Cloning and Characterization of the Rat Lysyl Oxidase Gene Promoter

IDENTIFICATION OF CORE PROMOTER ELEMENTS AND FUNCTIONAL NUCLEAR FACTOR I-BINDING SITES*

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Lysyl oxidase (LO)2 stabilizes the extracellular matrix by cross-linking collagen and elastin. To assess the transcriptional regulation of LO, we cloned the 5′-flanking region with 3,979 bp of the rat LO gene. LO transcription started at multiple sites clustered at the region from −78 to −51 upstream of ATG. The downstream core promoter element functionally independently of the initiator predominantly activated the TATA-less LO gene. 5′ Deletion assays illustrated a sequence of 804 bp upstream of ATG sufficient for eliciting the maximal promoter activity and the region −709/−598 exhibiting strongly enhancing effects on the reporter gene expression in transiently transfected RFL6 cells. DNase I footprinting assays showed a protected pattern existing in the fragment −612/−580, which contains a nuclear factor I (NFI)-binding site at the region −594/−580 confirmed by electrophoretic mobility supershift assays. Mutations on this acting site decreased both NFI binding affinity in gel shift assays and stimulation of SV40 promoter activities in cells transfected with the NFI-binding site-SV40 promoter chimeric construct. Furthermore, at least two functional NFI-binding sites, including another one located at −147/−133, were identified in the LO promoter region −804/−1. Only NFI-A and NFI-B were expressed in rat lung fibroblasts, and their interaction with the LO gene was sensitively modulated by exogenous stimuli such as cigarette smoke condensate. In conclusion, the isolated rat LO gene promoter contains functionally independent initiator and downstream core promoter elements, and the conserved NFI-binding sites play a critical role in the LO gene activation.

Lysyl oxidase (LO)2 (EC 1.4.3.13) is a copper-dependent enzyme secreted by fibrogenic cells such as fibroblasts (1). This enzyme catalyzes the initiation of cross-linking of collagen and elastin, major structural components of the extracellular matrix (ECM), by oxidizing peptidyl lysine residues within these proteins to peptidyl α-aminoadipic-δ-semialdehyde, leading to the formation of condensation products stabilizing polymeric collagen or elastin as insoluble fibers. Thus, LO plays a central role in ECM morphogenesis and tissue repair (1).

In addition to the major function in stabilizing the ECM, LO also exhibits other biological activities. As reported, expression of transfected LO cDNA inhibited Ha-ras-induced cell transformation indicating an anti-tumorigenic effect of LO (2). LO can oxidize lysine residues in various globular proteins other than collagen and elastin (1). Oxidation of basic fibroblast growth factor (bFGF) by LO blocks the proliferation of bFGF-stimulated cells and highly tumorigenic bFGF autocrine-transformed cells (3). Purified mature bovine LO displays chemotactic activity for monocytes and vascular smooth muscle cells (4, 5). LO and its oxidized substrates exist within the nuclei, potentially using histone H1 as a substrate and modulating the promoter activity of the collagen type III gene (6–8). Increased LO activity is associated with fibrotic diseases such as lung and liver fibrosis and atherosclerosis (1), whereas decreased LO activity is associated with lathyritic agent-induced emphysema in animal models (9) and with disorders of copper metabolism like Menkes syndrome (10).

LO gene expression is regulated at the mRNA level in response to intra- or extracellular agents or conditions. LO transcription is up-regulated by transforming growth factor-β1 and interleukin-1β (11, 12) but down-regulated by bFGF and interferon-γ (13, 14). Steady-state LO mRNA levels were diminished in some cancer and transformed cell lines (1, 2) but elevated in invasive breast cancer cells (15). In addition, environmental agents can also act upon the LO gene resulting in its transcriptional modification. For example, our recent studies indicated that cigarette smoke condensate (CSC), the particulate phase of smoke, inhibited synthesis of nascent LO transcripts leading to reduced levels of LO mRNAs in treated rat fetal lung fibroblasts (16). Transcription of the LO gene is driven by proximal promoter sequences, which interact with ubiquitous transcription factors. A region of 1,865 bp of the rat LO promoter upstream of ATG was found to direct luciferase reporter gene expression. CSC suppressed luciferase activity in serum; ChIP, chromatin immunoprecipitation; CSC, cigarette smoke condensate; F, forward; R, reverse.

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2 The abbreviations used are: LO, lysyl oxidase; DPE, downstream core promoter element; Inr, initiator; NFI, nuclear factor I; ECM, extracellular matrix; bFGF, basic fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT, reverse transcription; EMSA, electrophoretic mobility shift assay; M-MLV, Moloney murine leukemia virus; BSA, bovine serum albumin; RLM-RACE, RNA ligase-mediated rapid amplification of cDNA ends; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; ChIP, chromatin immunoprecipitation; CSC, cigarette smoke condensate; F, forward; R, reverse.
cells transiently transfected with the LO promoter-luciferase gene chimeric vector consistent with its effects on LO mRNA levels (16). Although several cis-elements have been characterized in the LO promoter region for human, mouse, and rat (17–19), the precise mechanisms for their activation remain to be understood. Because the rat LO gene is widely used as a model for studies on the expression and regulation of the LO gene, it is important and necessary to further define the rat LO gene promoter.

