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Functional Analysis of the Lysyl Oxidase Promoter in Myofibroblast-Like Clones of 3T6 Fibroblast

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Abstract Lysyl oxidase (LO), an extracellular enzyme catalysing the first step of collagen and elastin cross-linking, is transiently expressed by myofibroblasts during fibrosis. A cell model with features of myofibroblast was thus established for studying the regulation of LO. Two clones of the 3T6 fibroblast cell line were selected because 1) they produced a relatively high steady-state level of the three lysyl oxidase mRNAs with the same relative ratio similar to fibrotic tissue and 2) they stably displayed certain features of myofibroblast (α-smooth muscle actin cytoskeleton, bundles of cytoskeletal filaments beneath the cytoplasmic membranes). These clones synthesized predominantly type I collagen fibers and a small amount of type III collagen. Neither type IV collagen nor elastin were observed. The cloning and sequencing of 2,073 bp of the mouse Balb/C LO promoter was performed, allowing the identification around the initiation of transcription of consensus sequences which are found on the COL1 promoters. A series of deletion constructs containing the LO 5′-flanking region ligated to the luciferase gene were transiently transfected into 3T6-5 fibroblasts. The region allowing the maximal activity was found between positions −416 to −192, while the more upstream region negatively regulated the promoter. The −898 to −865 sequence (called LOcol1) displayed 79% of homology with a conserved sequence of murine, rat, and human COL1A1 promoters. This sequence participated to the binding of several nuclear factors within a region (−970 to −784) allowing 50% of inhibition of the LO promoter. Therefore, the level of LO transcription is regulated in 3T6-5 fibroblast by positive and negative cis-acting regulatory elements which might have common features with the COL1A1 promoter. J. Cell. Biochem. 64:328–341.

Key words: Lysyl oxidase; type I collagen; myofibroblast; fibrosis; mRNA

Lysyl oxidase (LO) is the key enzyme involved in the cross-linking of fibrillar collagens and elastin [Kagan, 1986]. It catalyzes the post-translational oxidative deamination of the "ϵ"-amino group of some lysyl and hydroxylysyl residues of collagen and of lysyl residue of elastin. The enzyme has a critical role in the formation of connective tissues. This involvement has been highlighted by the induction of lathyrism in animal models through inhibition of the enzyme by β-amino propionitrile (β-APN) or copper deficiency and clinically in Menkes' disease and X-linked cutis laxa [Siegel, 1979; Kagan, 1986]. The regulation of LO in fibrosis might determine the evolution of the fibrotic deposit, as the ratio of LO-dependent cross-linked collagen was higher in a nonreversible model of fibrosis than in a reversible fibrosis [Ricard-Blum et al., 1992a,b] and as cross-linking decreased the degradation rate of collagen [Vater et al., 1979].

Different LO isoforms were purified from various cells and tissues, but only two related genes, LO and lysyl oxidase–like (LO-L), were identified [Trackman et al., 1990; Wu et al., 1992; Hämäläinen et al., 1993; Contente et al., 1993; Kenyon et al., 1993]. A high level of conservation was established between the murine, rat, and human LO and LO-L genes. Both LO and LO-L genes have a seven exon structure, with divergences mainly occuring at the exon I and VII levels. The transcription of LO gene allowed the synthesis of three mRNAs (5.5 kb, 4.5 kb, and 2.0 kb) by using alternate polyadenylation signals [Jourdan-Le Saux et al., 1994]. The 4.5 kb mRNA was the most abundant in myofibroblasts of fibrotic tissues, while the 5.5 kb

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mRNA was predominantly found in cultured myofibroblasts from the same origin, indicating a loss of regulation at the 3′ end part of LO gene in cell culture [Jourdan-Le Saux et al., 1994]. The LO mRNA encoded a 46.6 kD peptide processed as a 32 kDalton mature enzyme [Trackman et al., 1990], while the LO-L gene is transcribed as a 2.4 kb messenger [Kenyon et al., 1993]. On the other hand, the LO or a related gene product might have a tumor suppressor activity, as partial LO cDNA (*ras recision gene, rrg*) was picked up by subtraction between cDNA libraries from tumorigenic and nontumorigenic fibroblasts [Contente et al., 1990; Kenyon et al., 1991]. However, although the tumorigenic phenotype was clearly correlated to downregulation of the LO^{rrg}, the mechanism of LO in the oncogene suppression (*ras, erb2*) is unknown [Hajnal et al., 1995; Krzyzosiak et al., 1992].

LO was transiently expressed by myofibroblasts during the development of schistosomal liver granuloma [Sommer et al., 1993]. In this fibrosis model, LO appeared to be mainly synthesized with type I collagen. Thus, for study of LO expression, a cell model with a high steady-state synthesis of LO and cross-linked type I collagen fibers had to be set up. For this purpose, primary or secondary cultures of myofibroblasts from fibrotic liver were studied but discarded because they did not maintain a stable phenotype, and they did not express a high steady-state level of type I collagen mRNA and well-formed collagen fibres or maintain the ratio of the two main LO mRNAs as an indication of a correct regulation at the 3′ end level [Jourdan-Le Saux et al., 1994]. A spontaneous cell line derived from fibrotic liver myofibroblasts was also discarded because it exhibited downregulation of the LO gene [Jourdan-Le Saux et al., 1994], a situation also found in transformed cells [Kuivaniemi et al., 1986]. However, the characteristics reported by Lamandé and Bateman [1993] concerning the 3T6 fibroblast cell line exhibited important features for our study: 1) these cells deposited a well-organized extracellular matrix in vitro in presence of ascorbic acid, 2) they expressed mainly type I collagen, and 3) cross-linking of type I collagen was found in long term culture, using the LO-dependent pathway. Furthermore, the use of 3T6 fibroblasts for transfection has already been reported [Lamandé et al., 1993].

This study was undertaken to test the usefulness of 3T6 fibroblasts for studying LO expression in a cell model. Stable clones from the heterogeneous population of 3T6 fibroblasts were isolated. The first criteria used for selection was a myofibroblastic phenotype (a well-developed cytoskeleton positive for smooth muscle–specific proteins, the presence of cytoplasmic bundles of microfilaments [Sappino et al., 1990]), expecting that a steady expression of myofibroblast features would be correlated to a high LO expression level. The myofibroblast-like clones with the highest steady-state level of LO transcription were finally selected. Their expression of LO substrates (type I, type III, and type IV collagens and elastin) was determined, and the activity of the LO promoter was assessed. For this purpose, the cloning of 2 kb of the 5′ flanking region of the mouse LO promoter was undertaken, permitting the determination of the activity of different regions of the LO promoter in the 3T6 model. The regulatory sequences of the LO promoter were then compared to the equivalent regions of collagens and elastin promoters in order to raise a model for studying the coregulation of the enzyme with its substrates.

