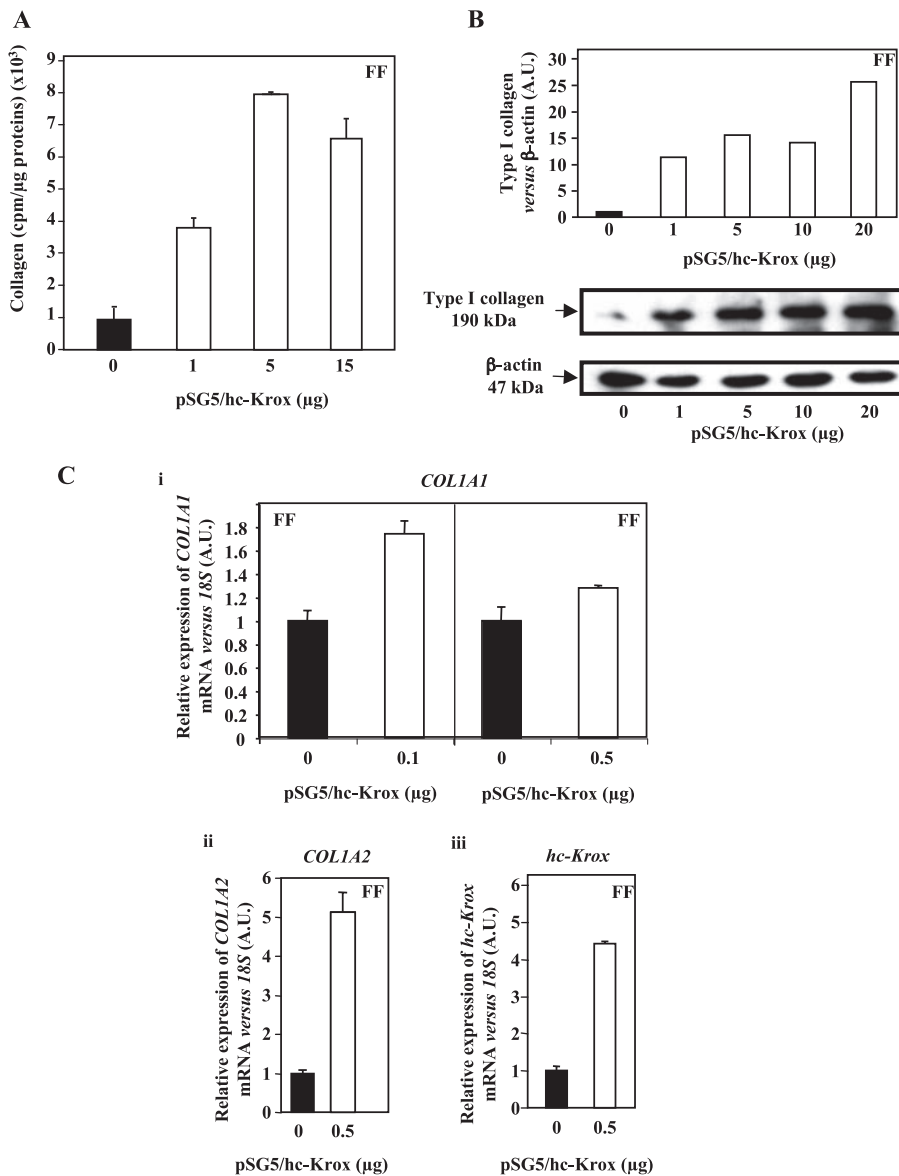
FIGURE 1. **hc-Krox increases type I collagen synthesis and *COL1A1* and *COL1A2* mRNA steady-state levels in FF.** *A,* FF were transiently transfected by the calcium phosphate co-precipitation method with 1, 5, and 15 μg of pSG5/hc-Krox expression vector. For each amount of hc-Krox transfected, the insertless pSG5 vector was used as a complement to 15 μg, to transfect each sample with the same amount of DNA. 15 h later, the cell culture medium was changed, and a bacterial collagenase assay was realized as described under "Experimental Procedures." The values, normalized to total protein amount, are expressed as counts/min/μg of protein and represent the means ± S.D. of triplicate dishes. *B,* FF were transfected as in *A*, with a total amount of DNA equal to 20 μg; total proteins were extracted and used in Western blotting experiments as described under "Experimental Procedures" to detect type I procollagen and β-actin using specific antibodies. The histograms represent the relative expression of type I collagen *versus* β-actin, estimated after densitometric analysis of the electrophoregram. *C,* FF were transiently transfected using the AMAXA Nucleofector with 0.1 or 0.5 μg of pSG5 containing or not the hc-Krox cDNA. 18 h after transfection, total RNAs were extracted, and 1 μg was reverse-transcribed into cDNA. 2 μl of cDNAs were diluted 100 times and used in real time PCR to amplify *COL1A1* (*panel i*), *COL1A2* (*panel ii*), *hc-Krox* (*panel iii*), and 18 S cDNAs, with specific forward and reverse primers. *COL1A1*, *COL1A2*, and *hc-Krox* mRNAs relative expression was normalized to 18 S rRNA according to $2^{-\Delta Ct}$ method (*A.U.,* arbitrary units).

(acrylamide/bisacrylamide at 30:1) and electrotransferred on a polyvinylidene difluoride membrane (PerkinElmer Life Sciences). After blocking with TBST (TBS, 0.1% Tween 20) containing 10% nonfat dry milk, membranes were incubated with primary antibodies (rabbit anti-human type I collagen, dilution 1:1500 (Novotec, Lyon, France), or mouse anti-human β-actin, dilution 1:500 (Tebu-Bio)) in TBST, 2% nonfat

dry milk. Membranes were then rinsed with TBST and incubated with secondary antibodies (horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody) (Tebu-Bio). Type I collagen and β-actin protein bands were revealed using a Western blot detection kit (WestPico kit, Pierce) and quantified with Adobe Photoshop 7.0.

Immunoprecipitation Assays—10 μl of antibody raised against c-Krox were incubated with 40 μl of anti-rabbit IgG conjugated to agarose beads (Sigma) in cold 1× phosphate-buffered saline for 2 h at 4 °C. The samples were then centrifuged for 15 s at 1200 rpm at 4 °C. The supernatants were discarded, and the pellets were rinsed twice with RIPA buffer (1% IGEPAL CA-630, 1% (w/v) sodium deoxycholate monohydrate, 0.1% SDS, 0.15 M NaCl, 40 μg/ml aprotinin). Cellular extracts (corresponding to 2–3 × 10^6 FF) were added to the substrates, incubated for 2 h at 4 °C, and centrifuged for 15 s at 1200 rpm. Supernatants were removed, and pellets were rinsed four times with an IP buffer (0.05 M Tris-HCl, pH 7.4, 0.1% Triton X-100, 0.15 M NaCl) and once with cold phosphate-buffered saline. Then 20 μl of 2× sample buffer were added to each immunoprecipitation reaction, and samples were denatured at 95 °C for 5 min and submitted to Western blot assays as described above. Primary antibodies against Sp1 (rabbit anti-human Sp1, 1:300 dilution in TBST, 2% nonfat milk) and Sp3 (rabbit anti-human Sp3, 1:300 dilution in TBST, 2% nonfat milk) were purchased from Tebu-Bio. c-Krox antibody (rabbit anti-mouse c-Krox) was used at a dilution of 1:250 in TBST, 5% nonfat milk.

Glutaraldehyde Cross-linking Experiments—10 μg of nuclear extracts were incubated in the presence of 2 μl of 0.1% glutaraldehyde or not, in a protein binding buffer (43) for 10 min at room temperature. Then 5 μl of 5× sample buffer were added, samples were denatured for 5 min at 95 °C and subjected to Western blot assays as described previously.

Chromatin Immunoprecipitation Assay—Chromatin immunoprecipitation assays were performed by using a commercially
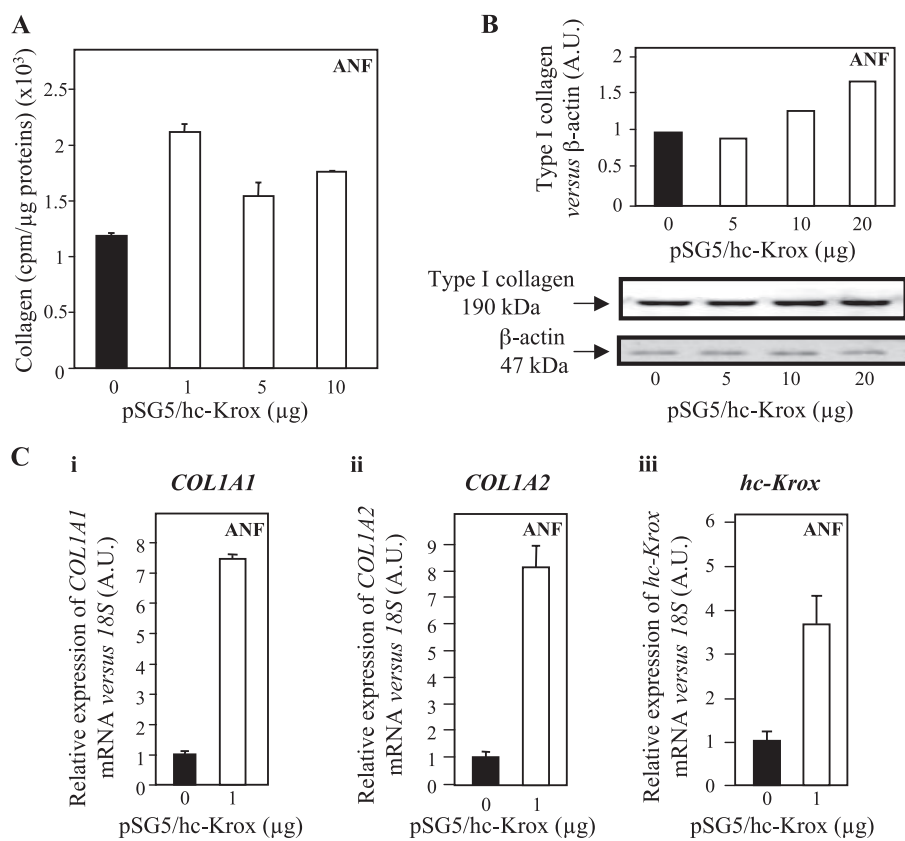
FIGURE 2. **hc-Krox increases type I collagen protein synthesis, and *COL1A1* and *COL1A2* mRNAs steady-state levels in ANF.** *A,* ANF were transfected as in Fig. 1*A* with a total amount of DNA equal to 10 μg. 15 h later, the culture medium was changed, and a bacterial collagenase assay was realized, as described under "Experimental Procedures." The values, normalized to total protein amount, are expressed as counts/min/μg of protein and represent the mean ± S.D. of triplicate dishes. *B,* ANF were transfected as in *A,* with a total amount of DNA of 20 μg, and total proteins were extracted and used in Western blotting experiments as described under "Experimental Procedures" to detect type I procollagen and β-actin using specific antibodies. Quantification of type I collagen expression was performed as described in Fig. 1. *C,* ANF were transiently transfected by the AMAXA Nucleofector with 1 μg of pSG5 containing or not the hc-Krox cDNA. 18 h after transfection, total RNAs were extracted, and 1 μg was reverse-transcribed into cDNA. 2 μl of cDNAs were diluted 100 times and used in real time PCR to amplify *COL1A1* (*panel i*), *COL1A2* (*panel ii*), *hc-Krox* (*panel iii*), and 18 S cDNAs. *COL1A1, COL1A2,* and *hc-Krox* mRNAs relative expression was normalized to 18 S rRNA according to $2^{-\Delta Ct}$ method (*A.U.,* arbitrary units).

ing amounts of an expression vector containing the hc-Krox cDNA, and the collagen neosynthesis was determined after tritiated proline labeling. We found that hc-Krox increased the amount of newly synthesized collagen, which includes type I isotype as the major form in the dermis (~80% of the total collagen) (Fig. 1*A*). Western blotting confirmed that this stimulating effect did affect type I collagen synthesis, indicating that hc-Krox was acting as an activator on this protein production (Fig. 1*B*).

