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# Lysyl Oxidase Activates the Transcription Activity of Human Collagene III Promoter

## POSSIBLE INVOLVEMENT OF Ku ANTIGEN*

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**Monia Giampuzzi‡, Gerardo Botti‡, Marco Di Duca‡, Lorenza Arata‡, GianMarco Ghiggeri‡, Rosanna Gusmano§, Roberto Ravazzolo¶, and Armando Di Donato‡‖**

*From the ‡Department of Nephrology and §Fondo Malattie Renali del Bambino, Gaslini Children's Hospital and ¶Laboratory of Molecular Genetics, Gaslini Children's Hospital, Genova and Department of Oncology, Biology and Genetics, University of Genova, 16147 Genova, Italy*

**Lysyl oxidase is an extracellular enzyme that controls the maturation of collagen and elastin. Lysyl oxidase and collagen III often show similar expression patterns in fibrotic tissues. Therefore, we investigated the influence of lysyl oxidase overexpression on the promoter activity of human COL3A1 gene. Our results showed that when COS-7 cells overexpressed the mature form of lysyl oxidase, the activity of the human COL3A1 promoter was increased up to an average of 12 times when tested by luciferase reporter assay. The effect was specific, because other promoters were not affected. Moreover, lysyl oxidase effect was abolished by β-aminopropionitrile, a specific inhibitor of its catalytic activity. Electrophoretic mobility shift assay showed a binding activity in the region from −101 to −77 that was significantly increased by lysyl oxidase overexpression. The binding was specifically competed by the cold probe, and the mutagenesis of this region abolished both the binding activity in gel retardation and lysyl oxidase stimulation of COL3A1 promoter in transfection experiments. We identified the binding activity as Ku antigen in its two components: Ku80 and Ku70. This study suggests a new coordinated mechanism by which lysyl oxidase might control the development of fibrosis.**

Lysyl oxidase (LOX)[1] (protein-6-oxidase; EC 1.4.3.13) is the key enzyme that controls collagen and elastin maturation (1, 2). It catalyzes the oxidative deamination of peptidyl-lysine and hydroxylysine to peptidyl-α-aminoadipic-δ-semialdehyde into elastin and collagen chains. The consequent aldheydes lead to a spontaneous condensation forming inter- and intra-chain cross-links into these important extracellular matrix (ECM) components. This post-translational modification of the ECM molecules seems to have a very important role both for collagen and elastin structural aspects and likely triggers some still unknown signal transduction pathways. Because of its action on ECM, deficit of normal LOX expression has been described in many polygenic and monogenic disorders such as atherosclerosis, type IX Ehlers-Danlos syndrome, and Menkes disease (3, 4). On the other hand, an enhanced level of LOX as well seems to be involved in human pathology. Several reports have recently suggested a clear association between organ fibrosis and increased LOX activity. This has been described in several chronic human liver diseases (5), in rat experimental hepatic fibrosis ($CCl_4$) (6), in idiopathic and experimental lung fibrosis (7), in adriamycin-induced kidney fibrosis in rat (8), and in other pathologies resulting in fibrosis (9–13).

Another aspect regarding LOX activity refers to its putative cell phenotype control and/or tumor suppressor activity. In many naturally occurring and oncogene-induced tumors and cellular models, LOX is down-regulated, whereas on the other hand is one of the main genes induced in concomitance with the reversion process (14–18). In particular, it seems that LOX is down-regulated in cells transformed by *ras* or *ras*-related oncogenes, so that it was first identified as a "*ras* recision gene" (rrg) (14, 15, 17). Moreover, LOX expression and activity are proved to be regulated by several growth factors, including transforming growth factor-β1, interleukin-1, prostaglandins, insulin-like growth factor 1 (19–25), and even the well defined tumor suppressor interferon responsive factor-1 (26), which puts this protein in a critical pathway for cell growth and phenotype control. Despite all these intriguing findings, there are no hypotheses so far about the mechanisms through which LOX might actually work as a tumor suppressor.

The localization of the enzyme is mainly extracellular, where its acknowledged substrates are located. Yet, this does not imply that regulation of ECM cross-linking is the only biochemical role of LOX. It is not known whether LOX has substrates other than collagen and elastin, although it has been proved that *in vitro* LOX can catalyze the oxidative deamination in several peptides and complex proteins (27, 28). Recently, it has been confirmed that processed LOX is localized intracellularly and inside the nucleus as well. Therefore, LOX might have an intracellular substrate(s), which would mediate its ability to control the cell phenotype (29, 30).

Considering the critical role of collagen III in organ fibrosis (31–34) and that its expression level changes are often preceded or paralleled by similar modifications of LOX activity (17, 35, 36), we investigated the effects of LOX overexpression on the activity of human collagen III α1 (COL3A1) promoter. To address this issue we used a reporter gene approach, transfecting COL3A1 promoter cloned upstream of luciferase gene into COS-7 cells. The influence of LOX expression was evaluated comparing the same cells co-transfected with the expression vector alone or carrying LOX coding sequence for the mature

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‖ To whom correspondence should be addressed: Laboratory of Nephrology, Istituto G. Gaslini, Largo G. Gaslini, 5, 16147 Genova, Italy. Tel.: 39-010-380742; Fax: 39-010-395214; E-mail: a-dido@usa.net.

[1] The abbreviations used are: LOX, lysyl oxidase; ECM, extracellular matrix; PCR, polymerase chain reaction; RT, reverse transcriptase; EMSA, electrophoresis mobility shift assay; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; MMTV, mammary mouse tumor virus; DNA-PK, DNA-dependent protein kinase.