MATERIALS AND METHODS

Cell Culture

NIH 3T3 fibroblasts, 3T6 fibroblasts, human skin fibroblasts, and myofibroblasts from schistosome-infected mouse liver [Jourdan-Le Saux et al., 1994] were grown in Dulbecco's Eagle's medium (DMEM) Gibco-BRL, Cergy Pontoise, France) complemented with 10% fetal calf serum (FCS), penicillin (100 U/ml), and streptomycin (100 µg/ml). L-ascorbic acid (Sigma Chemical Company, St. Louis, MO) was added daily at 100 µM (20 µg/ml) or 250 µM (50 µg/ml). The LO-specific inhibitor β-APN (Sigma) was used at 50 µg/ml. Clones were generated from the 3T6 cell line by dilution plating [Reid et al., 1979].

Immunocytochemistry and Electron Microscopy

For immunofluorescence, fibroblasts grown for 48 h in DMEM supplemented with 10% FCS were incubated for 15 min at 37°C with 0.25% Triton X-100 and fixed for 15 min at 4°C with acetone. Monoclonal anti-α-smooth muscle actin antibody (Sigma) was used at dilution of 1:200. For standard electron microscopy, the

3T6 cell cultures were fixed for 10 min with 2% glutaraldehyde in culture medium, for 20 min with 2% glutaraldehyde–0.1 M Na cacodylate/HCl, pH 7.4, washed three times with 0.1 M Na cacodylate/HCl, postfixed in 1% OsO_4–0.15 M Na cacodylate/HCl for 30 min, dehydrated in graded ethanols, and embedded in epon. Ultrathin sections were contrasted with methanolic uranyl acetate and lead citrate. For immunoelectron microscopy, the 3T6 cell cultures were fixed for 30 min at 4°C with 4% paraformaldehyde in PBS, dehydrated in graded ethanols, and embedded in Lowicryl K4M. Ultrathin sections were first incubated with polyclonal antibovine type I collagen antibody (Institut Pasteur de Lyon, Lyon, France) diluted 1:10 in Tris buffer saline (TBS) and then with colloidal gold anti-rabbit IgG (BioCell, Cardiff, UK) diluted 1:25 in TBS, pH 8.2. The sections were contrasted with ruthenium red 1%, methanolic uranylacetate, and lead citrate.

Hybridization Procedure

Northern and Southern blotting were performed as previously described [Sambrook et al., 1989; Sommer et al., 1993] using Hybond N+ (Amersham, Les Ullis, France) or positively charged nylon membrane (Boehringer Mannheim, Meylan, France), respectively. RNAs were extracted from cultured cells [Chirgwin et al., 1979], electrophoresed in standard conditions, and transferred by capillarity. Hybridizations were achieved using as probes the 1.2 kb PstI fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the 0.42 kb BamHI-HincII fragment of the mouse LO cDNA [Sommer et al., 1993], the 1.5 kb EcoRI fragment of the human COL1A1 procollagen (pHF677; provided by Dr. Ramirez, Piscataway, NJ), the 0.4 kb HindIII-HincII fragment of the mouse COL3A1 collagen (pHFS3; provided by Dr. Vuorio, Turku, Finland), a 0.75 kb BamHI-HincII fragment of human COL4A1 collagen (pC4A1H; provided by Dr. Clement, Rennes, France), and the 0.55 kb EcoRI-SstI fragment of the human elastin (plasmid HDE-3, provided by Dr. Davidson, Nashville, TN). Purified fragments (50 ng) were labeled using the Random Priming labeling system (Boehringer) with α-^{32}P dCTP (Amersham) or with digoxigenin-dUTP (Boehringer). The DNA and RNA molecular weight markers were from Gibco-BRL.

Genomic Map

Two clones (pLO10 and pLO12) were selected from a mouse Balb/C genomic library (provided by Pr. Chambon, Strasbourg, France) as described [Jourdan-Le Saux et al., 1994]. The Balb/C LO genomic map was determined by restriction mapping, Southern blotting with different probes generated from the mouse LO cDNA, and partial sequencing of the two clones, using standard procedures [Sambrook et al., 1989]. Different subclones carried by pBluescript II SK+ (Stratagene, La Jolla, CA) were generated from pLO10 (pLO15, SalI subcloning, residues about −5 kb to +14 kb) and pLO12 (pLO14 and pLO20, SalI subcloning, residues −2,073 to about +10,000 and about +10,000 to +13,000 respectively; pLO24 and pLO23, SmaI-SalI subcloning, residues −2,073 to +1,040 and +1,708 to about +10,000, respectively). The sequence of the genomic fragment including the promoter LO region and the beginning of the first intron was determined. Sequencing was performed on both strands of various subclones of pLO10 and pLO12 covering the 2,073 bp before the initiation of transcription. The Sequenase 7-deaza-dGTP DNA Sequencing Kit (Amersham) was used with ^{35}S-dATP and the T3 and T7 sequencing primers. The sequence analysis and the consensus sequences for nuclear binding factors [Faisst et al., 1992] were analyzed using GeneWorks software (IntelliGenetics, Oxford Molecular, Oxford, United Kingdom).

Construction of Genelight Plasmids

The promoter region (positions −2,073 bp to +434) was transferred between the KpnI and BglII sites of a genelight plasmid (pGl2-basic; Promega Corporation, Madison, WI) upstream of the luciferase gene, resulting in the pPl1 plasmid. Serial deletions of the promoter region were obtained by using restriction mapping and Exonuclease III digestion on pPl1 with the Exonuclease III Digestion System (Promega). The full-length promoter or deletions were subcloned in pGl3-basic vector (Promega): pGl3 (−2,073), pGl3 (−964), pGl3 (−912), and pGl3 (−416). The constructs pGl2 (−416), pGl3 (−622), and pGl3 (−193) were generated by digestion of DNA fragments from pPl1 or pGl3 (−2,073) digested with EcoRV, EcoRI, and StyI, respectively and subcloning on the appropriate cloning sites of pGl2-basic and pGl3-basic vec-

tors. The putative enhancer or silencer elements were inserted in the SalI-BamHI sites of pGl3 (−416) downstream the promoter-luciferase unit.

Transient Transfection and Luciferase Assay

The cells were seeded in DMEM–10% FCS 15 h before transfection at a density of 4.10^5 cells, at the same P7 passage in 60 mm diameter plastic dishes. Transient transfections of 3T6-5 cells were performed using Lipofectamine reagent (Gibco-BRL). The cells were transfected with 6 µg of pGl2 or pGl3 DNA mixed with 2 µg of pRSV-β-galactosidase plasmid DNA (gift of A. Mauviel, Philadelphia, PA) in order to monitor the transfection efficiencies and with 2 µg of pBluescript II SK+. The cells were harvested 44 h after transfection, lysed in Promega's lysis buffer, and assayed for luciferase activity using the Luciferase Assay Kit (Promega). The reported value was correlated to the β-galactosidase activity measured using the Galacto-light PlusTM system (Tropix, Bedford, MA). The enzyme activities were monitored for 30 s on a luminometer (BCL, SEE Automation, Clermont-Ferrand, France).