We then asked whether hc-Krox was exerting the same effect at the transcriptional level. To this end, hc-Krox was overexpressed in FF, and real time RT-PCR analysis was performed on total RNA extracts. hc-Krox always increased moderately *COL1A1* mRNA steady-state levels (Fig. 1*C, panel i*), whereas it up-regulated more importantly *COL1A2* (Fig. 1*C, panel ii*) and *COL3A1* (data not shown) mRNA steady-state levels. Effective forced expression of hc-Krox was verified in FF by measuring the steady-state levels of its mRNA (Fig. 1*C, panel iii*). Similar experiments were performed on ANF to determine whether hc-Krox regulates type I collagen expression in this type of dermal fibroblasts in the same way, because contradictory data have been published.

available chromatin immunoprecipitation kit (Active Motif). Briefly, fibroblasts (10 150-cm² flasks) were cross-linked, scraped, and lysed according to the manufacturer's instructions. The DNA was then sheared using enzymatic digestion, and specific protein-DNA complexes were immunoprecipitated using either anti-hc-Krox, anti-Sp1, or anti-Sp3 antibodies. Following overnight immunoprecipitation, cross-linking was reversed, and the proteins were removed after treatment with proteinase K, and the DNA was purified and used as a template in PCR assay. The primers used for the amplification of the *COL1A1* promoter were as follows: forward, 5′-CAGAGCTGCGAAGAGGGGA-3′, and reverse, 5′-AGACTCTTTGTGGCTGGGGAG-3′. The amplicon corresponds to a 300-bp fragment that covers the core *COL1A1* gene promoter (−200/+100 bp).

RESULTS

hc-Krox Increases Type I Collagen Expression in FF, ANF, and SF—We first studied the effect of hc-Krox on total collagen synthesis in FF. Cells were transiently transfected with increas-

When increased amounts of hc-Krox were overexpressed in ANF, collagen neosynthesis was also up-regulated, in a dose-independent manner (Fig. 2*A*). This activation of total collagenous protein neosynthesis reflects increased type I collagen expression, as demonstrated in Western blot analysis using a specific anti-type I collagen antibody (Fig. 2*B*). However, the increase in type I collagen synthesis appeared relatively weak, as compared with the effect on total collagen neosynthesis. This could be explained by the fact that Western blot analysis revealed the total collagen produced by the cells and not only the newly synthesized fraction. Consequently, hc-Krox effect on the neosynthesized fraction could be underevaluated.

hc-Krox-induced activation of collagen synthesis was accompanied with increased *COL1A1*, *COL1A2* (Fig. 2*C, panels i* and *ii*), and *COL3A1* (data not shown) mRNA steady-state levels. Fig. 2*C, panel iii*, shows that hc-Krox is effectively overexpressed in ANF after transient transfection.

In this study, we show for the first time that hc-Krox is an activator of type I collagen expression in normal FF and ANF.

A

B
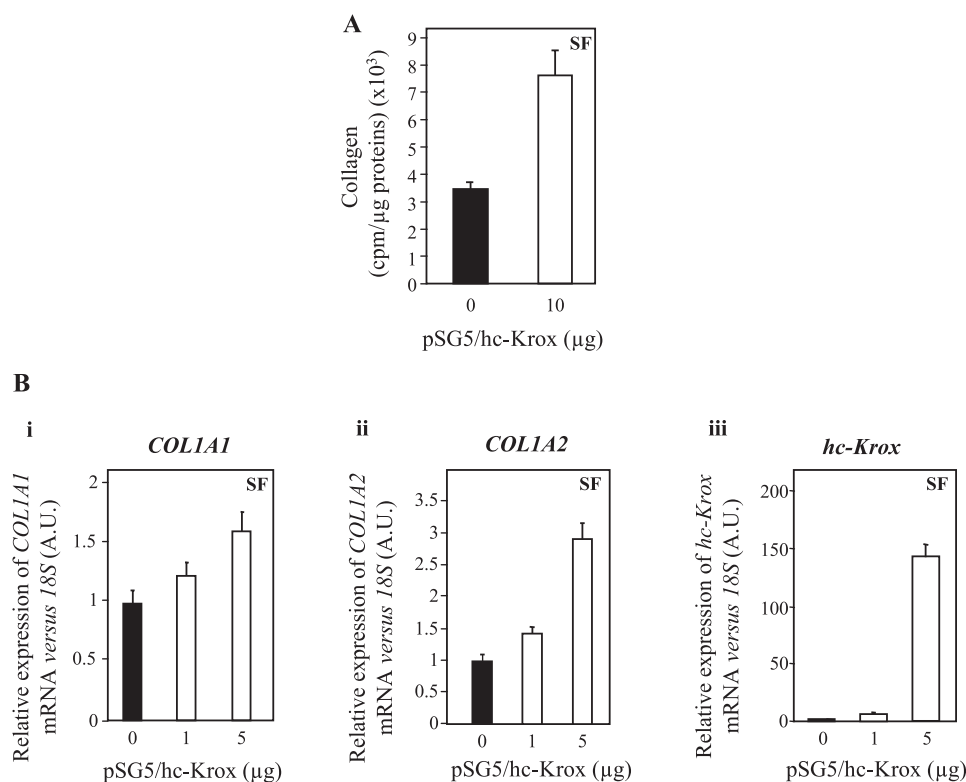
FIGURE 3. **hc-Krox increases type I collagen neosynthesis as well as *COL1A1* and *COL1A2* mRNAs steady-state levels in SF.** *A,* SF were transiently transfected by the calcium phosphate co-precipitation method with 10 μg of pSG5 containing or not the hc-Krox cDNA. 15 h later, the cell culture medium was changed, and a bacterial collagenase assay was carried out, as described under "Experimental Procedures." The values, normalized to total protein amount, are expressed as counts/min/μg of protein and represent the mean ± S.D. of triplicate dishes. *B,* SF were transiently transfected by the calcium phosphate co-precipitation procedure with 1 and 5 μg of pSG5/hc-Krox. For each amount of hc-Krox transfected, the insertless pSG5 vector was used as a complement to 5 μg, to transfect each sample with the same amount of DNA. 15 h after transfection, total RNAs were extracted, and 1 μg was reverse-transcribed into cDNA. 2 μl of cDNAs were diluted (1:100) and used in real time PCR to amplify *COL1A1* (*panel i*), *COL1A2* (*panel ii*), *hc-Krox* (*panel iii*), and 18 S cDNAs. *COL1A1, COL1A2,* and *hc-Krox* mRNAs relative expression was normalized to 18 S rRNA applying the $2^{-\Delta Ct}$ method (*A.U.,* arbitrary units).

These observations strongly suggest that hc-Krox could be also implicated in several fibrotic situations, such as scleroderma, where type I collagen expression is increased.

To elucidate this issue, hc-Krox was overexpressed in adult SF, and collagen neosynthesis was quantified. As expected, basal collagen neosynthesis of SF was greater, compared with ANF (~300%) (Fig. 3*A* compared with Fig. 2*A*). Fig. 3*A* shows that hc-Krox up-regulates collagen neosynthesis, and probably type I collagen neosynthesis.

To understand if this activating effect is due to a transcriptional regulation, SF were transiently transfected with increasing amounts of hc-Krox expression vector, and *COL1A1* and *COL1A2* mRNA were quantified. Overexpression of hc-Krox increased mRNA steady-state levels of both genes (Fig. 3*B, panels i* and *ii*), as well as those of *COL3A1* (data not shown). hc-Krox was clearly overexpressed in our experimental system (Fig. 3*B, panel iii*). These results indicate that hc-Krox is an activator of type I and III collagens under fibrotic conditions.

hc-Krox Up-regulates Human COL1A1 Gene Transcriptional Activity in FF, ANF, and SF by a Region Located between −112 and −61 bp—To investigate the potential hc-Krox effect on human *COL1A1* gene transcription, FF and ANF were tran-

siently transfected with plasmid constructs covering different regions of human *COL1A1* promoter, in the presence of an expression vector containing or not the hc-Krox cDNA. Our data showed that hc-Krox regulates human *COL1A1* gene in a similar fashion in both FF and ANF, because in both cases this transcription factor up-regulated transcriptional activity of all constructs tested, except for the shortest one containing only 61 bp of *COL1A1* promoter. Therefore, hc-Krox effect is mediated by a sequence located between −112 and −61 bp (Fig. 4*A, panels i* and *ii*).

Because hc-Krox was earlier reported as an inhibitor of human *COL1A1* gene expression (16, 17), it was important to demonstrate that the activating effect observed in our study was specifically due to hc-Krox. Therefore, FF and ANF were transfected with constructs containing four copies of the c-Krox *consensus* binding site cloned upstream of the 86 bp of the mouse *Col1a1* short promoter, containing only the TATA box as a *cis*-acting element, which was associated with the luciferase reporter gene. hc-Krox specifically increased the transcription of the pK1A4Luc construct containing four copies of the wild-type c-Krox DNA-binding site, whereas it did not exert any effect on pK1A4mut Luc transcription in which the four copies of the c-Krox-binding site were mutated (Fig. 4*B, panels i* and *ii*). Similar data were obtained for both FF and ANF and therefore confirm that hc-Krox is actually a transactivator. Moreover, pK1A4Luc basal transcriptional activity was higher compared with pK1A4mut Luc, which means that the endogenous hc-Krox itself acts as an activator (data not shown).

To determine whether the regulatory mechanisms of type I collagen expression are the same in SF compared with normal fibroblasts, the transcriptional function of hc-Krox was determined, as done in FF and ANF. As shown in Fig. 4*C*, forced hc-Krox expression increased transcription of *COL1A1* through the −112/−61 bp indicating that transcriptional regulatory mechanisms are similar in ANF and SF.

hc-Krox, Sp1, and CBF Interact Specifically with a Human COL1A1 Promoter Sequence Located between −112 and −61 bp—Subsequently to the previous data, nucleotide analysis of the −112/−61-bp sequence revealed the presence of one inverted CCAAT box described previously (−101/−97 bp (12)), two GC-rich sequences (−93/−84 bp, 5′ GC-rich box; −80/−71 bp, 3′ GC-rich box) and one putative hc-Krox bind-

A

	i		ii	
CONSTRUCT NAME	Control FF	pSG5/hc-Krox FF	Control ANF	pSG5/hc-Krox ANF
	RLU (%)		RLU (%)	
pGL2-2300 bp	100 ± 3.9	368.5 ± 32.7	100 ± 21	213 ± 7.5
pGL2-804 bp	100 ± 8.9	327 ± 3.2	100 ± 5	170 ± 24
pGL2-311 bp	100 ± 17.4	208 ± 5.3	100 ± 8.8	201 ± 26
pGL2-198 bp	ND	ND	100 ± 7.6	218 ± 29.5
pGL2-112 bp	100 ± 18.6	368 ± 0.8	100 ± 15	356 ± 28
pGL2-61 bp	100 ± 1.4	144 ± 15.1	100 ± 11.7	102 ± 24

B

	i		ii	
CONSTRUCT NAME	Control FF	pSG5/hc-Krox FF	Control ANF	pSG5/hc-Krox ANF
	RLU (%)		RLU (%)	
pK1A4Luc	100 ± 0.51	240 ± 19	100 ± 2.7	157 ± 11.5
pK1A4 mut Luc	100 ± 23.6	97.5 ± 8.6	100 ± 13	67 ± 4.8

C

CONSTRUCT NAME	Control SF	pSG5/hc-Krox SF
	RLU (%)	
pGL2-804 bp	100 ± 7.5	169.5 ± 19.3
pGL2-112 bp	100 ± 28	302.3 ± 45.6
pGL2-61 bp	100 ± 8.4	110 ± 2.5

FIGURE 4. **hc-Krox-induced activation of human *COL1A1* gene expression, in FF, ANF, and SF is mediated by a promoter region located between −112 bp and −61 bp.** *A*, FF (*panel i*) and ANF (*panel ii*) were transiently cotransfected, by the AMAXA Nucleofector method, with 2 μg of different *COL1A1* (*A*), pK1A4 Luc or pK1A4 mut Luc (*B*) reporter plasmids together with 1 μg of the pSV40/β-gal expression vector and 1 μg of pSG5 expression vector containing or not (*control*) the hc-Krox cDNA. 12 h after transfection, the medium was changed. 6 h later, the samples were harvested, and protein content, luciferase, and β-galactosidase activities were assayed. Each series of transfections was performed in triplicate. Transcriptional activity of each construct was expressed as relative luciferase activity (*RLU*), after correction for both protein amount and transfection efficiency, and expressed as % *versus* the respective control transfected with the insertless expression vector pSG5. Values represent the mean ± S.D. of three independent samples of a representative experiment. *ND,* not determined. *C*, SF were transiently cotransfected and treated as described *in A* and *B* with the indicated reporter constructs. Treatment of the samples and expression of the results are the same as in *A*.

migrating complex is also provided by antibody interference experiments (Fig. 6*B*).