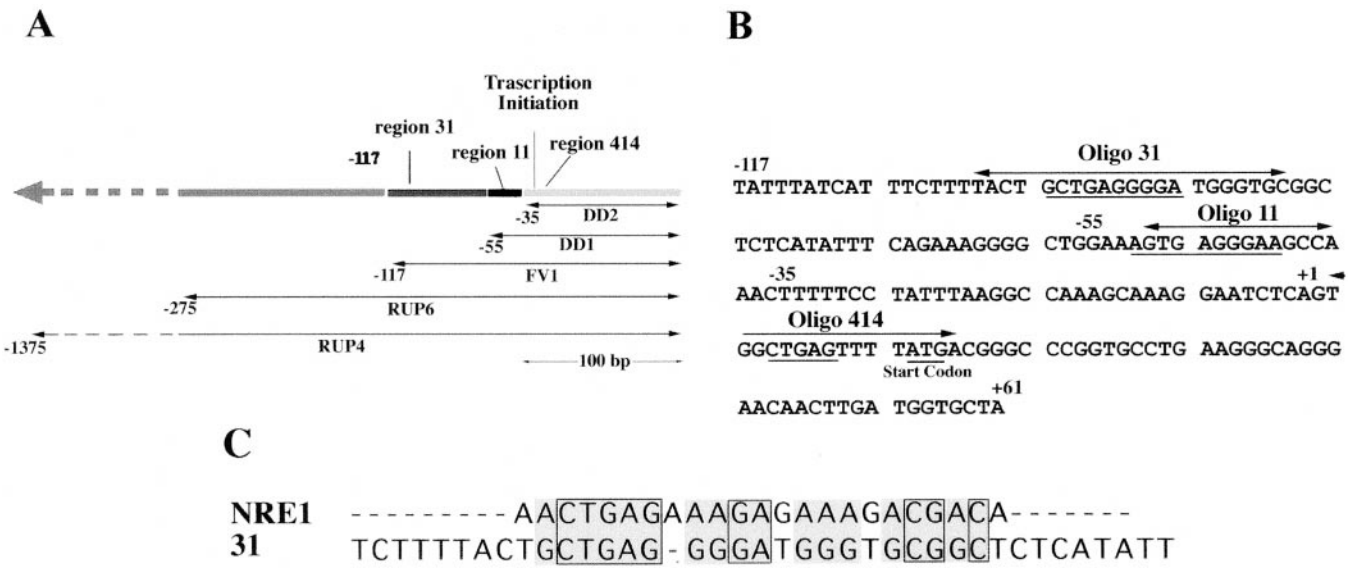


FIG. 1. *A*, the scheme shows the different fragments of COL3A1 promoter subcloned into pGL2-basic vector. *B*, FV1 fragment of Col3A1 promoter. The homology among oligonucleotides 31, 11, and 414 is *underlined*. *C*, alignment between NRE1 (nuclear regulatory element of the long terminal repeat of MMTV) and oligonucleotide 31.

32-kDa protein (30, 37, 38). Our results showed a dramatic increase in COL3A1 promoter activity when the recombinant LOX was overexpressed. Furthermore, we defined the region of COL3A1 promoter most likely involved in this LOX-dependent activation and some of the possible mechanisms involved.

## MATERIALS AND METHODS

*Cell Culture*—Monkey renal fibroblast COS-7 cell line was obtained from American Type Culture Collection (Manassas, VA) and was grown under humidified atmosphere of 5% $CO_2$ at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 1% glutamine, 100 $\mu$g/ml streptomycin, 100 units/ml penicillin. Human skin fibroblasts were a primary culture and grown in the same conditions as described above for COS-7 cells.

*Expression Vectors*—The different fragments of COL3A1 promoter described in the text were derived from the IdF8 clone kindly provided by Dr. F. Ramirez (Mt. Sinai School of Medicine, New York, NY) both by endonuclease restriction and PCR amplification. The fragments were then subcloned into pGL2-basic vector (Promega Inc., Madison, WI) upstream the luciferase reporter gene (39). The construct pFV1 was mutated into pFV1m by PCR, using overlapping primers containing the mutated nucleotide sequence. The mutation, encompassing the region from −97 to −75 in the COL3A1 promoter insert, transformed the original sequence 5′-GCT GAG GGG ATG GGT GCG-3′ into 5′-CAA TCT CCT GCC TAG ATT-3′.

Lysyl oxidase coding sequence for the mature 32-kDa protein, obtained by PCR, was previously cloned into pTrc-His vector (30). The fragment *Nco*I-*Bgl*II including the poly-His tag with LOX coding sequence was subcloned into pSG5 vector (kind gift of Dr. D. Guerini, Swiss Federal Institute of Technology, Zurich, Switzerland), using a *Nco*I-*Eco*RI adapter. All sequences were checked by restriction analysis and DNA sequencing (40).

*Transient Transfections*—COS-7 cells were transfected with 4 $\mu$g of the indicated plasmids by DEAE-dextran method (41). Briefly, for a 100-mm Petri dish 1–4 $\mu$g of each supercoiled plasmid resuspended in 1.2 ml of Dulbecco's modified Eagle's medium supplemented with 10% of NU-Serum™, 1% glutamine, 100 $\mu$g/ml streptomycin, 100 units/ml penicillin was added to the cells, previously washed with phosphate-buffered saline. An equal volume of the above medium buffered with 10 mM HEPES, pH 7.15, containing 1 mg/ml DEAE-dextran was then added to the dish. The cells were incubated for 30 min at the usual growth conditions, and after the addition of 6 ml of the above medium were incubated for 3 h more. To improve the efficiency of the transfection, the medium added for the second incubation contained also 100 $\mu$M chloroquine, which inhibits the lysosomal degradation of the DNA. The incubation was ended by aspirating the DNA/DEAE-dextran solution and washing the cells for 3 min with phosphate-buffered saline containing 10% of dimethyl sulfoxide. The cells were then incubated for 48 h with its normal medium and at the normal growth conditions, before being processed for luciferase assay. To normalize each experiment for transfection efficiency, 1–2 $\mu$g of pCMV-lacZ expressing the $\beta$-galactosidase enzyme was always co-transfected.

*Luciferase Assay Luciferase Assay*—Luciferase activity was determined by measuring luminescence in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA), according to the luciferase assay kit (Promega) directions. The results were normalized on the basis of $\beta$-galactosidase activity, which was assayed by spectrophotometric conversion of resorufin-$\beta$-galactopiranoside (Sigma) at 572 nm.

*Nuclear Extracts and Cellular Fractionation*—The cells, previously washed with phosphate-buffered saline, were collected by scraping with a rubber policeman. The cells were pelleted at 600 × *g* for 10 min and resuspended in 200 $\mu$l/100 mm plate of 20 mM HEPES, pH 7.9, 1 mM EDTA, 1 mM dithiotreitol, 0.5 mM phenylmethylsulfonyl fluoride, 1 $\mu$g/ml each of leupeptin, pepstatin, and aprotinin, 1 mM sodium vanadate, 10 mM sodium fluoride and kept at 4 °C for 15 min. The cell suspension was then added with ⅕ volume of 1% Nonidet P-40 to obtain a final concentration of 0.2% Nonidet P-40 and incubated at 4 °C for 15 min. The cell lysate was then centrifuged at 700 × *g* for 15 min. The supernatant (S1) was saved for further processing, whereas the pellet (P1), mostly containing the unbroken nuclei, was resuspended in the previous buffer, added with 0.2% Nonidet P-40, 0.4 M NaCl, 10% glycerol and incubated at 4 °C for 15 min. P1 was centrifuged at 20,000 × *g* for 30 min. The supernatant fraction (S2) was collected and used as nuclear extract for gel retardation assay. S1 was then centrifuged at 20,000 × *g* for 1 h, to obtain a partially purified cytosolic (S3) fraction. The protein concentration was determined using a classical Coomassie Blue-based assay (42).