Gel Mobility Shift Assay and DNAse Protection Assay

Nuclear proteins from 3T6-5 fibroblasts were extracted as described [Andrews and Faller, 1991], dialyzed for 2 h at 4°C against 25 mM HEPES buffer, pH 7.6, 0.1 mM EDTA, 40 mM KCl, 10% glycerol, and then stored at −70°C. Four DNA fragments spanning the regions −912 to −622, −970 to −784, −898 to −861, and −970 to −898 were used as probes or competitors. The sequences −970 to −784 and −970 to −898 were generated by polymerase chain reaction (PCR) using the Taq polymerase from Boehringer and subcloned on pBluescript II SK+, resulting in pPLO104 and pPLO108, respectively. The −912 to −622 sequence was digested from pGl2 (−912) and was subcloned on pSK+, resulting in pLO89. A 38 bp double-stranded oligomer (LOcol1 oligomer) was designed on the region −898 to −861 of the mouse LO promoter. The DNA probes were 3′ end-radiolabeled with α-^{32}P dATP (Amersham) using Klenow fragment enzyme (Boehringer) at the overhanging end created by linearization by EcoRI-HindIII digestion for pLO104 or by oligonucleotides match for LOcol1 oligomer. For gel shift mobility assays, the pLO104 insert (50,000 cpm) and LOcol1 oligomer (20,000 cpm) were incubated for 30 min at 4°C with 7 µg of nuclear protein extracts in 20 µl of binding reaction buffer (17.5 mM HEPES buffer, pH 7.6, 0.07 mM EDTA, 80 mM KCl, 5 mM $MgCl_2$, 0.7 mM DTT, 7% glycerol) supplemented with 2 µg of poly(dI-dC) · poly(dI-dC). The DNA-protein complexes were separed from unbound DNA fragment on 4.5% polyacrylamide (30:1) gels as described [Sambrook et al., 1989]. For the DNAse protection assays, the noncoding strands of pLO104 and pLO89 were used as probes and 3′ end-radiolabeled as described above. Labeled fragments were digested and purified on a 4.5% polyacrylamide gel. Different amounts of nuclear proteins were allowed to react for 30 min at 4°C with pLO89 (14,000 cpm) or pLO104 (28,000 cpm) in the binding reaction buffer supplemented with 4 µg of poly(dI-dC) · poly(dI-dC). Digestion of the nonprotected DNA was performed for 2 min at 20°C with 400 ng or 100 ng of DNAseI (Boehringer) when proteins were added or not, respectively. The DNAse reaction was stopped by incubating the DNA complexes for 45 min at 50°C with 60 µl of 0.17 M NaCl, 0.017 M EDTA, 0.83% SDS, 0.208 mg/ml tRNA, and 0.2 mg/ml proteinase K. The DNAs were loaded on a 6% sequencing gel, and the electrophoresis was carried out together with a Maxam-Gilbert G + A sequencing reaction performed on the equivalent DNA region [Sambrook et al., 1989].

RESULTS

Establishment of Myofibroblast-Like Clones of 3T6 Fibroblasts

Our first attempts to study the regulation of LO in a primary culture of myofibroblasts from schistosomiasis-infected liver were hindered by the very low growth rate and the phenotypic instability of such cultures (data not shown). As 3T6 fibroblast cells were known to deposit a cross-linked collagen matrix in culture, its usefulness for the LO study was investigated. Five clones, out of 96, were isolated from the original 3T6 heterogeneous cells, according to criteria currently used for characterizing myofibroblasts at the light microscopy level: elongated shape, full contact inhibition, and anchoring capabilities. The 3T6-2 and 3T6-5 clones were further characterized as they displayed the higher steady expression of LO mRNAs (Fig. 2a). These two clones were positive for α-smooth muscle actin (Fig. 1a) and vimentin (data not

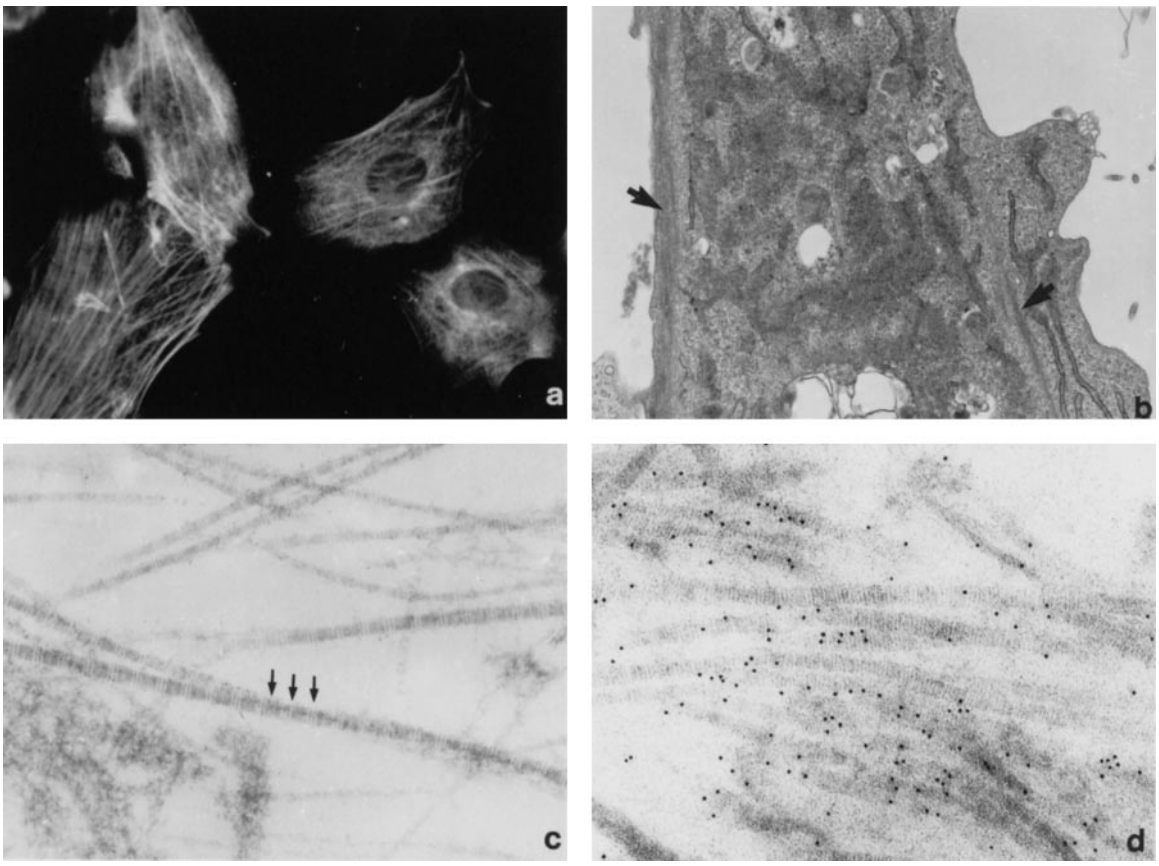

Fig. 1. Phenotype characterization of 3T6-5 fibroblasts. **a:** Immunofluorescent staining of α-smooth muscle actin. ×500. **b:** Ultrastructural localization of bundles of cytoskeletal filaments disposed beneath the cytoplasmic membrane and indicated with arrow. ×11,000. **c:** Collagen fibres staining with 67 nm periodic cross striation indicated with arrow. ×45,000. **d:** Type I collagen immunogold staining. ×50,000.

shown). They obviously differed from myofibroblasts by their immortalized status with inflated nuclei, but they exhibited the condensation of cytoskeletal microfilaments beneath the cytoplasmic membrane characteristic of myofibroblasts at the ultrastructural level (Fig. 1b).