Regarding the faster migrating complex, it most likely corresponds to hc-Krox, as demonstrated in Fig. 6*A*, in which a c-Krox antibody was used in EMSA experiments. Moreover, this complex has the same migration profile as hc-Krox complexed to an Aα$_1$(I)wt oligonucleotide (Table 2 and Fig. 6*C*), which is known to bind specifically c-Krox and was used to clone mc-Krox (mouse c-Krox) (14 and data not shown). As we demonstrated previously, hc-Krox migrates as a doublet, the higher migrating complex corresponding to hc-Krox monomer and the slower migrating band to hc-Krox homodimer (14, 15).

In parallel, we analyzed by EMSA the direct binding of the transcription factors to wild-type and mutated probes with nuclear extracts from FF and ANF. Fig. 5*B* shows that the binding of hc-Krox, Sp1, and CBF was not modulated when the 5′ GC-rich sequence was mutated. By contrast, the binding activity of these transcription factors was decreased when the 3′ GC box was mutated, indicating that this region presents more affinity for these *trans* factors, compared with the 5′ GC box. A significant decrease in the binding of hc-Krox, Sp1, and CBF was observed when the two GC boxes (−112/−61mut4), or only the hc-Krox-binding site (−112/−61mut61–62), or only the CCAAT box (−112/−61mut3), or when both the GC-rich sequences and CCAAT box were mutated (−112/−61mut) (Fig. 5*B*).

Overall, from this EMSA analysis, it can be concluded that hc-Krox and Sp1 bind to the 5′ and 3′ GC boxes and the −67/−61 c-Krox sequence. hc-Krox is the factor that binds with an extremely strong affinity to the −112/−61-bp promoter, compared with Sp1 and CBF. hc-Krox and Sp1 interact with the three sequences with a decreasing affinity order as follows: −67/−61 hc-Krox > 3′ GC box > 5′ GC box. Moreover, the interaction of CBF with the CCAAT box is strictly dependent on the presence of functional 5′ and 3′ GC boxes as well as the −67/−61 hc-Krox DNA-binding site. The binding of c-Krox and Sp1 is also dependent on the binding of CBF to the CCAAT box.

ing site (−67/−61 bp) (Fig. 5*A*), which could bind zinc finger transcription factors such as Sp1, Sp3, and hc-Krox.

To further investigate the interactions between hc-Krox and DNA in fibroblastic cells, EMSA experiments were performed. Fig. 5*B* (and also supplemental Fig. S1) shows that incubation of the −112/−61wt probe (Fig. 5*A* and Table 2), with nuclear proteins extracted from FF, produced three major complexes (*1st lane*). The slowest migrating correspond to complexes between DNA and Sp1 and CBF, according to the literature (43). Additional proof of the presence of Sp1 in the slowest
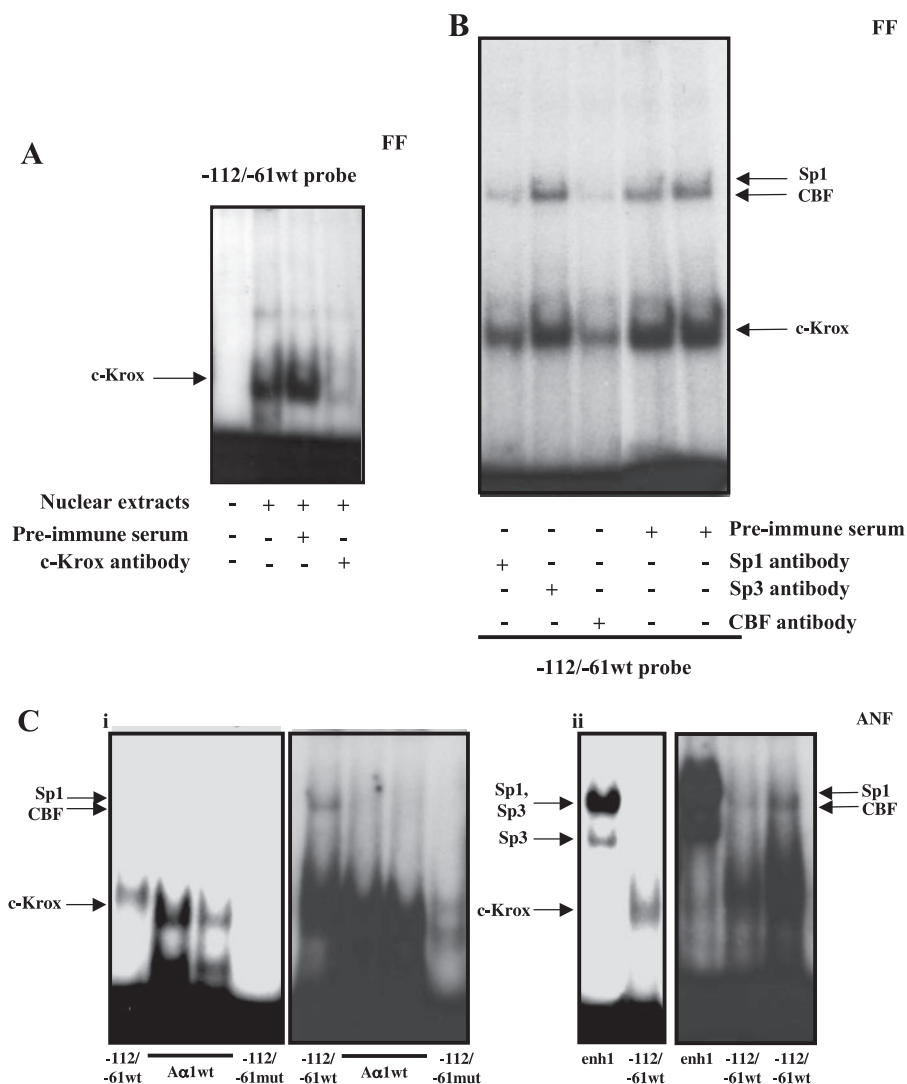
A

	CCAAT-box	GC-rich 5'	GC-rich 3'	hc-Krox

-112/-61 wt 5'-AGGCAGCTCTG**ATTGGC**TG**GGGCACGGGC**GGC**CGGCTCCCCC**TCT**CCGAGGG** -3'

-101 -97 -93   -84 -80   -71 -67   -61

-112/-61mut 5'- AGGCAGCTCTGA*AAA*GCTGG*TTTT*CGG*TTT*GCC*T*GC*AAA*CC*T*TCTCCGAGGG –3'

-112/-61mut1 5'- AGGCAGCTCTGATTGGCTGG*TTTT*CGG*TTT*GCCGGCTCCCCCTCTCCGAGGG –3'

-112/-61mut2 5'- AGGCAGCTCTGATTGGCTGGGGGCACGGGCGGCC*T*GC*AAA*CC*T*TCTCCGAGGG –3'

-112/-61mut3 5'- AGGCAGCTCTGA*AAA*GCTGGGGCACGGGCGGCCGGCTCCCCCTCTCCGAGGG –3'

-112/-61mut4 5'- AGGCAGCTCTGATTGGCTGG*TTTT*CGG*TTT*GCC*T*GC*AAA*CC*T*TCTCCGAGGG –3'

-112/-61mut61-62 5'-AGGCAGCTCTGATTGGCTGGGGGCACGGGCGGCCGGCTCCCCCTCTCCGAG*TT* -3'

B

FIGURE 5. **hc-Krox, Sp1, and CBF bind specifically to −112/−61 bp *COL1A1* sequence.** DNA binding was analyzed by EMSA. *A,* a schematic representation of the −112/−61-bp region of *COL1A1* gene, including the CCAAT box, the 5' and 3' GC boxes, and the putative hc-Krox-binding site. The different mutant probes in the −112/−61-bp sequence used in direct binding EMSA experiments are presented. The mutations are indicated in *italic* and are *underlined*. *B,* 5 μg of FF or ANF nuclear extracts were incubated in direct binding experiments with the indicated radiolabeled −112/−61 wild-type and mutant probes. *Arrows* indicate the complexes formed between DNA and nuclear proteins.

below Sp1 disappears, implying that CBF can bind efficiently on the −112/−61-bp region, and again, when this antibody was used, the binding of hc-Krox and Sp1 was slightly decreased, indicating that CBF facilitates the binding of the other two zinc finger transcription factors.

The absence of super-shift in these assays may be due to the fact that hc-Krox, Sp1, and CBF antibodies recognize the DNA binding domain of these transcription factors, preventing their interaction with DNA, and therefore the formation of the complexes.

Finally, additional experiments were performed with nuclear extracts from ANF. As shown in Fig. 6*C*, hc-Krox bound on the −112/−61 sequence of the *COL1A1* promoter with a stronger activity compared with Sp1 and CBF. Mutation of both GC boxes and of the CCAAT box prevented almost all of the binding of these three transcription factors, as shown previously in Fig. 5*B* (Fig. 6*C, panels i* and *ii*, two panels on the *left part* of the figure). Sp1 interacted with low affinity with the −112/−61 *COL1A1* sequence compared with a GC box, present in the first intron enhancer of the human *COL2A1* gene, which binds Sp1 and Sp3 (sequence called enh1 for "enhancer 1") (38). CBF bound with stronger affinity to the −112/−61 *COL1A1* sequence, compared with Sp1 (Fig. 6*C, panels i* and *ii*, panel in the *right part*). These data demonstrate that the −112/−61 sequence of *COL1A1* gene binds the same transcription factors (*i.e.* hc-Krox, Sp1 and CBF) in FF and ANF.

The Proximal Region of COL1A1 Harbors a Functional −67/−61-bp DNA-binding Site for hc-Krox in Dermal Fibroblasts—Because hc-Krox transactivates human *COL1A1* gene by binding to a sequence located between −112 and −61 bp, we wanted to validate these results in terms of transcriptional function. For that purpose, we used the −112/−61-bp probe and different mutants as decoy oligonucleotides in transfection experiments. FF were transfected with the pGL2–112-bp construct in the absence of decoy oligonucleotide or in the presence of a control decoy oligonucleotide, or the −112/−61wt double-stranded sequence. We found that the −112/−61wt decoy oligonucleotide decreased significantly the pGL2–112-bp transcriptional activity, confirming that this region contains activating *cis* elements, including binding sites for hc-Krox, Sp1, and CBF (supplemental Fig. S2).

Additional EMSA were performed to confirm the identity of the transcription factors involved in the formation of the different complexes (Fig. 6). Nuclear extracts prepared from FF were incubated in the presence of the radiolabeled −112/−61wt probe and an hc-Krox antibody. In this case, the hc-Krox-DNA complex formation was abolished (Fig. 6*A*), and an identical pattern was observed when nuclear extracts from ANF and SF were used (data not shown).

When an Sp1 antibody was added to the binding reaction, the Sp1-DNA complex also disappeared (Fig. 6*B, upper complex*), but it should be noticed that the binding of hc-Krox and CBF to the probe was decreased, suggesting that Sp1 binding is essential for the two other transcription factors.