*Electrophoresis Mobility Shift Assay*—The assay was performed as described previously (43), using 2–5 $\mu$g of nuclear extract proteins in a total volume of 20 $\mu$l containing 20 mM Tris-HCl, pH 7.5, 0.1 M NaCl, 0.35 mM dithiotreitol, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 10% glycerol, and 0.5 $\mu$l of 2 mg/ml of double-stranded poly(dI-dC) (1 $\mu$g/reaction). Where indicated the poly(dI-dC) was substituted with 5 $\mu$g/reaction of calf thymus DNA (Sigma), heat denatured at 95 °C for 5 min, and then rapidly cooled on ice. The sequences of the oligonucleotide used were the following: 31, 5′-TAC TGC TGA GGG GAT GGG TGC GGC-3′; 11, 5′-AGG GGC TGG AAA GTG AGG GAA GCC A-3′; and 414, 5′-TGG CTG AGT TTT ATG ACG-3′. All oligonucleotides employed were labeled at their 5′ ends with [$\gamma$-$^{32}$P]ATP (PerkinElmer Life Sciences) using T4 polynucleotide kinase. The binding to the double-stranded oligonucleotides corresponding to the different regions of COL3A1 promoter was performed by incubation at 4 °C for 30 min with 10 ftmol of $^{32}$P-labeled probes with the previous nuclear extract mix. The DNA-protein complexes were separated by electrophoresis on a 5% polyacrylamide gel and detected by autoradiography of the dried gel. Competitions were performed by the addition of 100–300-fold molar excess of unlabeled double-stranded oligonucleotide competitor to the incubation mixture. Where indicated, the nuclear extracts were prein-

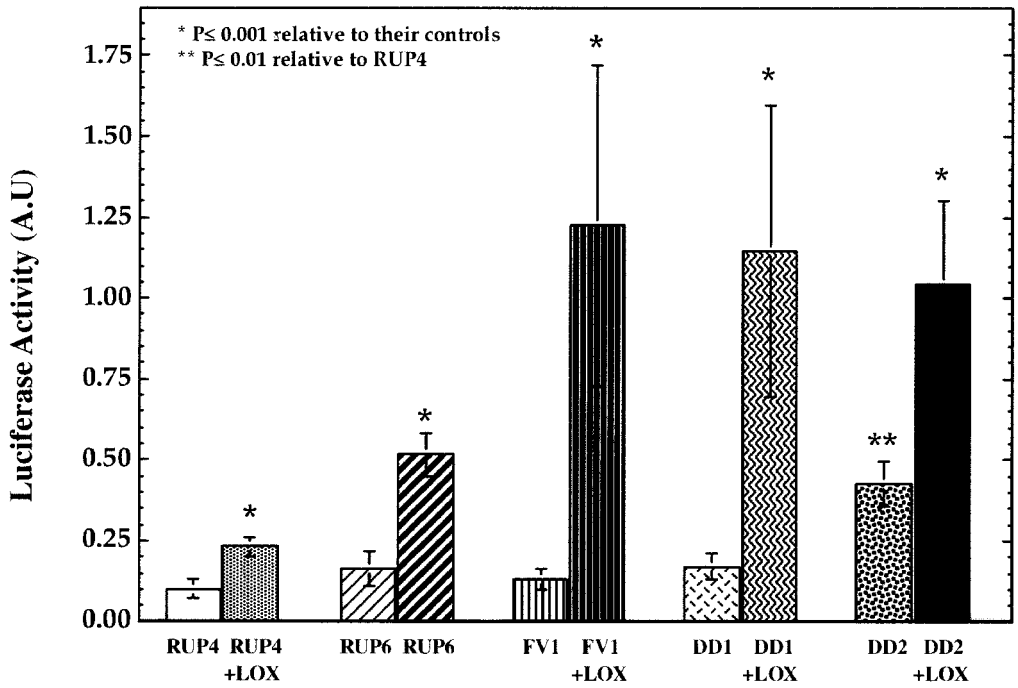


FIG. 2. **Luciferase activity of the indicated pGL2 constructs carrying different fragments of COL3A1 promoter in the absence or presence of recombinant LOX co-expression.** Luciferase activity is expressed as arbitrary units (*A.U.*). The results are the averages of at the least three independent experiments performed in triplicate ± S.E. The significance of the difference between the activities in presence of LOX expression and their relative controls has been evaluated by *t* test analysis.

cubated with the specified antibody for 30 min at 4 °C and then used for the gel retardation assay. The antibodies were the same used in Western blot analysis.

*Microcircle Construction*—To introduce the oligonucleotide 31 in a circular DNA structure, it was modified, adding at its ends *Xba*I and *Pst*I restriction sites and subsequentely subcloned into pSK$^+$ plasmid digested in the corresponding restriction sites. After selection of the pSK$^+$ clones carrying the oligonucleotide 31 insert, the plasmid was cut in *Eco*RI and *Pvu*II restriction sites external to oligonucleotide 31, and the released fragment was purified from 3% agarose gel. Then the fragment was circularized by T4 ligation, producing a 280-bp circularized DNA molecule (44). The circular product was purified again from agarose gel after digestion with Bal31 to remove the remaining linear DNA. The obtained microcircles were used to compete the binding of Ku to the oligonucleotide 31. Microcircles obtained from wild type pSK$^+$ (without oligonucleotide 31) were used as negative control.

*Protein Analysis*—Approximately 30 $\mu$g of proteins from the indicated cell fractions were separated by SDS-PAGE on a 10% gel (45) and blotted to a Hybond-Super C nitrocellulose membrane (Amersham Pharmacia Biotech). The blots were probed with Omni-Probe/M21, an anti-(His)6 tag polyclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA) to detect recombinant LOX. To detect Ku80 and Ku70 in the indicated cell extracts, anti-Ku80 polyclonal (M-20) and anti-Ku70 polyclonal (C-19) antibodies (Santa Cruz Biotechnology Inc.) were used. The blots were developed using an alkaline phosphatase-conjugated species-specific anti-immunoglobulin antibody.