Level of LO Steady-State Transcription by 3T6 Fibroblasts

The mRNAs from the 3T6 clones were hybridized with a LO probe covering the LO and LO-L conserved region (exons II to VI). The four clones expressed the 5.5 kb, 4.5 kb, and 2.0 kb mRNA transcribed by the LO gene. The 3T6-2 and 3T6-5 synthesized the highest amount of LO mRNAs, between two to five times more than the other clones (Fig. 2a) and much more than the human skin fibroblasts which displayed no LO/LO-L signal in the same conditions of autoradiography (Fig. 2b). The steady-state level of LO mRNAs in the 3T6-5 cells decreased when ascorbic acid was used at concentrations higher than 100 μM, a concentration which also decreased α1(I) collagen mRNA synthesis (Fig. 2b).

The 3T6-2 and 3T6-5 clones produced predominantly the 4.5 kb mRNA (Fig. 2a). The positive ratio of 4.5 kb vs. 5.5 kb mRNA transcription is characteristic of myofibroblasts in fibrotic tissue, while the 5.5 kb mRNA was relatively more abundant in the NIH-3T3 fibroblast and in primary culture of myofibroblast [Jourdan-Le Saux et al., 1994]. As this ratio was conserved in the 3T6 clones, it indicated that the use of different polyadenylation signals on the same transcript was correctly performed. On the other hand, the LO-L 2.4 kb mRNA was either not or poorly detected on the 3T6 clones.

Collagens and Elastin Synthesis by the 3T6 Clones

In presence of 250 μM ascorbic acid, the 3T6 clones deposited well-formed and 67 nm banded

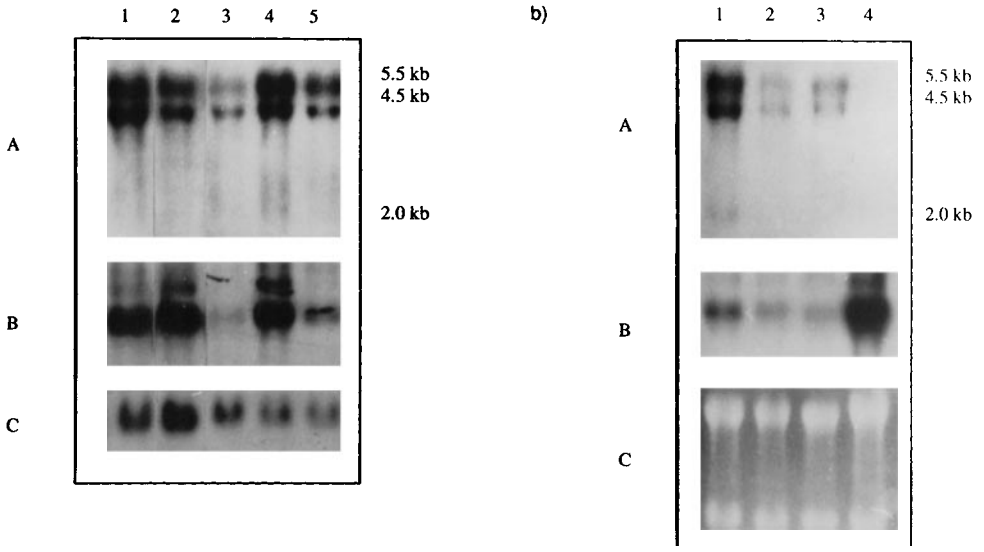

Fig. 2. Northern blotting analysis of pro α1(I) collagen and LO. **a:** Total RNAs (20 μg) were obtained from 3T6-2, 3T6-3, 3T6-4, 3T6-5, and 3T6-6 fibroblasts (lanes 1–5). The RNAs were hybridized with LO (*A*), human procollagen I (*B*), and rat GAPDH (*C*) probes. Autoradiographs were obtained after one day of exposure. **b:** Total RNAs (10 μg) were obtained from 3T6 fibroblasts (*lane 1:* 3T6-5; *lane 2:* 3T6-5 + 100 μM of ascorbic acid; *lane 3:* 3T6-5 + 250 μM of ascorbic acid) and from human skin fibroblasts (*lane 4*). The RNAs were hybridized with LO (*A*) and human procollagen I probe (*B*). *C* is the same gel stained with ethidium bromide. Autoradiographs were obtained after 1 day of exposure for procollagen I and 3 days for LO.

long collagen fibers (Fig. 1c) immunoreactive with anti-type I collagen antibody (Fig. 1d). This required LO activity, as its specific inhibition by β-APN resulted in a lack of deposition of type I collagen fibres (data not shown). The 3T6 clones produced a high steady-state level of messengers for α1(I) collagen, the higher amount being produced by the 3T6-5 clone. No fiber was observed in absence of ascorbic acid, while the steady-state level of type I collagen transcription was decreased by raising the concentration of ascorbic acid up to 100 μM (Fig. 2b). A small amount of α1(III) collagen transcript was observed in the different 3T6 clones, and the detection of type III collagen fibers by immunogold labeling was obtained only in long-term culture (more than 10 days) (data not shown). Messengers for elastin and type IV collagen, two other putative substrates of LO, were not detected. Thus, the characteristics of the 3T6-2 and 3T6-5 fibroblasts fulfilled the requirement for studying LO regulation: they exhibited the most important features of myofibroblast phenotype, they expressed a high level of LO and type I collagen mRNAs, and they synthesized in vitro an extracellular matrix mainly composed of type I collagen cross-linked by LO.

Cloning and Sequencing of the 5′ Flanking Region of the Mouse Balb/C LO Gene

Two clones were isolated from a genomic mouse library [Jourdan-Le Saux et al., 1994]. This Balb/C mouse LO gene appeared to be similar to the Swiss mouse LO gene (Fig. 3), with a seven exon structure, a long first exon, and a seventh exon encoding only the last amino acids of the LO peptide [Contente et al., 1993]. A major difference between both maps occurred within the fourth intron which appeared to be 4 kb longer in the Balb/C mouse gene than in the Swiss mouse gene. The significance of this intronic variation was not further investigated in this study.