Addition of an Sp3 antibody (Fig. 6*B*) or an NF-1 antibody (data not shown) did not modify the interaction pattern of Sp1, CBF, and hc-Krox to the wild-type probe, suggesting that this transcription factor does not interact with the −112/−61-bp sequence of *COL1A1* in FF. On the other hand, in the presence of a CBF antibody, the complex migrating just

A

B

C

FIGURE 6. Characterization of the transcription factors that bind to the −112/−61 bp *COL1A1* sequence. DNA binding was analyzed by EMSA. *A* and *B,* double strand radiolabeled −112/−61 wild-type oligonucleotide was incubated with FF nuclear extracts (5 µg) in the presence or absence of a c-Krox antibody (5 µl), an Sp1 antibody (2 µl)), an Sp3 antibody (2 µl), or a CBFb-antibody (2 µl). *C,* 5 µg of ANF nuclear extracts were incubated with the indicated labeled double-stranded probes. Enh1 probe represents the +2817/+2842-bp sequence of human *COL2A1* gene that we previously demonstrated to be highly specific for Sp1/Sp3 (31). This probe has been used as a positive control to indicate the migration pattern of Sp1. *C, panels i* and *ii,* two exposures of the autoradiograms are presented; on the *left* a short exposure, and on the *right* a longer time of exposure. *Arrows* indicate the complexes formed between DNA and nuclear proteins.

mutations in the 3′ GC-rich sequence (112mut−73/−74) or in the hc-Krox-binding site located between −67/−61-bp (112mut−61/−62). As shown in Fig. 7, the 112mut−73/−74 reporter plasmids in FF, ANF, and SF displayed a decreased transcriptional activity compared with the 112wt reporter construct, indicating that the 3′GC-rich sequence (−71/−80-bp site) binds *COL1A1* transactivators (Sp1 and hc-Krox, *i.e.* Fig. 5*B*). Mutation of the −61/−67 hc-Krox-binding site in the −112-bp *COL1A1* reporter plasmid further decreased transcription activity indicating that most part of the hc-Krox functional activity is essentially mediated by the −67/−61-bp sequence.

However, it should be noticed that the inhibition percentages in the 112mut−73/−74 reporter constructs compared with their respective wild-type promoter plasmids are lowered in SF compared with ANF, and this is also observed between ANF and FF. Similar conclusions can be deduced concerning the 112mut−61/−62 constructs. These data may suggest that a partial functional redundancy could take place, and it is linked to the endogenous levels of the major *COL1A1* transactivators (*i.e.* hc-Krox, Sp1, and CBF). In other words, the effect of the mutations in the 3′GC-rich sequence or in the −61/−67 hc-Krox-binding site can be partly minimized if the endogenous levels of activators are higher.

Mutation of both −61 and −62 nucleotides in the −67/−61 hc-Krox-binding site was able to prevent the inhibition induced by the −112/−61wt decoy oligonucleotide, indicating that this hc-Krox *cis* element is absolutely critical for the transactivation potential of this short *COL1A1* promoter and for the recruitment of the other activating transcription factors (supplemental Fig. S2). These data suggest that the integrity of −112/−61-bp promoter region of human *COL1A1* gene is crucial for the preservation of its basal transcriptional activity and that hc-Krox and Sp1, as well as CBF, are playing the main roles in this regulatory mechanism.

To examine whether the recognition sites for hc-Krox in the −112/−61-bp region were able to bind specifically the cognate transcription factor in human fibroblasts and to effectively modulate transcriptional activity, transient transfections were performed with reporter constructs bearing

Additionally, the functionality of −67/−61-bp hc-Krox sequence has also been validated in forced hc-Krox expression experiments in the context of the −804-bp *COL1A1* promoter in FF, ANF, and SF (data not shown). These data suggest that the −67/−61-bp region is required for the recruitment and full hc-Krox transactivation potential. All together, these experiments demonstrate that hc-Krox activates the transcription of *COL1A1* gene essentially by the −67/−61-bp region of the gene.

A hc-Krox siRNA Prevents hc-Krox-induced Transactivation of COL1A1 Gene and Type I Collagen Neosynthesis in FF, ANF, and SF—To better characterize the transactivating potential of hc-Krox in fibroblastic cells, the siRNA strategy was used. RNA interference designed to silence specifically endogenous hc-Krox expression was used in transient transfection experiments performed in the three fibroblast cell types. Cells were
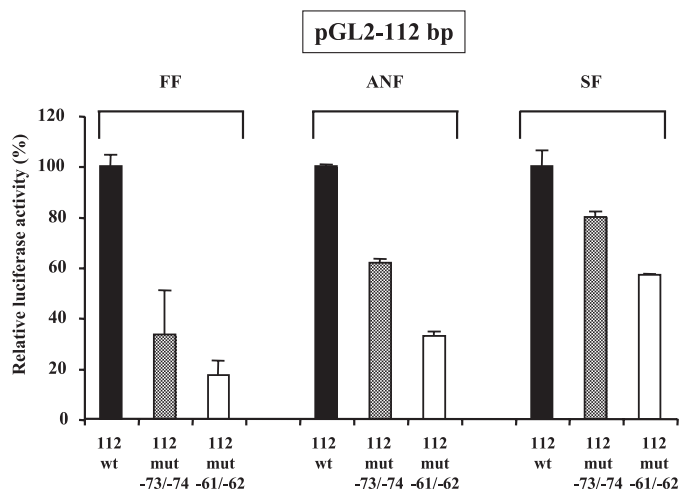
FIGURE 7. **hc-Krox-induced activation of human *COL1A1* gene expression in FF, ANF, and SF is mediated by the −80/−71- and −67/−61-bp sequences.** FF, ANF, and SF were transiently cotransfected, by the AMAXA nucleofector, with 2 μg of different *COL1A1* reporter plasmids (pGL2-112 bp wild-type (*wt*); pGL2–112 bp mutant (*mut −73/−74*); pGL2–112 bp mutant (*mut −61/−62*)) together with 1 μg of the expression vector pSV40/β-gal. 12 h after transfection, the medium was changed. 6 h later, the samples were harvested, and protein content, luciferase, and β-galactosidase activities were assayed. Each series of transfection was performed in triplicate. Transcriptional activity of each construct was expressed as relative luciferase activity after correction for both protein amount and transfection efficiency. Values represent the mean ± S.D. of three independent samples of a representative experiment.

first transfected only with a control siRNA or with a hc-Krox siRNA, and total RNA was extracted, to check hc-Krox siRNA efficiency on *hc-Krox* expression and to determine the resulting effect on *COL1A1* mRNA steady-state levels and transcription. Real time RT-PCR analysis revealed a significant decrease of *hc-Krox* mRNA steady-state levels in FF, ANF, and SF (∼50–85%), which ascertained the siRNA efficiency (Fig. 8, *A, panel i, B, panel i,* and *C, panel i*). Endogenous hc-Krox silencing was accompanied by an important down-regulation of *COL1A1* mRNA steady-state levels (∼45–85%), providing further evidence for the hc-Krox activating role on *COL1A1* gene transcription in the three types of fibroblasts (Fig. 8, *A, panel ii, B, panel ii,* and *C, panel ii*). Next, the three types of fibroblasts were transfected with the plasmid construct pGL2–112 bp, which includes the *COL1A1* gene domain mediating hc-Krox effect, in the presence of a control or hc-Krox siRNAs. The data demonstrate that the hc-Krox siRNA inhibits pGL2–112 bp transcriptional activity in FF, ANF, and SF, suggesting that hc-Krox interacts with transactivating *cis* elements present in the −112/+1 bp sequence, through which it induces increased transcriptional activity of the human *COL1A1* gene (Fig. 8, *A, panel iii, B, panel iii,* and *C, panel iii*).

Finally, we determined if the observed effects at the transcriptional level in the hc-Krox knockdown experiments were also detected at the protein level. For that purpose, FF, ANF, and SF were transiently transfected with control or hc-Krox siRNAs, and the collagen neosynthesis was determined. As shown in Fig. 9, the production of newly synthesized collagens is decreased by ∼50–65% when an hc-Krox knockdown siRNA was transfected in the three fibroblastic cell types, confirming that this transcription factor is an activator of type I collagen expression. Overall, these data demonstrate that hc-Krox has

the same behavior in FF, ANF, and SF, because the same transcriptional regulatory mechanisms affecting the same target sequences are involved.

hc-Krox, Sp1, and Sp3 Physically Interact in Primary FF— Our previous data showed that hc-Krox and Sp1 can bind to the same *COL1A1* promoter region, whose integrity is important for the maintenance of basal transcriptional activity of the gene. Knowing that hc-Krox contains a BTB-POZ domain allowing dimerization and interactions with other transcription factors, we asked whether hc-Krox could interact physically with Sp1 or/and Sp3 transcription factors. To answer this issue, co-immunoprecipitation experiments were performed with nuclear extracts prepared from FF, using an antibody directed against c-Krox. Immunoprecipitated proteins were subjected to Western blotting, and the membranes were incubated with c-Krox, Sp1, or Sp3 antibodies. Immunoblotting with c-Krox antibody revealed a band corresponding to a complex of ∼70 kDa, which matches the hc-Krox apparent molecular weight (67 kDa) and indicates that hc-Krox was correctly immunoprecipitated (Fig. 10*A, panel i*). The other two bands of higher electrophoretic mobility could correspond to β- and γ-hc-Krox homologues.

Similarly, immunoblottings with Sp3 (Fig. 10*A, panel ii*) and Sp1 antibodies (Fig. 10*A, panel iii*) revealed two bands of 85 and 95 kDa corresponding to Sp3 and Sp1 apparent molecular masses, respectively. These results indicate that Sp3, as well as Sp1, can interact physically with hc-Krox in primary FF. Moreover, in these experiments, two bands of lower mobility appeared after treatment with Sp3 antibody; these bands probably correspond to Sp3 longer isoforms or to Sp family homologues. On the other hand, incubation in the presence of Sp1 antibody produced also two bands migrating less than the 95-kDa band, which could be identified as the Sp1-phosphorylated form and/or Sp family homologues.

To verify c-Krox antibody specificity, especially with respect to Sp1, a Western blot experiment was performed. As presented in Fig. 10*B*, the c-Krox antibody does not cross-react with the human recombinant Sp1 protein, therefore validating the *in vivo* interaction of hc-Krox and Sp1 as demonstrated in the immunoprecipitation experiments (Fig. 10*A, panels i* and *iii*).

To corroborate the above data, protein cross-linking experiments were also carried out. Nuclear extracts prepared from FF were incubated, in the presence or absence of glutaraldehyde, and then submitted to Western blot. After blotting, membranes were incubated with antibodies against c-Krox, Sp3, and Sp1.

Immunoblotting with c-Krox antibody and without glutaraldehyde treatment produced essentially one band of 70 kDa, corresponding to c-Krox apparent molecular weight (Fig. 10*C, panel i*). By contrast, in the presence of glutaraldehyde, the 70-kDa band disappeared, and several bands of lower electrophoretic mobility were revealed, suggesting that hc-Krox from FF is involved in multimeric protein complexes (Fig. 10*C, panel i*).

Similarly, when an Sp3 antibody was used and in the absence of cross-linker, two bands corresponding to Sp3 isoforms appeared (Fig. 10*C, panels ii* and *iii*), whereas in the presence of glutaraldehyde, the intensity of both bands decreased, and complexes of higher apparent molecular weight appeared.