*RT-PCR Analysis*—Total RNA was extracted (RNA Fast, Molecular Systems, San Diego, CA) from about 1–2 million subconfluent human skin fibroblast cells after 2 days from the transfection of pSG5 or pSG5-LOX vector. 1 $\mu$g of total RNA was reverse-transcribed by Maloney murine leukemia virus reverse transcriptase, using random hexamers as primers (cDNA 1$^{st}$ Strand synthesis kit, CLONTECH, Palo Alto, CA). The resulting cDNA was diluted to a ratio of 1:5 and used as template for the following PCR. The PCR was performed in 10 mM Tris-HCl, pH 8.2, containing 1.5 mM $MgCl_2$, 50 mM KCl, 1 $\mu$M of each primer, 200 $\mu$M dNTP, 5% $Me_2SO$, and 1.5 units of *Taq* Polymerase reactions (*Taq* DNA Polymerase from Termus Aquaticus strain YT1; Promega). The primers used to amplify Col3A1 messenger were: forward, 5′-CTG AAA TTC TGC CAT CCT GAG-3′; reverse, 5′-CCA AGG TCC ACA CCA AAT TC-3′. The PCR conditions used to amplify the Col3A1 were: preincubation for 5 min at 95 °C and then 24 cycles of 1 min at 95 °C, 30 s at 57 °C, and 1 min at 72 °C, followed by a final step of 5 min at 72 °C. The expected product was of 590 bp long. Parallel PCR reactions were performed to amplify the housekeeping gene G3PDH

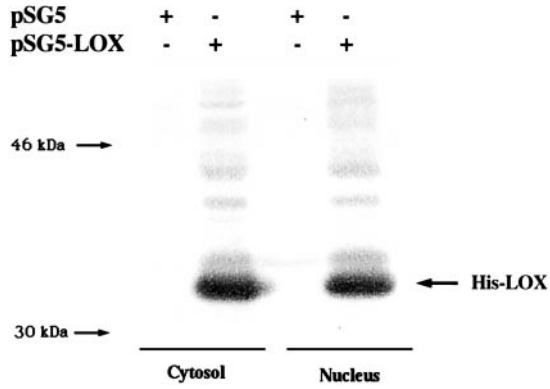


FIG. 3. **SDS-PAGE of fractionated cell extracts from COS-7 transfected with LOX (pSG5-LOX) or with the vector alone (pSG5).** The recombinant LOX was detected by Western blot with polyclonal anti-His tag antibodies.

and to normalize the results obtained for Col3A1. The primers used for the amplification of G3PDH were the following: forward, 5′-TGA AGG TCG GAG TCA ACG GAT-3′; reverse, 5′-CAT GTG GGC CAT GAG GTC C-3′. To amplify the G3PDH we used the same conditions as above, but a temperature of 60 °C for the annealing and 90 s of elongation at 72 °C. The expected product was 980 bp long. Several amplifications for both messengers were performed to define the range of template amount and cycle number that would give the best linear results. The PCR products were quantified by densitometric analysis (National Institutes of Health Image v1.62 for Macintosh), and Col3A1 product was expressed as a Col3A1:G3PDH ratio.

## RESULTS

Several fragments of COL3A1 promoter, ranging at their 5′ ends from −1375 to -35 bp from the transcription start site and extending up to +61 bp, were cloned into pGL2-basic vector. In length order the constructs are: pRUP4, pRUP6, pFV1, pDD1, and pDD2, respectively starting at −1375, −257, −117, −55, and −35 (Fig. 1, *A* and *B*).

All of the above constructs showed a comparable basal activity, except for pDD2, which was significantly two times higher

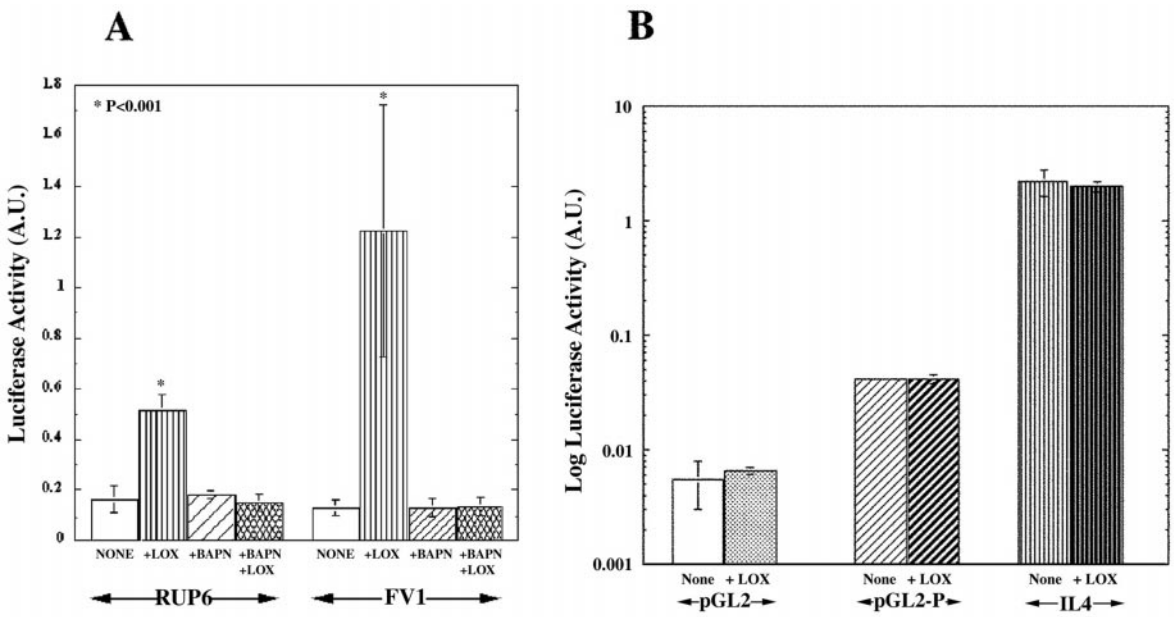


FIG. 4. *A*, luciferase activity of the indicated pGL2 constructs in the absence or presence of LOX expression and of 0.1 mM BAPN where indicated. *B*, luciferase activity in the absence or presence of LOX expression tested in the indicated constructs. *pGL2*, pGL2 basic vector; *pGL2-IL4*, pGL2 carrying interleukin 4 minimal promoter; *pGL2-P*, pGL2 carrying SV40 promoter; *pFV1*, pGL2 carrying pFV1 COL3A1 Fv1 promoter fragment. Luciferase activity is expressed as arbitrary units (*A.U.*). The results are the averages of at the least three independent experiments performed in triplicate ± S.E. The significance of the difference between the activities in presence of LOX expression and their relative controls has been evaluated by *t* test analysis.