The full-length sequence of the 2,073 bp upstream of the putative initiation of transcription is presented in Figure 4. The main and most upstream initiation of transcription in myofibroblasts from fibrotic tissue was used as +1 of transcription (position −392 before the AUG), although different starts were localized in myofibroblasts or NIH-3T3 fibroblasts (ranging from residues −392 to −270 before the ATG [Contente et al., 1993; Jourdan-Le Saux et al., 1994]). The −635 to +392 region was almost identical to the sequence of the Swiss mouse LO

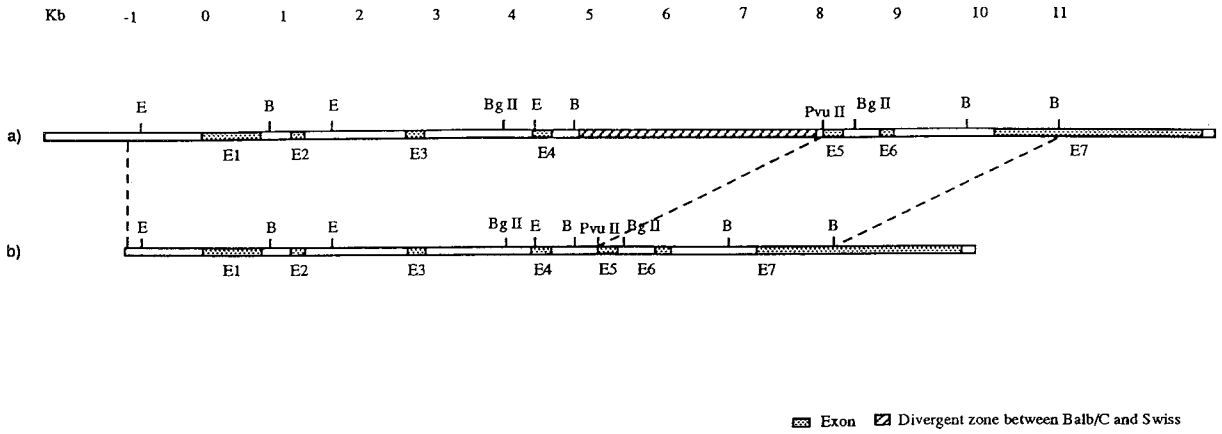

Fig. 3. Comparison of Balb/C and Swiss LO genes. **a:** The restriction map of Balb/C LO gene was determined on the pLO10 and pLO12 genomic clones. **b:** The restriction map of the Swiss LO gene was reported by Contente et al. [1993]. The restriction sites of the following enzymes were indicated: B, BamHI; Bg II, BgIII; E, EcoRI; Pvu II, Pvu II.

promoter [Contente et al., 1993]. A search for consensus sequences determined the presence of different potential binding sites for nuclear factors. TATA box and Initiator (Inr) consensus sequences were found, but their positions (−510 and −274, respectively) excluded them from a putative function in initiation. The consensus sequences for the recognition sites of AP1 and SP1 binding factors were also detected (positions −320 and −43, respectively). Around the initiation of transcription, the LO promoter displayed a number of sequences which have functional equivalents involved in the regulation of murine COL1A1 and COL1A2 promoters. For instance, the consensus sequence for the CCAAT factor binding was found at position +12, while SP1 binding sites or the consensus sequence for C-Krox A binding site were found at positions −43 and −33, respectively. More upstream, the motif $TC(X)_4GCCAA$ (position −1,534 to −1,523) could correspond to the transforming growth factor b responsive element (TbRE) described at position −1,623 to −1,610 on the rat COL1A1 promoter ($TGC(X)_5GCCAAG$) [Ritzenthaler et al., 1991] or at position −307 to −295 on the mouse COL1A2 promoter ($TCG(X)_5GCCAAG$) [Rossi et al., 1988]. The region −888 to −865 also displayed a significative similarity (79%) with the −1,525 to −1,502 sequence of the murine COL1A1 promoter.

Deletion Analysis of the Mouse LO Promoter

To identify the sequences determining the steady-state level of mouse LO transcription, we constructed a chimaeric gene containing the LO promoter fused to the luciferase gene. Differential deletions of the LO promoter were obtained using the exonuclease III strategy in pGl2-basic vector. The activities of some promoter regions were then checked in the more efficient pGl3-basic vector. The luciferase activities of the 3T6-5 fibroblasts transiently transfected by the different constructs are summarized in Figure 5. The highest luciferase activity was observed with both pGl2 and pGl3 constructs harboring LO promoter sequences deleted upstream the position −416. The transcriptional activity of pGl2 (−416) and pGl3 (−416) corresponded to 274% and 269% of the full-length promoter, respectively. A further deletion until position −193 induced a drastic decrease of the luciferase activity, indicating that the sequence located between −416 and −193 positively regulated the LO promoter. On the other hand, the regions −1,933 to −622 downregulated the LO promoter. The lowest activity was obtained with the pGl2 (−1,933) construct, while a large zone of inhibition was observed between −964 to −416. This latter region behaved differently according to the plasmid used: the region giving maximum inhibition was attributed to positions −912 to −548 in pGl2 and to −964 to −622 in pGl3. As similarities with the mouse COL1A1 promoter were found at positions −888 to −865, the sequence −970 to −784 was subcloned in an enhancer position on pGl3 (−416) in order to decipher if it should be considered as a negative regulator. This insertion resulted in a 50% decrease of the luciferase activity of pGl3 (−416), clearly demonstrating its negative effect on LO promoter

Fig. 4. Nucleotide sequence of the 5′ flanking region of the LO promoter. Sequencing was performed on pLO10 and pLO12 plasmid derivatives. The initiation of transcription at position −392 before the ATG was used as +1 and boxed. The consensus sequences for putative TATA and Inr recognition sites were also boxed. The restriction sites for some restriction enzymes and the position of some consensus binding factor sites are indicated with arrowheads. The sequence of high similarity with the COL1A1 promoter is underlined.

activity. We thus decided to determine whether or not the conserved motives were involved in nuclear factors binding.