Ai

Aii

Aiii

Bi

Bii

Biii

Ci

Cii

Ciii
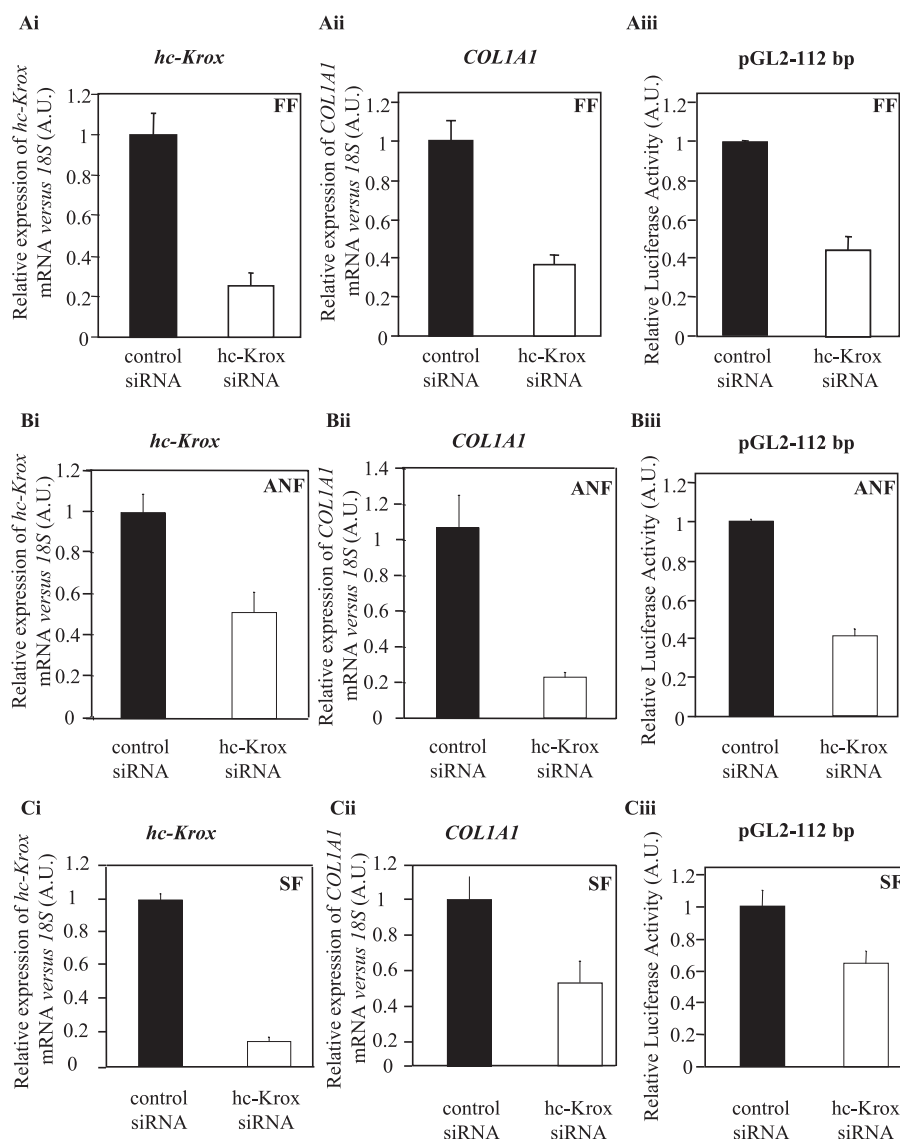
FIGURE 8. **hc-Krox siRNA inhibits the hc-Krox-induced activation of type I collagen expression in FF, ANF, and SF.** *A–C,* FF (*A*), ANF (*B*), and SF (*C*) were transiently transfected by the AMAXA nucleofector with 1 μg of a control or hc-Krox siRNA. 12 h after transfection, the cell culture medium was changed, and 6 h later, the samples were harvested. 1 μg of total RNAs extracted was reverse-transcribed into cDNA. 2 μl of cDNA were diluted (1:100) and used in real time PCR to amplify *hc-Krox* (*A, panel i*; *B, panel i*; and *C, panel i*), COL1A1 (*A, panel ii*; *B, panel ii*; and *C, panel ii*), and 18 S cDNAs. The relative expression of *hc-Krox* and *COL1A1* mRNAs was normalized to 18 S rRNA according to $2^{-\Delta Ct}$ method (*A.U.,* arbitrary units). In parallel, fibroblasts were transiently transfected using the AMAXA nucleofector with a control or a hc-Krox siRNA (1 μg) together with 2 μg of pGL2-112-bp construct and 1 μg of the expression vector pSV40/β-gal. 12 h after transfection, the medium was replaced. 6 h later, the samples were harvested, and transcriptional activities have been analyzed as described in Fig. 4.

These data show that Sp3 can also multimerize in FF. Finally, incubation of the control sample in the presence of an Sp1 antibody revealed one band of ~95 kDa, which corresponds to Sp1 apparent molecular weight. Addition of glutaraldehyde markedly reduced the 95-kDa band intensity and produced a multicomplex of high molecular weight, showing that Sp1 interacts with other proteins in FF. These results show that in FF, hc-Krox, Sp3, and Sp1 form multimeric protein complexes and interact between each other.

hc-Krox, Sp1, and Sp3 Bind to the COL1A1 Promoter in Vivo—To determine whether hc-Krox, Sp1, and Sp3 are effectively involved in the regulation of *COL1A1* gene activation, and to validate our *in vitro* experimental data, chromatin immunopre-cipitation assay was performed. As shown in Fig. 11, these three zinc finger proteins bind to the −200-bp promoter region of *COL1A1* in FF. These factors could therefore interact and could be recruited on the same region of *COL1A1* gene to favor optimal transactivation.

The Levels of hc-Krox DNA Binding Activity Are Correlated with Type I Collagen Expression in Fibroblasts—In this study, we show for the first time that hc-Krox is an activator of type I collagen expression in normal and scleroderma fibroblasts. We next evaluated whether the endogenous levels of hc-Krox binding activity could be correlated with the amounts of type I collagen produced by fibroblasts. Thus, we compared side by side the endogenous expression of type I collagen (steady-state mRNA, protein levels, and transcriptional activity) in FF, ANF, and SF, as well as the corresponding hc-Krox binding activity on the *COL1A1* −112/ −61 bp promoter. As shown in Fig. 12*A*, the hc-Krox binding activity is higher in SF compared with ANF, which also present a much more higher DNA binding activity compared with FF. Collagen neosynthesis evaluated in parallel on the same cells was shown to be approximately 3- and 4-fold higher in SF compared to ANF and FF, respectively (Fig. 12*B*). These observed effects on type I collagen expression resulted from a transcriptional control because the transcription of the pGL2– 112-bp *COL1A1* construct is higher in SF compared with ANF, and also increased in ANF compared with FF (Fig. 12*C*). These transcriptional effects were also confirmed at the level of *COL1A1*, *COL1A2*, and *COL3A1* steady-state mRNA amounts (supplemental Fig. S3).

It can be concluded that hc-Krox activates type I collagen expression in dermal fibroblasts, and the DNA binding activity of this transcription factor determines, at least in part, the levels of the endogenous type I collagen expression. Knowing that increased collagen synthesis is a major event observed in scleroderma, we may assume that hc-Krox is one of the transcription factors involved in the *COL1A1* up-regulation.

DISCUSSION

This study focuses on molecular mechanisms controlling human *COL1A1* gene transcription in physiopathological con-
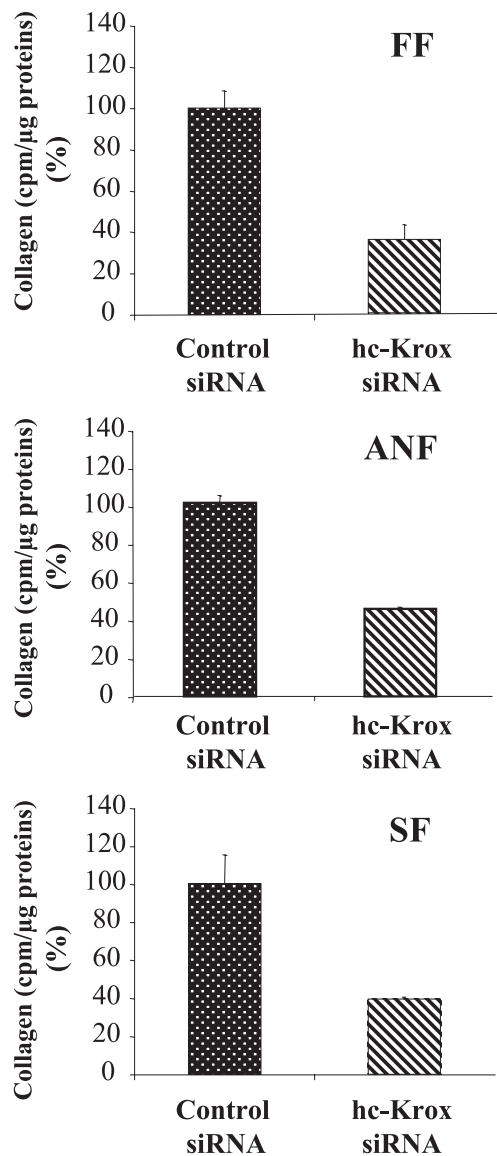
FIGURE 9. **hc-Krox siRNA inhibits the hc-Krox-induced increase of type I collagen neosynthesis in FF, ANF, and SF.** FF, ANF, and SF were transiently transfected by the AMAXA nucleofector with 1 μg of a control or of hc-Krox siRNA. 12 h after transfection, the cell culture medium was replaced, and the cells were incubated in the same fresh medium supplemented with β-aminopropionitrile (50 μg/ml) and ascorbic acid (50 μg/ml) for a further 24-h period. The same medium was then renewed, and [^{3}H]proline (2 μCi/ml) was added. After 24 h, the culture medium was collected and the labeled collagen was assayed, using the bacterial collagenase method. The values, normalized to total protein amount, are expressed as counts/min/μg of protein and represent the mean $\pm$ S.D. of triplicate dishes.

ditions. We particularly investigated the role of hc-Krox transcription factor in human adult and foreskin (from children) normal fibroblasts and in scleroderma fibroblasts. This approach should later on facilitate the clarification of events potentially implicated in the manifestation and/or evolution of the fibrotic reaction. Our results showed that hc-Krox is able to activate type I collagen expression, at the protein, transcriptional, and gene activity levels, in all types of primary fibroblastic cells. The data also demonstrate that this transcription factor interacts physically with Sp1 and Sp3 and that each of these *trans* factors binds *in vivo* on the core promoter of *COL1A1*.
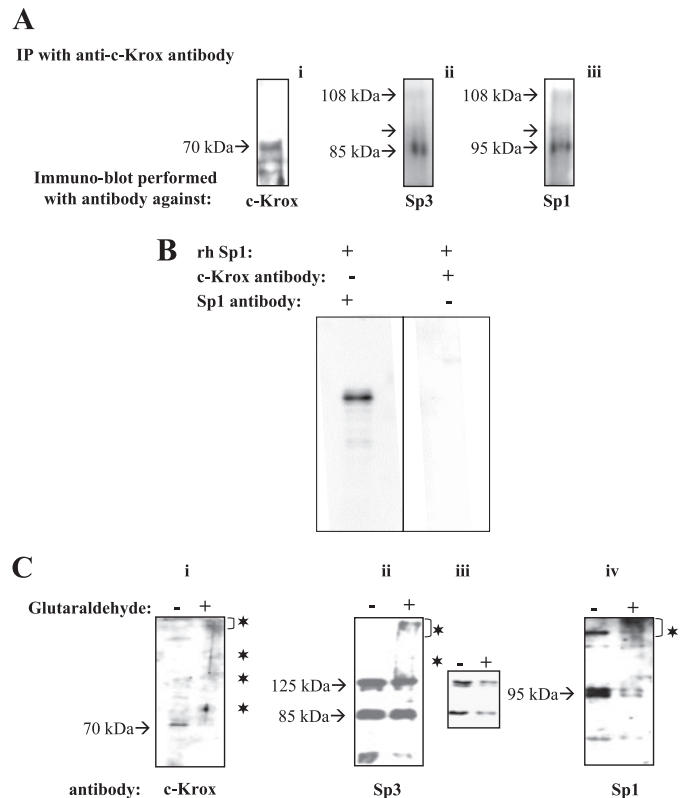
FIGURE 10. **hc-Krox, Sp1, and Sp3 interact physically in FF.** *A,* hc-Krox was immunoprecipitated (*IP*) in total cellular extracts prepared from 2.5 × 10^6 FF. Proteins isolated after immunoprecipitation were denatured and separated by SDS-PAGE. Three samples were transferred to polyvinylidene difluoride membranes and reacted with polyclonal antibodies against hc-Krox (*panel i*), Sp3 (*panel ii*), and Sp1 (*panel iii*). Proteins were revealed with a peroxidase-coupled secondary antibody (anti-rabbit IgG) using a West-Pico Western detection kit. *Arrows* indicate the molecular weight of proteins recognized by antibodies, as estimated by prestained marker proteins. *B,* 1 μl of human recombinant Sp1 protein (Promega) was separated by SDS-PAGE. The samples were then transferred to polyvinylidene difluoride membranes, which were reacted with polyclonal antibodies directed against hc-Krox (1:100 dilution) and Sp1 (1:300 dilution). Protein was revealed with a peroxidase-coupled secondary antibody (anti-rabbit IgG) using a West-Pico Western detection kit. *C,* 10 μg of FF nuclear extracts were incubated with or without 2 μl of glutaraldehyde 0.1%, during 10 min at room temperature. Proteins were then denatured and separated by SDS-PAGE. Samples were transferred to polyvinylidene difluoride membranes and reacted with antibodies against hc-Krox (*panel i*), Sp3 (*panels ii* and *iii*), and Sp1 (*panel iv*). Proteins were revealed as in *A*. *Arrows* indicate the molecular weight of proteins recognized by antibodies, as estimated by prestained marker proteins. *Panel iii* represents a shorter film exposure as compared with *ii*.