(Fig. 2). COS-7 cells were transfected with pSG5-HLOX, a SV40-driven mammalian expression vector that expresses the mature form of LOX tagged with poly(His)$_6$ at the $NH_2$ terminus. Interestingly, the recombinant LOX protein, although expressed in the cytoplasm as expected, was also largely expressed in the nuclear compartment, which is in agreement with the most recent findings (29) (Fig. 3). We were unable to detect by any means the recombinant LOX in the extracellular compartment (data not shown), confirming the importance of the pro-peptide sequence in the secretion of the enzyme. This suited our aim of studying the putative intracellular activity of LOX.

When COS-7 cells were co-transfected with pSG5-LOX and the other different COL3A1 promoter constructs, the activity of the promoter was increased up to an average of 12 times, depending on the construct. In Fig. 2 a summary of the responses of the different promoter fragments to LOX expression is shown. The FV1 and DD1 regions are the most responsive, although pDD2 construct still retains a significant 2–3-fold inducibility upon LOX expression. The Fig. 4*A* shows that LOX induction of COL3A1 promoter is dependent on LOX catalytic activity, being completely suppressed by its inhibitor, the β-amino-propionitrile (7, 46, 47). Additionally, to rule out any generic effects on the basic transcription machinery, we co-transfected pSG5-LOX with pGL2-control vector, which is deprived of any promoter region, or with two short minimal unrelated promoters, the SV40 contained in pGL2-promoter vector and the interleukin-4 minimal promoter, also subcloned into pGL2 plasmid. Fig. 4*B* shows that in all cases, except with pFV1, LOX expression did not cause any increase in luciferase activity. Based on these functional findings (Fig. 2), we guessed that a LOX-responsive sequence must be present in repeated copies in the FV1 fragment, because we observed the highest response to LOX expression in all three tested fragments, FV1, DD1, and DD2. Moreover, the response decreased with the shortening of COL3A1 fragment, probably due also to an increased basal activity. We therefore analyzed the homology among the three fragments. The homologous regions are shown

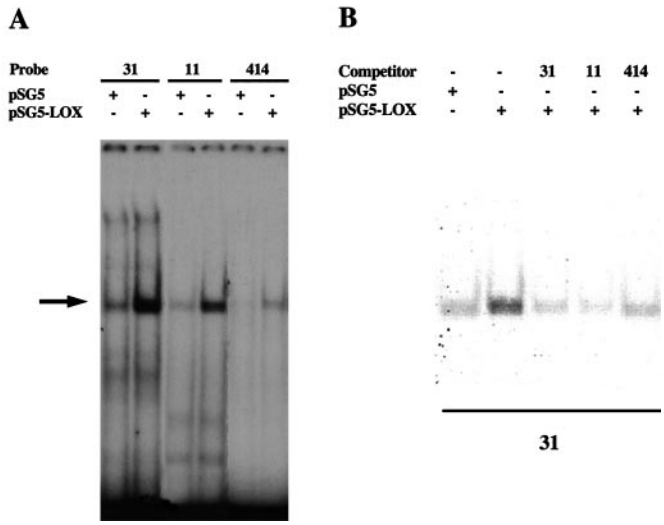


FIG. 5. *A*, EMSA using oligonucleotides 31, 11, and 414 as $^{32}$P-labeled probes in presence of nuclear extracts from COS-7 cells transfected with pSG5 expression vector alone or pSG5-LOX vector carrying the coding sequence for mature LOX. *B*, as above but using oligonucleotide 31 as $^{32}$P-labeled probe. Where indicated the reaction mix was preincubated with a 100-fold molar excess of the indicated cold oligonucleotides.

*underlined* in Fig. 1*B*. The resulting consensus sequence can be indicated as: (A/G)CT(G/A)A(G/A)GG(A/G)A. Although there are some differences within the three sequences, there is a strict conservation of the purines, which accounts for 80% of the overlapping sequences. To probe the ability of this consensus sequence to bind some transcription factor(s), we performed a classic EMSA analysis approach. The experiment was performed using the nuclear extracts from COS-7 transfected with the vector expressing the recombinant LOX or with the empty vector as negative control, and the three consensus DNA sequences, respectively named 31, 11, and 414, were used as probes. Fig. 5*A* shows clearly a retarded DNA-protein(s) complex that is increased 3–4 times when LOX is overexpressed.

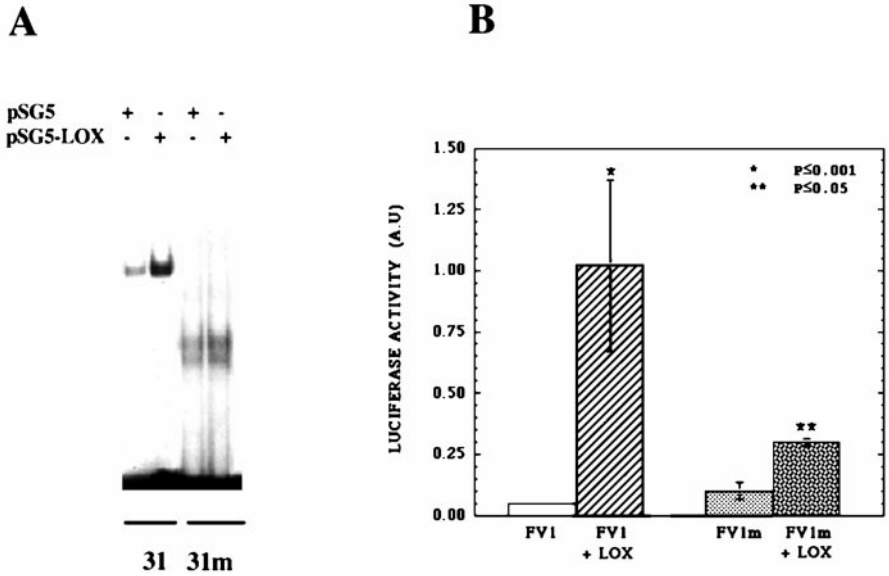


FIG. 6. *A*, EMSA using the mutated version of the oligonucleotide 31 (31m) as $^{32}$P-labeled probe in presence of nuclear extracts from COS-7 cells transfected with pSG5 expression vector alone or pSG5-LOX vector carrying the coding sequence for mature LOX. *B*, luciferase activity of the indicated pGL2 constructs in absence or in presence of LOX expression. FV1m represents pFV1 construct carrying the mutated version of the region 31. Luciferase activity is expressed as arbitrary units (*A.U.*). The results are the averages of at the least three independent experiments performed in triplicate ± S.E. The significance of the difference between the activities in the presence of LOX expression and their relative controls has been evaluated by *t* test analysis.