Nuclear Factor Binding to the Inhibiting Region of the LO Promoter

In order to determine whether nuclear factors bound the negatively active −970 to −784 region of the LO promoter, DNase I footprinting experiments were carried out using crude nuclear extracts from 3T6-5 fibroblasts. A large protected area at approximately −890 to −836 was highlighted when the segments −912 to −622 was used as probe (Fig. 6a). The protection was observed around the positions −921 to −850 when the segment −970 to −784 was used as probe (data not shown). The overlapping sequence protected in both experiments spanned the region −888 to −865 (called LO-col1) which was similar to the region −1,525 to −1,502 of the murine COL1A1 promoter (Fig. 6b). Another protected area was observed down-

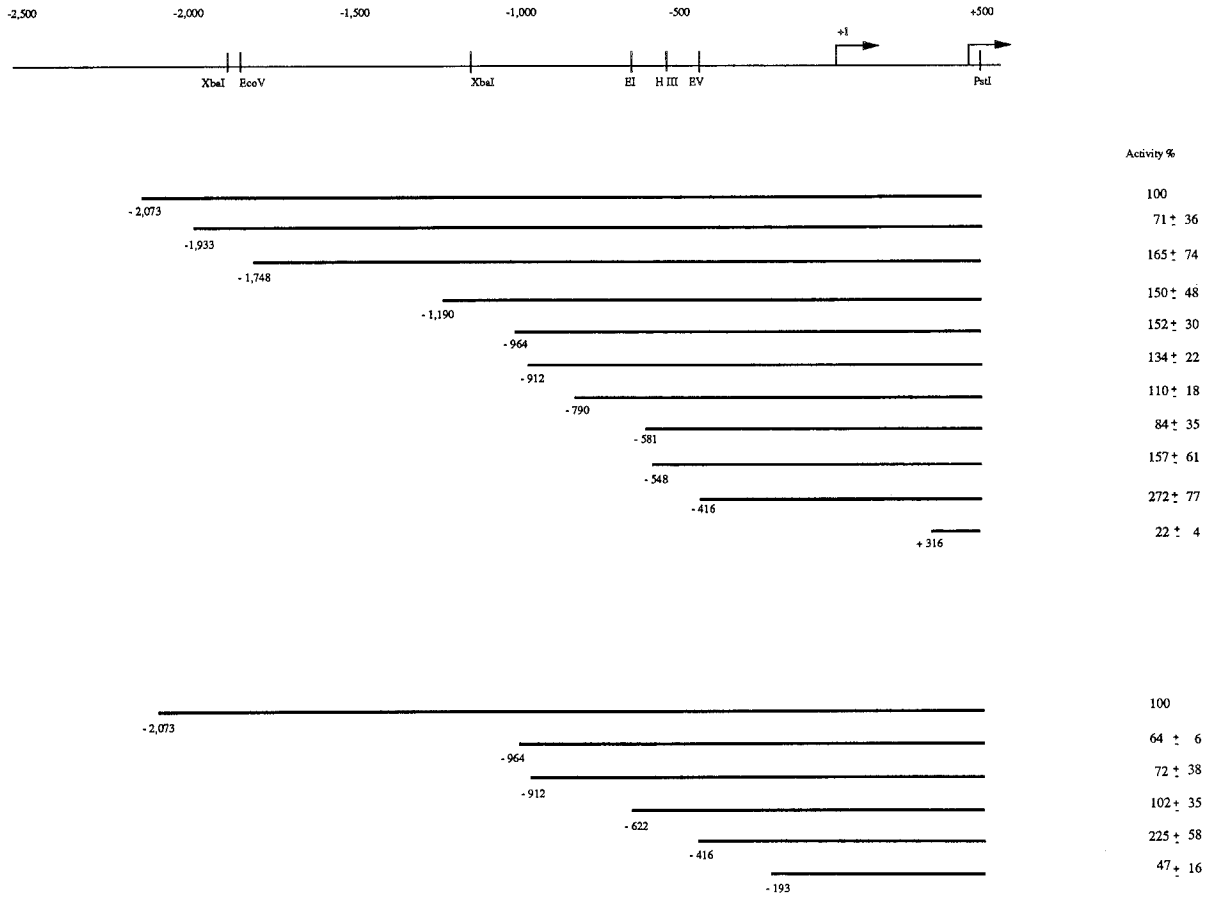

Fig. 5. Deletion analysis of LO promoter. A schematic linear map of LO promoter is represented with the relative position of the EcoRI, EcoRV, DraI, HindIII, NdeI, PstI, and XbaI restriction sites used for the generation of pGI constructs. The sizes were indicated in bp. The sites of initiation of transcription and translation are localized with arrows. The various lengths of promoter sequences were obtained by deletion of the −2,073 bp LO promoter coupled to the luciferase gene carried out by pGl2 (top) or pGl3 (bottom) basic vectors. 3T6-5 fibroblasts were transiently cotransfected with the pGl constructs and pRSV-β-galactosidase plasmid. Values of luciferase assays were corrected for β-galactosidase activity in the same extracts and were expressed as a percentage of activity relative to the luciferase activity of full-length promoter constructs. The average value (three to ten experiments) was given for each constructs.

stream of the LOcol1 sequence at positions and −827 to −774.

To further investigate the involvement of the LOcol1 segment in the binding of nuclear factors, gel mobility shift assays were performed. Six bands with retarded mobility (C1 to C6) were obtained when the −970 to −784 region was incubated with crude 3T6-5 nuclear extracts prior electrophoresis (Fig. 7a, lane 3). A 100-fold molar excess of the LOcol1 oligomer (−898 to −861) prevented the formation of the complexes C3 and C5 (Fig. 7a). This result demonstrated that the LOcol1 sequence directly bound nuclear factors from the 3T6-5 cells. This was further confirmed as two major and one minor bands with retarded mobility were obtained when the LOcol1 oligomer was used as probe (Fig. 7b). The large size of the protected area (−921 to −836) around the LOcol1 binding site and the formation of three complexes indicated the participation of numerous factors. Furthermore, the directly upstream −970 to −898 segment interfered with the formation of the C5 complex as preincubation of this segment with nuclear extracts partially decreased the amount of LOcol1–nuclear factors C5 complex. However, at least a 200-fold excess was required, indicating that the competitive fixation of this surrounding sequence might be indirect.

DISCUSSION

The purpose of this study was to characterize the molecular mechanisms of LO regulation. A