Our particular interest for hc-Krox function in human skin fibroblasts comes from the fact that this factor has been described previously as an inhibitor of type I collagen expression in NIH-3T3 mouse fibroblastic cells (16). These data suggested that hc-Krox could be involved in the fibrotic process and make it a motivating target to better understand scleroderma. The present results revealed in fact that hc-Krox is a potent activator of type I collagen expression in normal foreskin and adult fibroblasts but also in scleroderma cells. The discrepancy between our study and previous results can be due to the cell type and/or to different experimental procedures used. For example, the transfection method employed here is the Amaxa nucleofection, which is, in our hands, the more appropriate method to obtain high transfection efficiency and proper expression of the reporter gene, especially for high size reporter vectors.
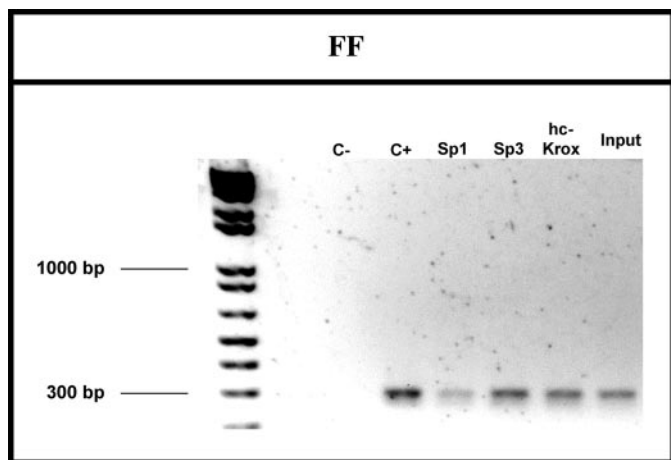
FIGURE 11. **hc-Krox, Sp1, and Sp3 bind *in vivo* to the *COL1A1* promoter.** Ten flasks of FF cultures were fixed, digested, and immunoprecipitated overnight by hc-Krox, Sp1, and Sp3 antibodies. These steps were followed by PCR amplification with primers flanking the $-112/-61$ sequence of the *COL1A1* promoter. As positive control (*Input*), the same primers were used on the genomic DNA. Samples were also immunoprecipitated with anti-RNA polymerase II used as positive control (*C+*) and with a nonspecific immunoglobulin used as a negative control (*C−*).

hc-Krox inhibiting effects have been associated to its BTB/POZ domain. In the case of promyelotic leukemia zinc finger, the BTB/POZ domain is located at the N-terminal extremity and interacts with co-repressors, like silencing mediator for retinoid and thyroid hormone receptors, and nuclear receptor co-repressor. Co-repressors recruit histone deacetylase 1 that interacts with the target promoter and inhibits its transcriptional activity, modifying locally the chromatin structure (44). However, all transcription factors carrying BTB/POZ domains are not *trans*-repressors. For example, *Drosophila* CAGA transcription factor, encoded by Trithorax-like gene, enhances transcription activity by preventing chromatin condensation. In this case, the BTB/POZ domain provokes the oligomerization of CAGA factor, and this structure allows direct interaction with many sites of DNA to the detriment of the nucleosome structure in a way that makes the promoter accessible to transactivators (45).

Moreover, it is not very surprising that hc-Krox behaves as an activator of collagen in skin fibroblasts because we demonstrated that its mouse homologue, mc-Krox, is an activator of mouse *Col1a1* gene in NIH-3T3 fibroblasts (14). Additionally, we showed that mc-Krox is highly expressed in skin and that its expression during development precedes at least 3 days type I collagen expression (14). This element strongly suggests that c-Krox is a factor that could initiate and enhance type I collagen synthesis.

Even if the mc-Krox cDNA at that time was an incomplete clone lacking the BTB/POZ domain, we have shown that the transactivating effects exerted on target gene transcription were mediated by its C-terminal extremity and the zinc finger domain (15). This is in agreement with the observations of Widom *et al.* (16), who showed that the truncation of hc-Krox N-terminal domain (but not of BTB/POZ domain alone) leads to an activation of target genes transcription, thus preventing the inhibiting effect.

Currently, no identified mechanism characterizing c-Krox function is known. Nevertheless, an interesting example of
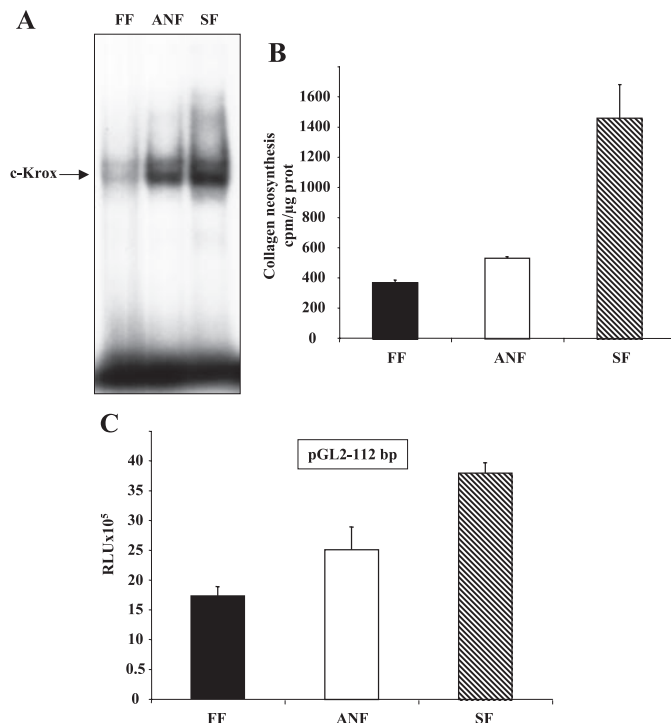
FIGURE 12. **The endogenous hc-Krox DNA binding activity to *COL1A1* gene in fibroblasts is correlated with type I collagen synthesis.** *A*, DNA binding was analyzed by EMSA. 7.5 μg of FF or ANF or SF nuclear extracts were incubated with the radiolabeled $-112/-61$ wild-type probe, in direct binding experiments. *Arrow* indicates the complexes formed between DNA and hc-Krox. *B*, confluent cultures of FF, ANF, and SF were preincubated for 24 h in Dulbecco's modified Eagle's medium + 10% fetal calf serum supplemented with β-aminopropionitrile (50 μg/ml) and ascorbic acid (50 μg/ml). The same medium was then renewed, and [^{3}H]Proline (2 μCi/ml) was added. After 24 h, the cell culture medium was collected, and the labeled collagen was assayed, using the bacterial collagenase method, as described under "Experimental Procedures." The values, normalized to total protein amount, are expressed as counts/min/μg of protein and represent the mean ± S.D. of triplicate dishes. *C*, FF, ANF, and SF were transiently transfected using the AMAXA nucleofector with 2 μg of pGL2–112-bp construct and 1 μg of the expression vector pSV40/β-gal. 12 h after transfection, the medium was replaced. 6 h later, the samples were harvested, and transcriptional activities have been analyzed as described in Fig. 4.

transcription factors having a dual activation or repression domain has been reported for YY1 ("Yin Yang-1"), another Krüppel-like factor. Thus, YY1 can activate or inhibit target gene transcription depending on its relative concentration, of the promoter, and/or of the cellular context (46–49). The mechanism proposed to explain its bifunctional effect is related to the interaction of the factor with histone acetyltransferases, such as CBP, p300, and PCAF (CBP/p300 associated factor) and histone deacetylases 1-, -2, and -3 (50, 51). These findings, together with the capacity of YY1 to interact with Sp1, GATA-1, and c-Myc (52, 53) suggest an hypothesis for hc-Krox function.

This hypothesis could be supported by the fact that c-Krox can homo- or heterodimerize through its BTB/POZ or C-terminal domains (15, 17). Although the proteins interacting with this factor are not yet all identified, it seems very plausible that the transactivating effects of hc-Krox could depend on the spatial configuration of the protein, on the promoter sequences surrounding c-Krox-binding sites and, on the context of the transcriptional partner.

Indeed, previous results showed that hc-Krox inhibits human *COL2A1* and *UGDH* genes transcriptional activity in differentiated and de-differentiated rabbit articular chondrocytes by binding close to Sp1/3-binding sites (18, 38),[6] whereas it activates the human *COL1A1* gene in skin fibroblasts by interacting with Sp1 and Sp3 (this paper). Thus, at the same time hc-Krox seems to be a pro-arthrotic and a pro-fibrotic factor, suggesting its involvement in inflammatory or/and immune responses.

It has been shown recently that the full-length mc-Krox plays a key role in the differentiation of thymocytes in CD4 helper rather than in CD8 killer T lymphocytes (19, 20). Constitutive expression of c-Krox in helper-deficient mice led to the differentiation of class I-restricted thymocytes in CD4+ cells, whereas overexpression of a mutant form of this transcription factor appeared functionally inert (20). Interestingly, during the first stages of scleroderma disease, an accumulation of CD4+ lymphocytes is observed, whereas CD8+ population is decreased. CD4+ cells migrate in vascular epithelium and dermis connective tissues and lead to an activation of target cells and to an increased production of pro-fibrogenic growth factors and cytokines (22, 54). Taken together, these data suggest that hc-Krox could be one of the first actors involved in the manifestation of scleroderma, playing a role in the immune reaction but also in the remodeling of dermis ECM.

This study provides some evidence about this last issue, because hc-Krox was found to increase *COL1A1* transcriptional activity and not only *COL1A1*, *COL1A2*, and *COL3A1* mRNA steady-state levels but also *Sp1* and *Sp3* transcription (data not shown), two other activators of type I collagen genes (6, 10, 11, 28, data not shown). These three zinc finger factors interact with the same human *COL1A1* promoter region as demonstrated in chromatin immunoprecipitation experiments, through which they exert their activating effects (this study and data not shown). Artlett *et al.* (55) showed that this region is sufficient to confer maximal transcriptional activity of the human *COL1A1* gene *in vitro*. From a transcriptional point of view, nucleotide analysis of the 198-bp sequence revealed four potential binding sites for hc-Krox ($-168/-141$, $-93/-84$, $-80/-71$, and $-54/-47$ bp), all of them overlapping with Sp1/Sp3 potential binding sites. EMSA experiments showed that hc-Krox can bind with high affinity to $-168/-141$- and $-80/-71$-bp sequences, whereas its affinity for the other two sites is very low (data not shown). However, from our EMSA analysis and transient transfection assays, we have identified a new $-67/-61$-bp sequence binding hc-Krox that clearly mediates a great part of its activating effect in FF, ANF, and SF.

Our data also demonstrate that in terms of binding affinity and amount, hc-Krox is the major transcription factor interacting with the $-112/-61$-bp region, compared with CBF and Sp1. Moreover, as demonstrated by Cicchillitti *et al.* (43), we confirmed that Sp1 and CBF interact with this sequence, and the latter one binds much more efficiently compared with Sp1.

CBF interacts with the $-100/-96$-bp sequence, enhancing *COL1A1* transcriptional activity, providing further evidence

that the $-198/+1$-bp proximal promoter, and particularly the $-112/-61$-bp sequence, plays a critical role in the regulation of human *COL1A1* gene in human skin fibroblasts. Besides, it is noteworthy that the binding of each factor is dependent of the binding of the others.