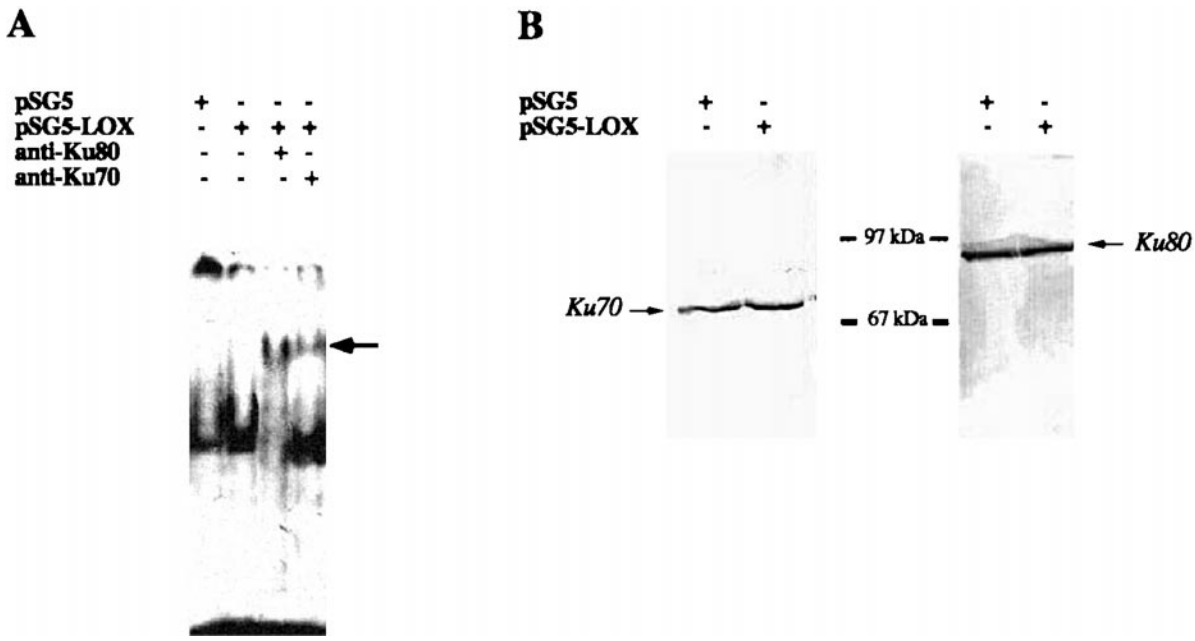


FIG. 7. *A*, EMSA using oligonucleotide 31 as $^{32}$P-labeled probe in presence of pSG5; nuclear extracts from COS-7 cells transfected with pSG5 expression vector alone or pSG5-LOX vector carrying the coding sequence for mature LOX. Anti-Ku80 and anti-Ku70 indicate the presence of the specific antibodies in the incubation mixture. The *arrow* points to the supershift. *B*, 10% SDS-PAGE; Western blot analysis of Ku80 and Ku70 levels in COS-7 cells, with and without overexpression of mature LOX.

Although LOX induces the highest binding with probe 31, the relative increase seems within the same range for all three sequences. Fig. 5*B* shows the specific competition of the DNA-protein complex bound to probe 31 by a molar excess of the cold oligonucleotide 31 or the other two consensus sequences, 11 and 414. The mutation of oligonucleotide 31 (31m) resulted in the abolition of the above described complex (Fig. 6*A*). The same mutation was also introduced in the pFV1 construct (FV1m) to test its effect in the luciferase gene reporter assay. The basal promoter activity of FV1m was almost double the one of the FV1, but, as expected, there was a much lower response to LOX expression (Fig. 6*B*).

Although we did not find any similarity between our consensus sequence and the ones described in a transcription factor data base (MatInspector version 2.1), we performed a homology search against the EMBL DNA data base. Among the many possible nucleotide sequences that partially matched the oligonucleotide 31 sequence, our attention was drown by NRE1 (nuclear regulatory element), which is a sequence in the long terminal repeat of MMTV responsible for the inhibition of its induction by glucocorticoids (Fig. 1*C*). Recent investigations (44, 48–51) showed that NRE1 is able to bind the Ku antigen heterodimer complex (Ku80 and Ku70). Ku antigen usually binds to DNA-free ends or nicked double-stranded DNA and mediates the binding of the DNA-dependent protein kinase (DNA-PK). The whole complex is involved in some of the main

DNA repair and recombination processes (52–54). Based on this evidence, we tested the presence of Ku in the DNA-protein complex that appears in our EMSA experiments, using anti-Ku80-specific and anti-Ku70-specific antibodies. Fig. 7*A* shows, indeed, that both antibodies were able to induce a supershifted complex. In the case of anti-Ku80, the antibody supershifted the entire complex. This might indicate a special role of Ku80 in the binding of the whole complex to the target DNA sequence. The antibodies alone did not produce any retarded complex (data not shown).

Because Ku can bind in a nonspecific manner to the ends of double-stranded DNA, we wanted to test the extent of the specificity of the binding of Ku to oligonucleotide 31. First, we performed the EMSA in higher stringency conditions, substituting the poly(dI-dC) with a higher concentration of shredded calf thymus DNA to compete the nonspecific binding (44). In Fig. 8, the first two lanes show that the Ku complex is barely visible in the control transfection, whereas it appears clearly in the presence of LOX expression. Moreover, to eliminate any possibility of nonspecific binding to the DNA ends, oligonucleotide 31, modified with suitable restriction sites, was subcloned into $pSK^+$ plasmid, excised with *Eco*RI/*Pvu*II, and circularized by T4 ligase, producing a 280-bp circularized DNA molecule (44). The obtained microcircles were used to compete Ku binding to oligonucleotide 31. Similar microcircles obtained from wild type $pSK^+$ (without oligonucleotide 31) were used as negative control. In Fig. 8, we can see that only the microcircles containing the sequence 31 were able to compete the retarded band (*third* and *fourth lanes*). Taken together these results prove a specific binding of Ku to the identified consensus sequence.

Looking for an explanation for the increased binding of Ku induced by LOX overexpression, we tested whether it was the result of a change in Ku expression level itself. That was not the case, because the Western blot shows that neither Ku80 nor Ku70 protein levels were affected by LOX expression (Fig. 7*B*). Also, there was no change in their intracellular distribution (data not shown).