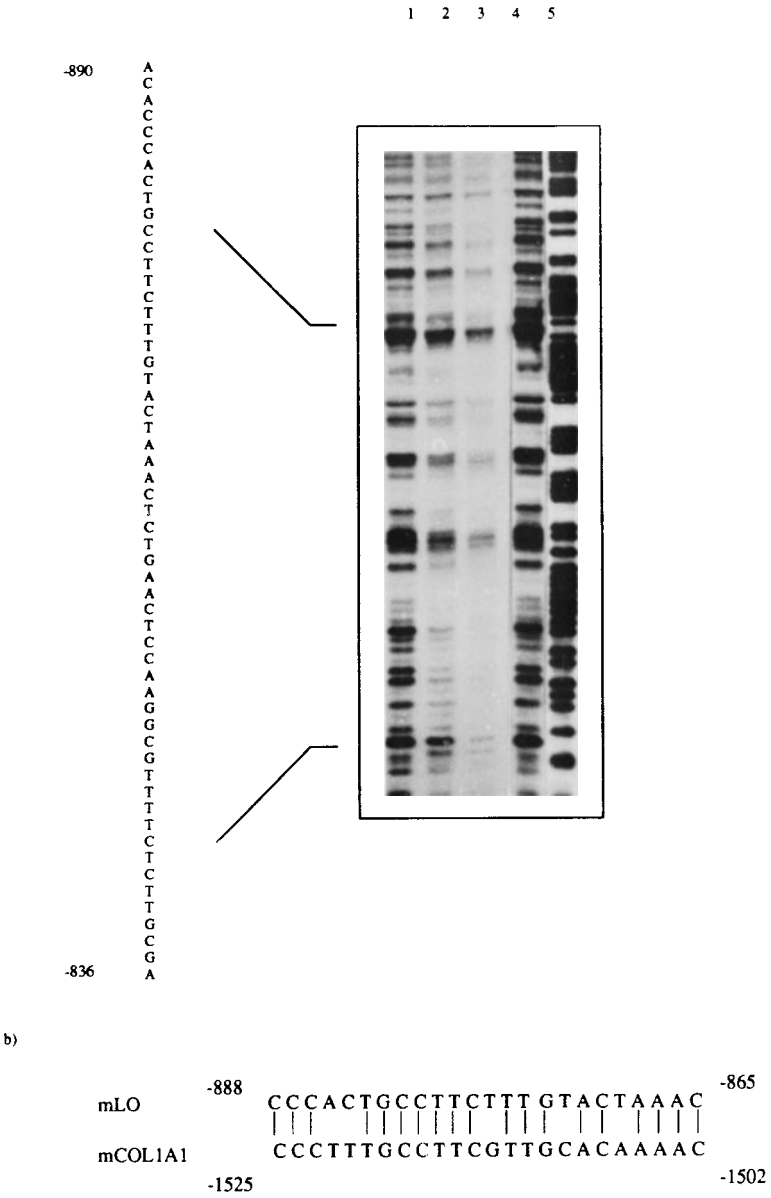

Fig. 6. DNAse footprinting of noncoding strand of pLO89. **a:** pLO89 fragment (−912 to −622) labeled at the 3′ end of the noncoding strand was incubated without (*lanes 1, 4*) or with different amount of crude nuclear extract of 3T6-5 fibroblast (*lane 2:* 3 µg; *lane 3:* 6 µg) and was treated with DNAse I before electrophoresis. The electrophoretic migration of pLO89 probe submitted to G + A chemical cleavage was used as marker (*lane 5*). The sequence of the protected region (−890 to −836) was indicated on the left. **b:** Sequences similarity between the LOcol1 sequence of mouse LO (−888 to −865) and murine Col1A1 (−1,525 to −1,502) promoters.

convenient model of myofibroblast was first established, as this cell is one of the main active cells in fibrosis and its role in LO expression was previously demonstrated [Sommer et al., 1993; Konishi et al., 1985; Jourdan-Le Saux et al., 1994]. The origin of myofibroblasts is still debated, and their heterogeneity reflects either different origins or different stages of differentiation [Schmitt-Gräff et al., 1994; Greenwel et al., 1993; Ronnov-Jessen et al., 1995]. This heterogeneity and the variable stability of myofibroblasts phenotype [Desmoulière et al., 1992; Kirk et al., 1995] are obviously limiting factors for their study. Nevertheless, a well-developed cytoskeleton displaying smooth muscle–specific proteins, the presence of bundles of filaments beneath the cytoplasmic membranes, and the synthesis of collagen fibers are used for