In this sense, transient transfections were performed with reporter constructs bearing mutations in the 3′ GC-rich sequence or in the hc-Krox-binding site located between $-67/-61$ bp. The data revealed that the extent of inhibition in the mutated reporter constructs compared with their respective wild-type promoter plasmids is lower in SF compared with ANF, and this effect is even more pronounced when compared with FF. We therefore hypothesize that a partial functional redundancy could be effective, and this is probably because of the fact that endogenous levels of the major *COL1A1* transactivators such as hc-Krox, Sp1, and CBF display a greater DNA binding activity in SF compared with the normal fibroblasts. This means that the effect of the mutations in the 3′ GC-rich sequence or in the $-61/-67$ bp hc-Krox-binding site can be partly counteracted if the endogenous levels of activators are higher.

Additionally, our results are in agreement with those obtained by Hitraya *et al.* (56) who reported that the maximal transcriptional activity of *COL1A1* is located within the -129- and -84-bp region. This region includes two tandem NF1/Sp1 elements ($-129/-107$-bp and $-104/-78$-bp) and the CCAAT-box, and the cognate transcription factors display a higher binding activity in nuclear extracts from SSc fibroblasts (12, 56). We demonstrate here that hc-Krox also appears to have a pivotal role in the activated expression of *COL1A1* in SSc fibroblasts, because its DNA binding activity is increased in SF nuclear extracts in comparison with the nuclear extracts from normal cells (ANF and FF). Moreover, our observations suggest that hc-Krox is involved in both basal and increased *COL1A1* transcription and that the level of hc-Krox DNA binding activity is closely correlated with the type I collagen expression in fibroblasts, as demonstrated for Sp1 and CBF (12, 56).

To explain that the $-112/-61$-bp sequence binds mainly hc-Krox in our EMSA, and to a much more lower extent CBF and Sp1, we propose that our probe covers the oligonucleotidic sequence between -78 to -61 bp in which we have identified for the first time a new hc-Krox DNA-binding element (*i.e.* $-67/-61$ bp), which is responsible for the main part of the transactivating effect of this factor as demonstrated in mutagenesis functional assays (Fig. 7). By contrast, other studies have restricted their analysis to the $-104/-78$-bp region and have not focused on the downstream sequences.

Taken together, these data suggest that, in human skin fibroblasts, the $-112/-61$-bp region may interact with several transcription factors exerting activating effects on human *COL1A1* gene transcription, but also promoting interactions with TATA box and/or other promoter or intron sequences of the gene.

In conclusion, we have characterized the transcriptional function of hc-Krox on human *COL1A1* gene in healthy foreskin fibroblasts and correlated these findings with data obtained in adult normal and scleroderma fibroblasts. Given the relatively tissue-restricted expression of hc-Krox compared

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[6] B. Porée, E. Renard, G. Beauchef, and P. Galéra, unpublished observations.

with Sp1 and CBF, all these data make this factor an attractive candidate for setting up therapeutic strategies against fibrotic diseases. Additionally, we showed for the first time that hc-Krox is able to form macromolecular complexes with other transcription factors such as Sp1 and Sp3. This element, associated with the fact that all three factors exert their transactivating effects through a *COL1A1* 198-bp short promoter, will allow us to develop further analysis of human *COL1A1* gene regulation of expression, taking into account the complexity of molecular mechanisms controlled by direct or indirect interactions between endogenous nuclear factors under normal and pathological conditions.

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Figure S1. hc-Krox, Sp1, and CBF bind specifically to -112/-61 bp *COL1A1* sequence.
DNA binding was analyzed by EMSA.
Panel A.i – A double-strand radiolabeled -112/-61 wt oligonucleotide was incubated with FF nuclear extracts (5 µg) (*lanes* 2-13) or with human recombinant Sp1 protein (Promega, Charbonnières, France) (*lane* 14). Competition with molar excesses of cold wild-type and mutant oligonucleotides were performed (-112/-61wt: *lanes* 3-4, -112/-61mut: *lanes* 5-6, -112/-61mut1: *lanes* 7-9, -112/-61mut2: *lanes* 10-11, -112/-61mut3: *lanes* 12-13). *Lane* 1 represents the probe incubated without nuclear extracts. *Panel A.ii* represents a magnification of C1 and C2 complexes corresponding respectively to Sp1 and CBF. *Panel B* – 5 µg of FF nuclear extracts were incubated with a double-strand labeled -112/-61 wt oligonucleotide (*lanes* 1-6) in the presence of molar excesses of cold wild-type and mutant oligonucleotides (-112/-61wt (*lanes* 2-3), -112/-61mut4 (*lanes* 4-6)). *Arrows* indicate the complexes formed between DNA and nuclear proteins.

Fig. S1Ai shows that incubation of the -112/-61wt probe (Table I) with nuclear proteins extracted from FF, revealed two major complexes (*lane* 2). The slowest migrating two bands, called C1 and C2, are magnified in Fig. S1Aii, and correspond, according to the literature (43), to complexes between DNA and Sp1 (C1) or CBF (C2). To get more information about the identity of the C1 complex, the same oligonucleotide was incubated with the human recombinant Sp1 protein (*lane 14*) and only one complex, having an identical migration profile as C1, was detected. These data provide further evidence that C1 corresponds to Sp1. Another proof of the presence of Sp1 in C1 complex was also provided by antibody interference experiments (Fig. 6B).

The binding specificity of these two complexes (C1 and C2) was studied in competition experiments, using wild-type and mutated oligonucleotides presented in Table I and Fig. 5A. Thus, competition with an unlabeled -112/-61wt oligonucleotide decreased the binding of both complexes (*lanes 3, 4*), suggesting the specificity of the interactions observed between proteins and DNA. When the -112/-61mut was used as a cold competitor, in which both GC-rich sequences and the CCAAT box were mutated, a decrease of the binding activity of Sp1, CBF and hc-Krox to the probe was found (*lanes 5, 6*). This finding indicates that another *cis* element is able to bind or recruit the three transcription factors in the -112/-61-bp region. The data provide further evidence that mutation of the three boxes present on -112/-61 bp *COL1A1* sequence is not sufficient to prevent the binding of proteins interacting with the wild type sequence. Additionally, when the 5' GC-rich box was mutated (-112/-61mut1) and used as a competitor (*lanes 7-9)*, the formation of Sp1/CBF-DNA and hc-Krox-DNA complexes was reduced, demonstrating that the transcription factors are still able to directly interact with the inverted CCAAT box and the 3' GC-rich box which are not mutated, as well as with other domains of the -112/-61 bp sequence located in 3'.

Mutation only in the 3' GC-rich box (-112/-61mut2) competed efficiently the binding of Sp1, CBF and hc-Krox to the probe (*lanes 10, 11*). This suggests that an interaction of Sp1 and hc-Krox with the 5' GC-rich and with the putative hc-Krox *cis* element (-67/-61) is possible. When a cold competitor mutated only in the CCAAT box was used, it significantly reduced the binding of the three transcription factors (*lanes 12, 13*). The observation of lanes 12, and 13 reveals a significant decrease of the C2 complex (which corresponds to CBF), that normally should not be altered, and strongly suggests that CBF needs to interact with factors binding to the GC-rich boxes in order to bind efficiently on *COL1A1* promoter. Finally, competition with a -112/-61mut4 oligonucleotide, in which both GC-rich sequences were mutated, was able to slightly compete away the binding of hc-Krox, Sp1 and CBF to the wild-type probe, confirming that these transcription factors can be recruited probably through the -67/-61 hc-Krox site (Fig. S1B).

Figure S2. Decoy oligonucleotides corresponding to -112/-61 bp promoter region of *COL1A1* gene are able to reverse hc-Krox transactivating effect.
Panels A, B and C - FF were transiently transfected by the AMAXA nucleofector with 2 µg of pGL2-112 bp construct and 1 µg of the expression vector pSV40/β-gal. 30 µg of different oligonucleotides corresponding to the wild type -112/-61 bp sequence or mutant derivatives (indicated on the Fig.) were added in the culture medium immediately after transfection. 12 hrs after transfection, the medium was changed and supplemented with the same decoy oligonucleotide (30 µg). 6 hrs later, cells were harvested and relative transcriptional activities have been analyzed.

Having shown that hc-Krox can transactivate human *COL1A1* gene by binding to a sequence located between -112 and -61 bp, we tried to validate these results in terms of transcriptional function. We used the -112/-61 bp probe and different mutants as decoy oligonucleotides in transfection experiments. FF were transfected with the pGL2-112 bp construct in the absence of decoy oligonucleotide or in the presence of a control decoy oligonucleotide (a sequence of the human *COL2A1* intronic enhancer binding specifically SOX9 transcription factor), or the -112/-61wt double-stranded sequence. We found that the -112/-61wt decoy oligonucleotide decreased significantly the pGL2-112 bp transcriptional activity, confirming that this region contains activating *cis* elements, including binding sites for hc-Krox, Sp1, and CBF (Fig. S2A). To determine the possible involvement of the 5' and 3' GC-rich sequences and the CCAAT box in the transcription activity of the -112 bp *COL1A1* promoter, several mutants were used. As shown in Fig. S2A, transfection of a decoy oligonucleotide presenting a mutation in both 5' and 3' GC boxes, together with a mutation in the CCAAT box, allowed to maintain almost all of the basal transcription activity of the -112-bp *COL1A1* promoter, demonstrating that hc-Krox and Sp1 bind to the two GC boxes and CBF to the CCAAT sequence, and that all are transactivators.

In Fig. S2B, a hc-Krox consensus oligonucleotide (DAα1(I)wt), used as a decoy, inhibited the transcription of the pGL2-112 bp construct, confirming again that hc-Krox is an activator of *COL1A1* transcription. In this set of experiments, -112/-61 bp mutants were also used as competitors. D-112/-61mut1 (for decoy -112/-61 bp mut1, mutation in the 5' GC-rich sequence), which competes for CBF and partially for Sp1 and hc-Krox binding, slightly reduced the 112 bp promoter activity, suggesting that Sp1 and hc-Krox still bind respectively on the 5'-GC-rich and the hc-Krox regions, and are sufficient to maintain almost all the basal transcriptional activity of the promoter. However, D-112/-61mut2 (mutation only in the 3' GC-rich sequence) and D-112/-61mut3 (mutation in the CCAAT box) induced a greater decrease of the 112 bp promoter activity. These data demonstrate that the integrity of the 5' and 3' GC-rich sequence, but also the -67/-61 *cis* element and the CCAAT box are important for the maintenance of the basal activity of the promoter. Moreover these results are in agreement with our EMSA results which show that hc-Krox, Sp1 and CBF bind on the -112/-61 bp sequence in an interdependent way, and prove that all of them are activators of the *COL1A1* gene transcription. Moreover, the use of -112/-61mut4 decoy, which allowed a very weak Sp1 and hc-Krox binding to the promoter, abrogating CBF fixation and inducing a down-regulation of promoter activity comes as a further support to our previous findings according to which hc-Krox, Sp1 and CBF binding on -112/-61 bp sequence is interdependent.

Mutation of both -61 and -62 nucleotides in the -67/-61 hc-Krox binding site was able to prevent the inhibition induced by the -112/-61wt decoy oligonucleotide, indicating that this hc-Krox *cis* element is absolutely critical for the transactivation potential of this short *COL1A1* promoter and for the recruitment of the other activating transcription factors (Fig.

S2C). The same decoy strategy was also applied to ANF and similar data were obtained when -112/-61wt, -112/-61mut, and -112/-61mut-61-62 sequences were used as decoy oligonucleotides (data not shown).

These data suggest that the integrity of -112/-61 bp promoter region of human *COL1A1* gene is crucial for the preservation of its basal transcriptional activity and that hc-Krox and Sp1, as well as CBF, are playing the main roles in this regulatory mechanism.

Figure S3. The hc-Krox endogenous DNA-binding activity to *COL1A1* gene in fibroblasts is correlated with type I collagen synthesis.

Total RNAs were extracted from FF, ANF, and SF cultures. 1 µg of total RNAs was reverse-transcribed into cDNA. 2 µl of cDNAs were diluted 100 times and used in real time PCR to amplify *COL1A1* (*panel A*), *COL1A2* (*panel B*), *COL3A1* (*panel C*) and *GAPDH* cDNAs, with specific forward and reverse primers. *COL1A1*, *COL1A2,* and *COL3A1* mRNAs relative expression was normalized to *GAPDH* mRNA according to $2^{-deltaCT}$ method (A.U. Arbitrary Units).