Although our data strongly suggest a regulation of Col3A1 expression by LOX in COS-7, it is also true that these cells are not the best model for collagen production. Therefore, to test our hypothesis *in vivo* and on a more appropriate model, we decided to perform the same EMSA experiments using a primary culture of human skin fibroblasts. Although these cells expressed a significant amount of our recombinant LOX upon transfection of pSG5-LOX, the level of expression was much less than the one obtained in COS-7 cells (Fig. 9*B*). Nevertheless, as shown in Fig. 9*A*, we were able to reproduce the same results as with COS-7 cells, because even a milder expression of LOX was accompanied by a significant increase of binding of Ku antigen to the oligonucleotide 31. The supershift of the retarded DNA-protein complex induced by the anti-Ku80 antibody confirmed that we were dealing exactly with the same phenomenon (Fig. 9*A*, *third lane*). Next, we tested whether the presence of our recombinant LOX would really increased the level of Col3a1 messenger. In Fig. 9 (*C* and *D*) the results of a RT-PCR analysis show a significant 1.7-fold average increase of endogenous Col3A1 messenger in the fibroblasts expressing our recombinant LOX. Considering that the best transfection does not reach more than 20% of the target cells and that the conditions *in vivo* are rather different from those in the luciferase assay, these *in vivo* results seem to support our previous data. Moreover, as shown in Fig. 9*B*, we know that the transfection yield obtained with these human fibroblasts was quite lower than with COS-7, which should further reinforce the meaning of that quasi 2-fold increase in Col3A1.

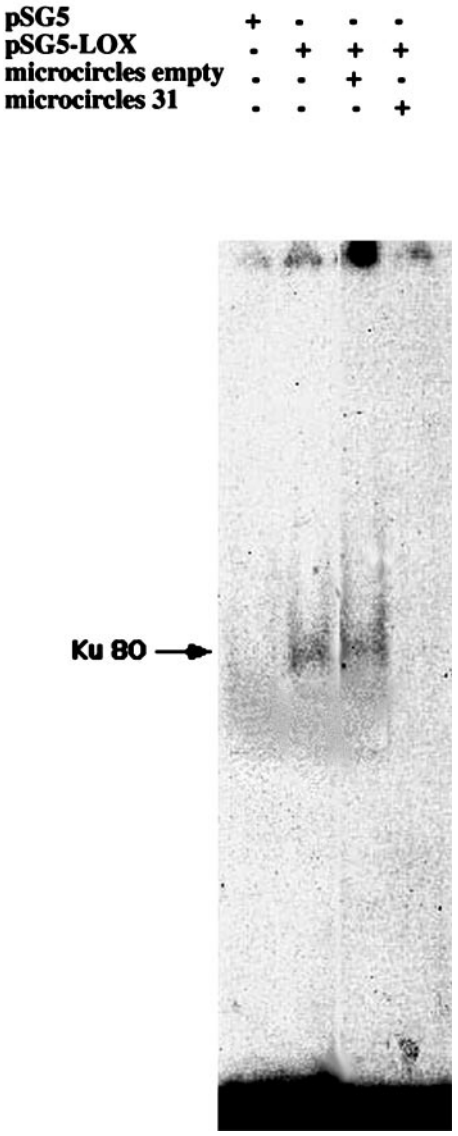


FIG. 8. **EMSA using oligonucleotide 31 as $^{32}$P-labeled probe in the presence of nuclear extracts from COS-7 cells transfected with pSG5 expression vector alone or pSG5-LOX vector carrying the coding sequence for mature LOX.** Where indicated 50-fold excess of cold microcircles empty or containing the sequence 31 was added to the incubation mix.

## DISCUSSION

LOX plays a critical role in the organization of extracellular matrix, stabilizing the molecules of collagen and elastin (1, 55–57). Because of that, LOX is considered one of the main actors in the development of many pathological fibrotic processes (5–8). Also, LOX has been proposed as the tumor suppressor gene, because of its down-regulation in many experimental and naturally occurring tumors and its anti-*ras* activity (14, 15, 17, 18, 30, 58). It has even been proved that LOX is up-regulated by the tumor suppressor interferon responsive factor-1 at the promoter level (26). This evidence suggests an additional role for LOX apparently not directly related to its collagen cross-linking activity. In support of this hypothesis there are observations that show a possible intracellular role for LOX (29, 30). Collagen III is one of the substrates of LOX and is also characteristic of specific organ fibrosis (31, 32, 34, 59–61). In many cases it has been shown that the level of LOX expression changes in parallel with collagen III (9, 17, 35, 62). Also LOX-like protein, a member of the LOX family, has been shown to be up-regulated together with collagen III in an