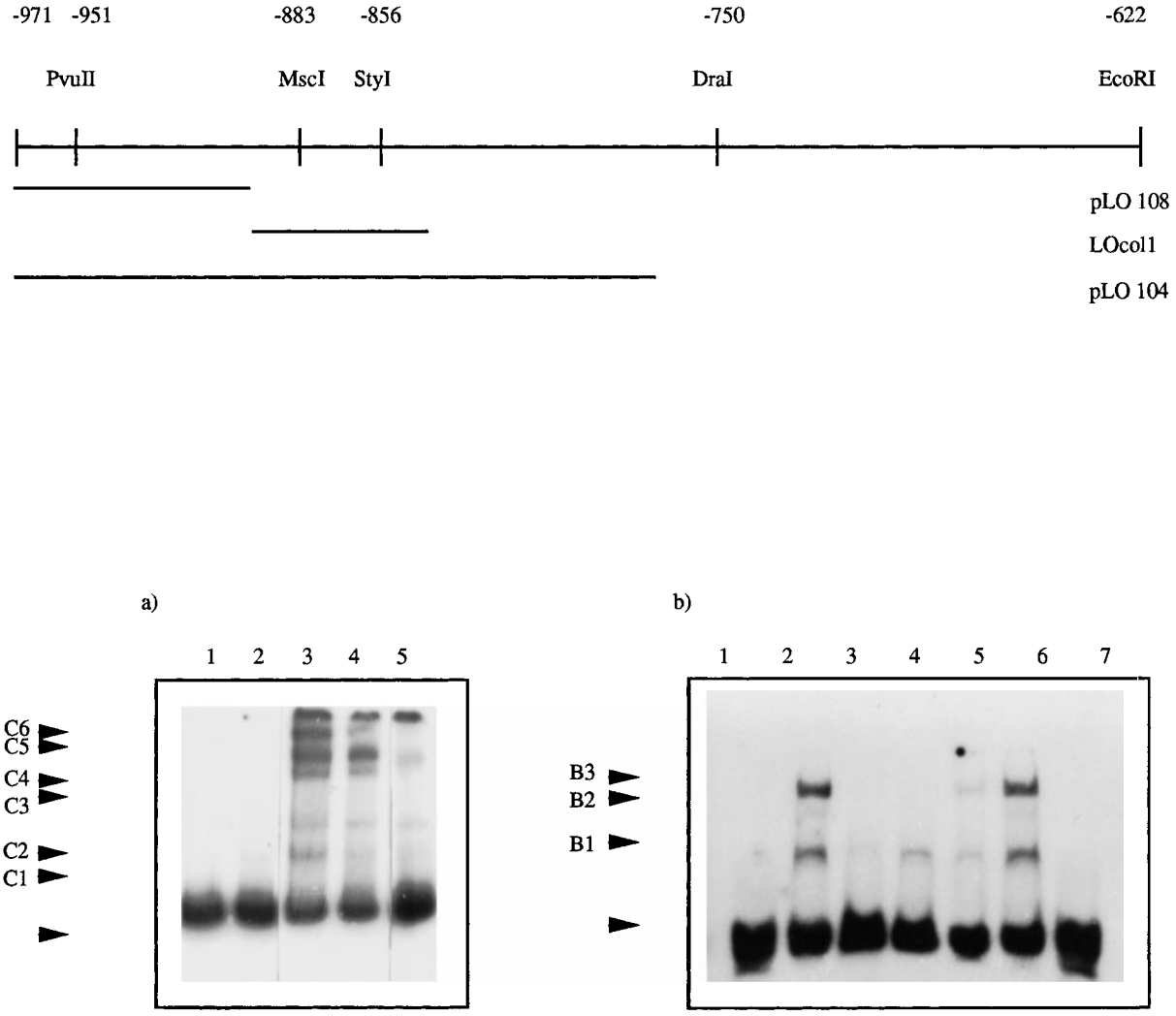

Fig. 7. Binding studies of LO promoter and protein extract from 3T6-5 fibroblasts. The upper schematic map represents the LO promoter regions (sequences −970 to −898 from pLO 108, −898 to −861 from LOcol1, and −970 to −784 from pLO104) and the sites of the PvuII, MscI, StyI, DraI, and EcoRI restriction enzyme. **a:** DNA binding was performed on pLO104 with 7 µg of crude proteins without (*lane 3*) or with competitions using a 100-fold molar excess of unlabeled pLO104 (*lane 2*), a 200-fold molar excess of the −970 to −784 DNA segment (pLO 108) (*lane 4*), and a 100-fold molar excess of LOcol1 oligomer (*lane 5*). The migration of the probe is shown in *lane 1.* **b:** Binding studies on the LOcol1 oligomer were performed with 7 µg of crude proteins without (*lanes 2, 6*) or with competitions using a 100-fold molar excess of unlabeled LOcol1 oligomer (*lane 3*), a 100-fold molar excess of pLO104 (*lane 4*), and a 200-fold molar excess of pLO108 (*lane 5*). The migration of the probe is shown in *lanes 1, 7.* Arrowheads indicate the position of migration of DNA-proteins complexes with retarded mobility.

determining the myofibroblast phenotype [Sappino et al., 1990; Kirk et al., 1995; Armendariz-Borunda et al., 1994]. All these properties were displayed by the 3T6-2 and 3T6-5 clones. They expressed a relatively high steady-state level of LO mRNAs and maintained the right posttranscriptional regulations as judged by the positive ratio of 4.5 kb mRNA vs. the 5.5 kb mRNA [Jourdan-Le Saux et al., 1994]. With respect to these parameters, the 3T6 clones fullfilled the characteristics needed for studying LO regulation, with the convenience that they are easily transfectable.

One feature emerging from the observation of LO is concerned with the coexpression of the enzyme with type I collagen. In this study, the myofibroblastic cells expressing the higher amount of LO also synthesized the larger amount of type I collagen, with low type III collagen and neither type IV collagen nor elastin synthesis. Such LO and type I collagen coexpression was also observed in myofibro-

blasts during the development of schistosomal liver granulomas [Sommer et al., 1993]. This icoregulation seemed to be specific to myofibroblasts, or at least some myofibroblasts, as some 3T6 clones and human skin fibroblasts expressed a high level of type I collagen but a low amount of LO (Fig. 2), and as acid ascorbic-induced downregulation of both LO and type I collagen was reported only in myofibroblasts (this study) but not in smooth muscle cells cultures or in nonfibrotic tissues [Faris et al., 1984; Quaglino et al., 1991]. However, there was a difference between the LO and the COL1 transcription. The COL1 promoters are known to be powerful promoters in numerous cells, in contrast to the LO promoter. The absence of a true TATA box and Inr box [O'Shea-Greenfield et al., 1992] on the LO gene might explain this difference, as almost all collagen genes are true TATA box genes. This difference is unexpected, as TATA boxes are displayed by other genes encoding enzymes of collagen postmaturation, like the prolyl hydroxylase [Tasanen et al., 1988]. The lack of a TATA box should thus control the level of LO and COL1 gene expression, while common factors qualitatively coordinate their expression. Indeed, the LO sequence around the initiation of transcription showed a significative concentration of motives equivalent to cis-acting elements which are involved in the coordinated regulation of the COL1A1 and COL1A2 genes. For instance, the CCAAT binding factor [Maity et al., 1988] activating the collagen promoters (position −100 of COL1A1, −34 of COL1A2) might recognize a putative binding site at position +12 of the LO promoter. The C-Krox A factor [Galéra et al., 1994], a transcriptional inhibitor of murine collagen (positions −190 and −146 for COL1A1 and −165 of COL1A2), might recognize GGGAGGG sequences at position −33 of the LO promoter. The C-Krox B factor [Karsenty and De Crombrugghe, 1990], an other inhibitor of murine collagen (positions −146 of COL1A1 and −165 of COL1A2) might be able to bind related consensus sequences at positions −59 of the LO promoter, and the SP1-like factor [Tamaki et al., 1995] involved in the inhibitory regulation of COL1 promoters might recognize the GGGCGGG sequence at positions −43 of LO promoter. The nonrandom distribution of these sequences around the initiation of transcription of the LO promoter argues in favor of a role of some of these COL1 regulators.

This work established that the steady-state level of LO transcription in the 3T6-5 myofibroblast resulted from a balance between positive and negative regulations. The sequence −416 to −193 maintained a positive regulation, while the more upstream part of the LO promoter acted negatively. One segment, the LOcol1 cis-element at position −888 to −865, was further examined because it presented a striking similarity to a highly conserved sequence of the COL1A1 promoter at positions −1,525/−1,502, −1662/−1639, and −1623/−1610 of the mouse, human, and rat COL1A1 promoters, respectively [Jimenez et al., 1994; Ritzenthaler et al., 1991]. The activity of the COL1A1 sequences was not studied, but they were close to the minimum sequences conferring either the high level osteoblast expression of human COL1A1 [Rossert et al., 1996] or the TGFβ responsiveness of the rat COL1A1 promoter [Ritzenthaler et al., 1991]. There are no equivalents to these minimum sequences in the vicinity of the LOcol1 box on the LO promoter. Only a consensus binding site for the specific COLF1 factor [Hamatochi et al., 1988] was found at position −962 to −941 of the LO promoter. The activity of the COLF1 factor is still unknown, but its binding activity on the COL1A2 sequence was considerably decreased in transformed NIH-3T3 compared to the level of nontransformed NIH-3T3. This putative binding site in the −964 to −898 segment of the LO promoter participated to the formation of a large and multifactorial DNA-protein complex with the downstream −898 to −861 sequence. The actual role of each cis-element on the LO regulation was not determined, but the binding of nuclear factors to the whole −964 to −784 region strongly inhibited the LO promoter, either in an upstream position or at a distance from the pGl3(−416) promoter–luciferase unit. Further studies will be required to understand the mechanisms of the LOcol1 binding factors and to identify the nuclear factors triggering the positive regulation of the LO promoter. It will be of particular interest to determine how the balance between activation and inhibition could be modified during the process of myofibroblast differentiation. During the fibrosis development, the steady-state level of LO mRNAs produced by myofibroblasts is increased more than five times [Sommer et al., 1993]. However, the differentiation process of resident mesenchymal cells toward myofibroblasts leads to heterogeneous cells

which differ from their expression level for TGFβ, type I collagen, and AP-1 binding proteins. This is a multistep process involving paracrine and autocrine stimulation by TGFβ and a concomitant regulation of AP-1 binding proteins [Armendariaz-Borunda et al., 1994; Inagaki et al., 1995; Bachem et al., 1992]. The 3T6 clones should provide a convenient standard for comparing the expression of the LO regulating nuclear factors during the process of myofibroblast differentiation.

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