The results indicate that SF express higher amounts of *COL1A1*, *COL1A2*, and *COL3A1* mRNA compared to ANF. The same conclusion is also true for ANF compared to FF.
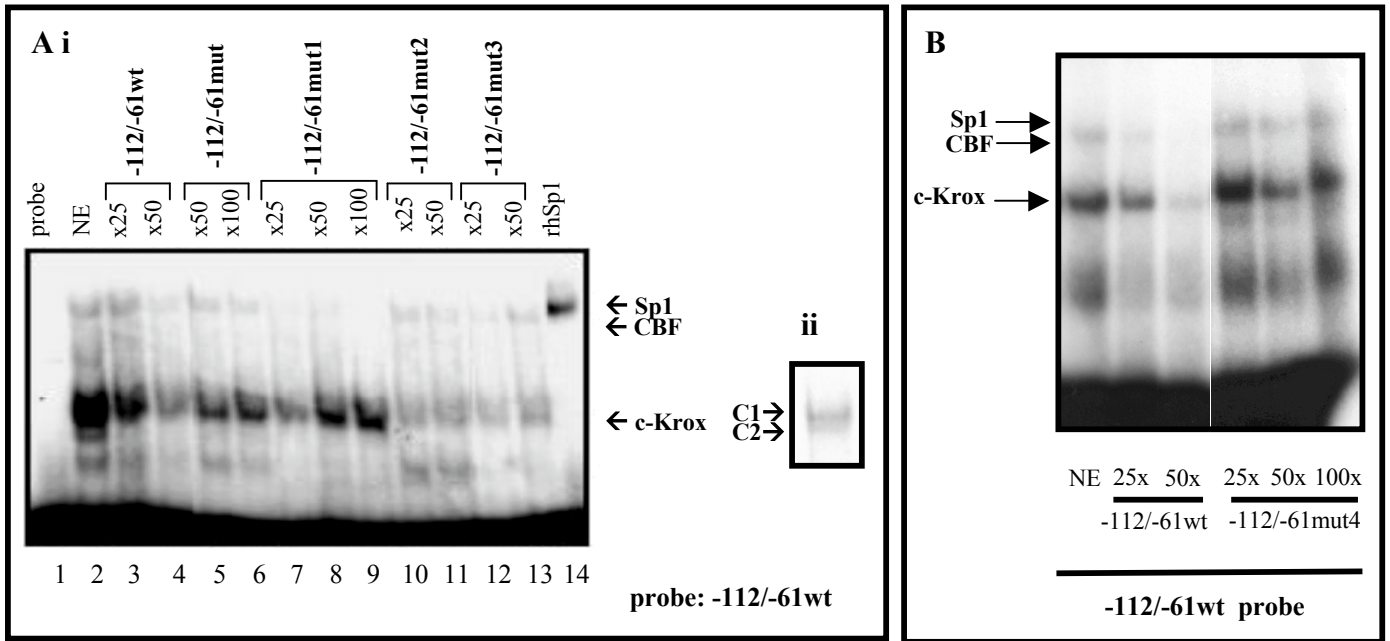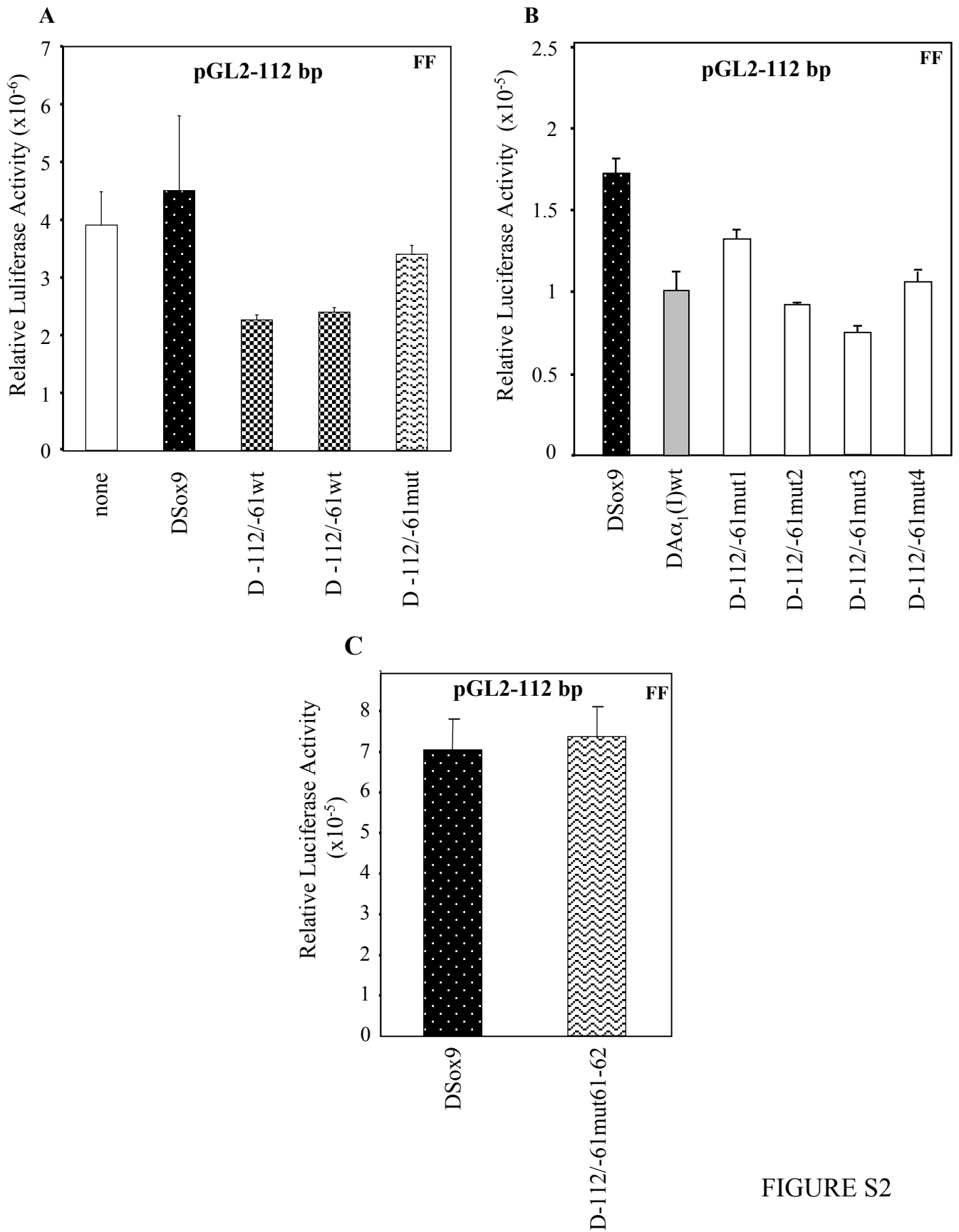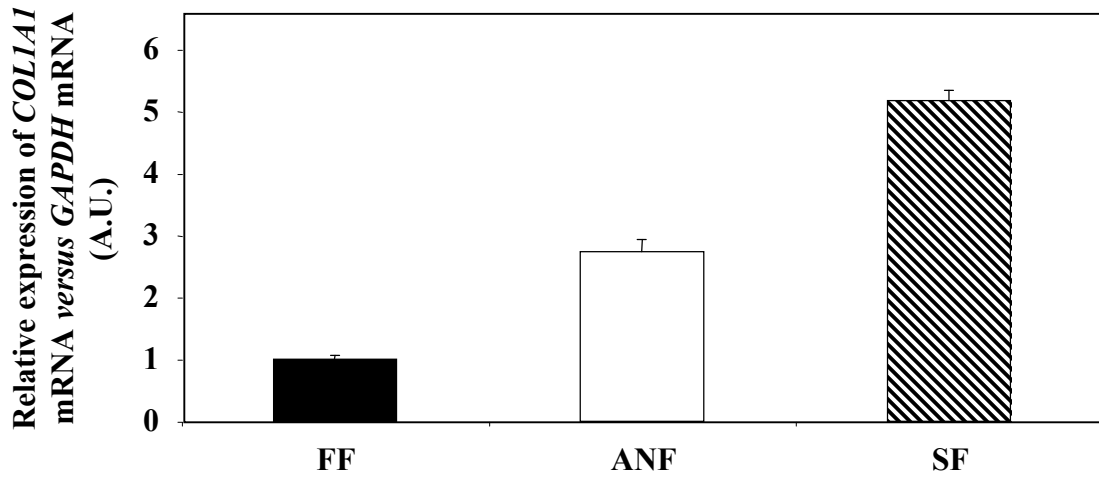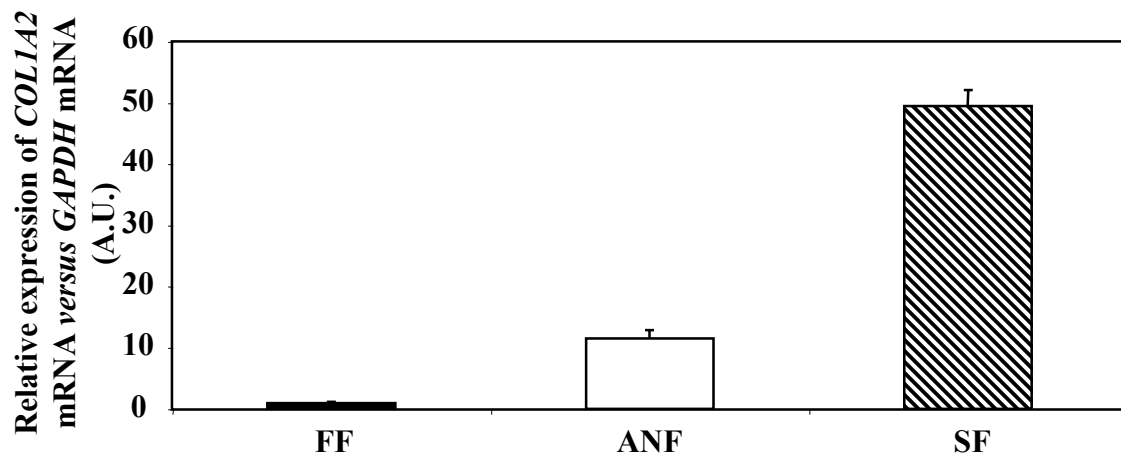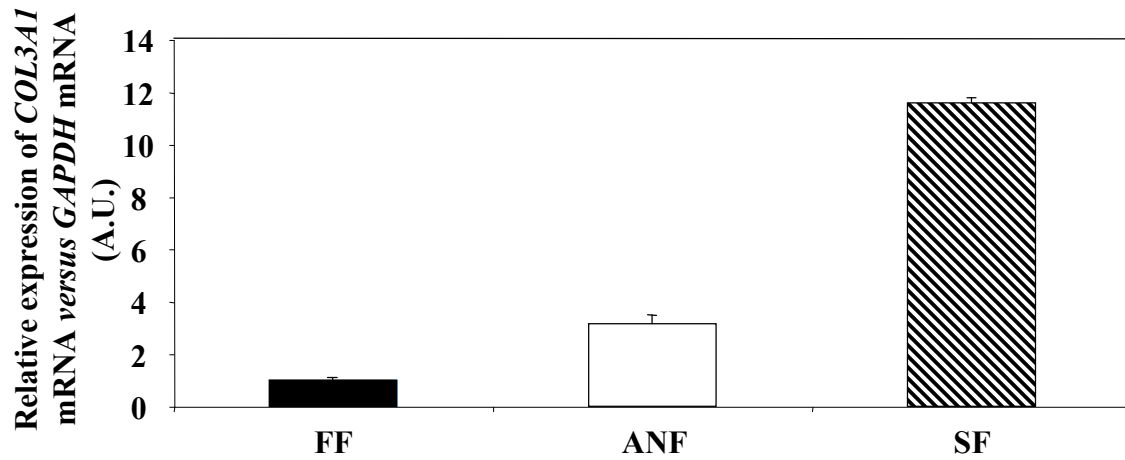
FIGURE S1

FIGURE S2

A

B

C

FIGURE S3

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