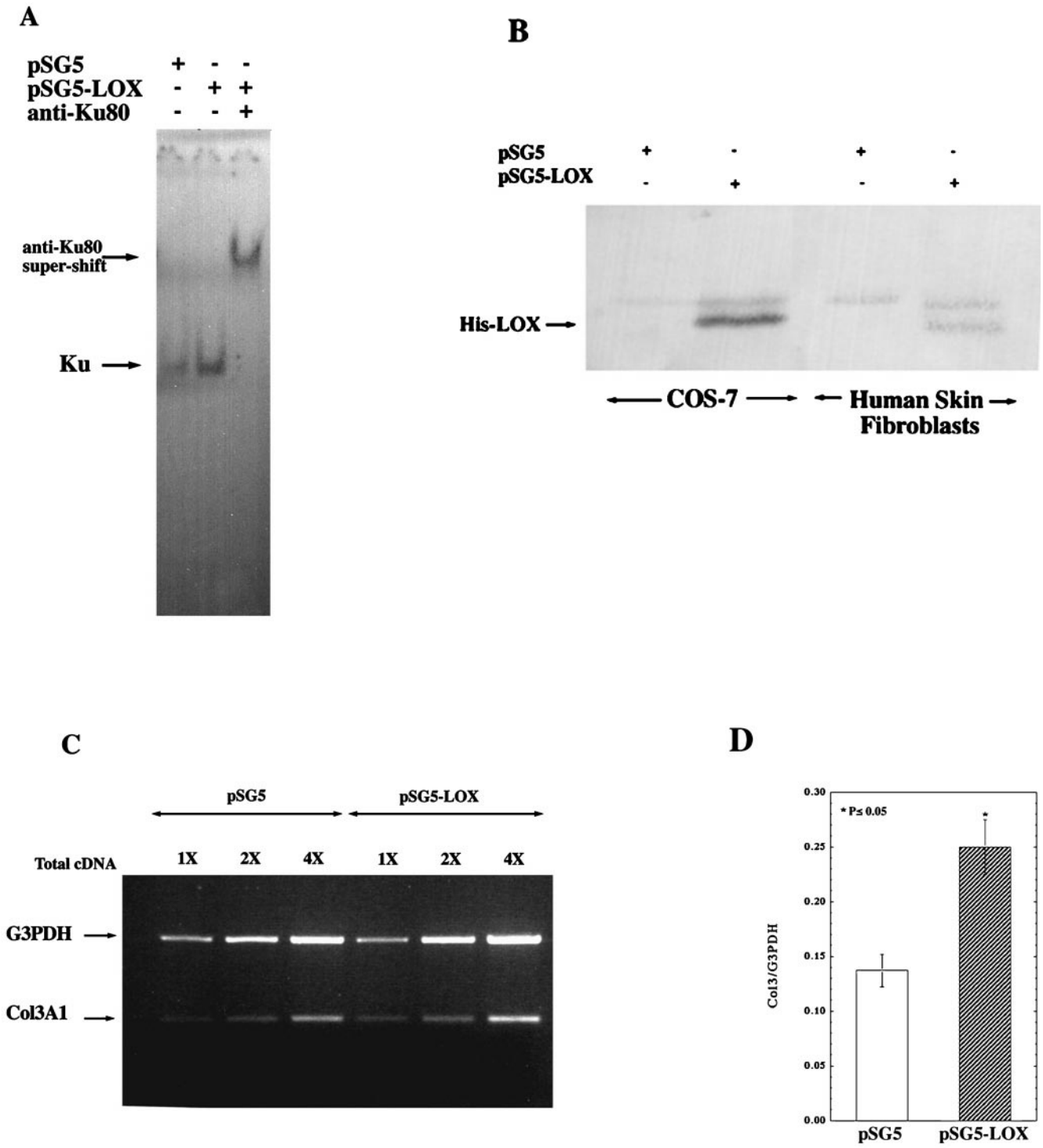


FIG. 9. *A*, EMSA using oligonucleotide 31 as $^{32}$P-labeled probe in presence of nuclear extracts from a primary culture of human skin fibroblasts transfected with pSG5 expression vector alone or pSG5-LOX vector carrying the coding sequence for mature LOX. Where indicated anti-Ku80 antibody was present in the incubation mix. *B*, SDS-PAGE of total cell extracts from COS-7 transfected with LOX (pSG5-LOX) or with the vector alone (pSG5). The recombinant LOX was detected by Western blot with anti poly-His tag polyclonal antibodies. *C*, agarose gel showing a typical RT-PCR amplification of a fragment of the human Col3A1 messenger using increasing amount of total cDNA as template. 1× is equivalent to 0.4 $\mu$l out of 100 $\mu$l of cDNA obtained from 1 $\mu$g of total RNA. In the same gel is shown the corresponding amplification of the messenger for G3PDH housekeeping gene. *D*, graphic results from the densitometric analysis of the at the least three independent RT-PCR experiments, expressing the mRNA level of Col3A1 gene normalized on G3PDH level (Col3A1:G3PDH ratio).

experimental liver fibrosis (36) and is found expressed in the intracellular compartment.[2] In the present paper we present evidence that the mature 32-kDa form of LOX intracellularly expressed (37, 38) can regulate the transcription activity of COL3A1 promoter. We showed that the region from −117 to + 61 of COL3A1 promoter is responsible for a positive transcriptional response to the overexpression of our recombinant mature LOX. The effect is specific for COL3A1 promoter, and it is dependent on LOX catalytic activity, which suggests some in-

[2] K. Csiszar, personal communication.

tracellular cross-linking process that mediates the observed effects. EMSA experiments and homology analysis of the indicated promoter region showed three highly conserved sequences that are able to bind the same proteic factor(s). By homology of our consensus sequences with NRE1, we inferred that the protein(s) complexing to our oligonucleotide probe could be Ku antigen, which, in fact, has been previously proved to bind to NRE1 in a specific manner (44, 48–50). Indeed, using specific antibodies for Ku antigen, we were able to compete and supershift the retarded band appearing in our EMSA analysis. Ku is a heterodimer (80–86 and 70 kDa) that forms a complex with the DNA-PK and brings it to the target DNA (63–65). A well described role for Ku/DNA-PK is to direct and control some DNA repair processes and V(D)J recombination (52–54, 66, 67). One of the consequences of such Ku activity is its role in controlling the general cell radiosensitivity (68–72). However, many different activities have been proposed for Ku antigen, from the physical interaction with EGF and glucocorticoid receptors (73, 74) or even somatostatin (75, 76) to the regulation of promoters activities (44, 48, 49, 77, 78). So far, the best documented alternative activity of Ku antigen is the regulation of NRE1, a sequence in the long terminal repeat promoter of MMTV, which is necessary to repress MMTV transcription induced by glucocorticoids (44, 48, 51). Furthermore, more recent evidence support Ku role in promoter regulation. DAS, a sequence similar to NRE1, which is downstream of the TATA box of the strict late ($\gamma$) UL38 promoter of herpes simplex virus type 1, has been reported to be able to bind Ku/DNA-PK and activate the promoter (49).

Our findings suggest that LOX can be responsible of alternative intracellular activities and that the activation of COL3A1 promoter can be one of those. In the specific case it is not surprising that an enzyme would up-regulate its own substrate, in a type of positive feedback fashion. Moreover, our data are supported by a previous observation suggesting a co-regulation of LOX and COL3A1. The finding that Ku antigen might be involved in such a regulation is completely novel. Presently it is difficult to define the possible mechanism by which LOX would increase Ku binding to its target sequence on COL3A1 promoter. We think that some LOX-dependent activation of Ku might occur, probably in terms of post-translational modification. It has already been described that Ku can be substrate of the DNA-dependent PK (79), and its phosphorylation seems related to its binding to an Epstein-Barr virus-responsive enhancer (78). Obviously, further studies are needed to completely understand the regulatory mechanism that links LOX to Ku. A very recent paper has pointed out that Ku can be placed in the class of the caretaker genes and, therefore, suppresses malignant transformation in certain conditions (80). It is intriguing to think that at the least some of LOX tumor suppressor activities might be explained by an improved Ku-dependent DNA repair activity and, therefore, a more stable genomic status of the cells.

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# Lysyl Oxidase Activates the Transcription Activity of Human Collagene III Promoter: POSSIBLE INVOLVEMENT OF Ku ANTIGEN

Monia Giampuzzi, Gerardo Botti, Marco Di Duca, Lorenza Arata, GianMarco Ghiggeri, Rosanna Gusmano, Roberto Ravazzolo and Armando Di Donato

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