Nuclear factor I (NFI) was originally described as a factor required for the replication of adenovirus DNA and then shown as a transcription factor widely involved in the regulation of constitutive or inducible gene expression, including both transactivation and repression (20). NFI encoded by four different genes (nfi-A, nfi-B, nfi-C/CTF, and nfi-X) binds to the consensus sequence TTGGC(N5)GCCAA (N indicates any nucleotide) on duplex DNA as a dimer. Notably, NFI can also bind to the individual half-site, i.e. TTGGC or GCCAA, with a somewhat reduced affinity. The highly conserved N-terminal residues contain the DNA binding domain, whereas the proline-rich C-terminal residues constitute the transcriptional regulation domain (20). Four cysteine residues are conserved in all DNA binding domains of NFI isoforms, but only three cysteines are required for the DNA binding activity. The fourth cysteine nonessential for DNA binding makes NFI proteins sensitive to oxidative damage. Exposure of cells to H$_2$O$_2$ inhibited the binding of NFI to its DNA consensus sequence (21). Mutation of the fourth cysteine induced NFI resistance to oxidative inactivation (22). The feature of oxidation sensitivity of NFI may play a critical role in the cellular response to oxidative stress (23). NFI has been reported to regulate the expression of a wide range of cellular and viral genes such as collagen (24), cytochrome P450 1A1 (21, 25), metallothionein-I (26), mouse mammary tumor virus, etc. (27).

To further understand the control of LO gene transcription, we have cloned the 5'-flanking region of the rat LO gene, mapped its transcription start sites and core promoter elements, characterized the regulatory activities in transcription of its different subsegments, and demonstrated NFI as a critical transactivator interacting with the cognate cis-element in the promoter region for LO gene activation.

**MATERIALS AND METHODS**

**Cell Culture**—The rat fetal lung fibroblasts (RFL6) obtained from the ATCC were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO$_2$ and 95% air incubator as described previously (28). Stock cultures were derived from the frozen cell line and passed every 4 days. A total of six passages was used for experiments.

**Primer Extension Analysis**—To identify start sites of the LO transcription, we first carried out a primer extension assay as described (29). Total RNA was isolated from RFL6 cells with TRIzol reagent (Invitrogen). An antisense oligodeoxynucleotide, 5’-ATGATGCTCCCCGGCTGTTCCCTGCTG-3’ (Integrated DNA Technologies, Coralville, IA), corresponding to positions from −23 to +2 in the rat LO sequence using the first nucleotide preceding the ATG codon as −1, was labeled with [γ-32P]ATP (PerkinElmer Life Sciences) by T4 polynucleotide kinase (New England Biolabs). Approximately 20,000–40,000 cpm of labeled oligonucleotides were annealed with 20 μg of total RNA in 5× M-MLV first strand buffer (Invitrogen) and adjusted to a final volume of 19 μl with diethyl pyrocarbonate-treated double distilled H$_2$O. Samples were heated at 95 °C for 1 min and subsequently incubated at 48 °C for 45 min. Then 2 μl of 0.1 M dithiothreitol, 8 μl of 2.5 mM dNTP, and 40 units of M-MLV RT enzyme (Invitrogen) were added into the reaction mixture. After incubation at 37 °C for 30 min, samples were mixed with 8 μl of a formamide sequencing gel loading buffer, heated at 90 °C for 1 min, and then chilled rapidly on ice. Aliquots of samples were analyzed on a 6% urea/acylamide sequencing gel. The transcription start sites were identified by the sequencing ladders directly parallel to the run-off reverse transcripts (29).

5’-RLM-RACE—The assay for 5’-RNA ligase-mediated rapid amplification of cDNA ends (5’-RLM-RACE) was performed to confirm the rat LO transcription start sites with the First Choice™ RLM-RACE kit (Ambion, Austin, TX) following the manufacturer’s instructions (30). Briefly, 10 μg of total RNA extracted from RFL6 cells was treated with calf intestinal phosphatase to remove the free 5’-phosphate group. Tobacco acid pyrophosphatase was then used to specifically remove the cap structure from the full-length mRNA, leaving a 5’-monophospho-"note. Note that non-tobacco acid pyrophosphatase-treated RNAs were also included in experiments as negative controls. An RNA oligonucleotide adaptor was next ligated to the newly decapped mRNA by T4 RNA ligase. With the ligated RNA as a template, LO cDNA was synthesized by reverse transcription using M-MLV reverse transcriptase and (dT)$_{15}$ primers. The resulting cDNA was then amplified by nested PCR using Platinum® Taq High Fidelity DNA polymerase (Invitrogen) as well as the rat LO gene primers (reverse) and the adaptor primers (forward) provided by the manufacturer. The gene-specific antisense inner primer 5’-GACTTCTCGAGGTTGTCACCGACGCGAGAATGG-3’ and the nested PCR outer primer 5’-CAGATGGGCTTGGAGTGCTCCTC-3’ were designed for the RLM-RACE assay based on the sequence of rat LO cDNA (16, 31). The 5’-RLM-RACE PCR products were analyzed on agarose gels and cloned into the pBluescript II SK (−) vector for sequencing as described (30).

**Construction of Reporter Plasmids and Cell Transfection**—The full length of the rat LO gene promoter and various subregions thereof was obtained by PCR using the rat genomic DNA extracted from RFL6 cells as a template. One common reverse primer and eight different forward primers were used for generation by PCR of progressive deletions of the 5’-flanking region of the LO gene, which were subsequently cloned into the SacI/Xmal sites of the promoterless and enhancerless firefly luciferase reporter vector pGL3-Basic. Each of the 5’ primers contained four nucleotides, GTCA, a restriction site, i.e. SacI or Xmal (labeled with underline), and a specific primer sequence, followed by a number in parentheses indicating the 5’ end position of each primer in the LO promoter region as follows: reverse, 5’-GTCACCCCGGATGATGCTCCCCGGCTGTC-3’, corresponding to positions from −23 to +2 in the rat LO gene sequence using the first nucleotide preceding

Regulation of the Rat Lysyl Oxidase Gene Promoter
Regulation of the Rat Lysyl Oxidase Gene Promoter

The ATG codon as −1; forward 1, 5′-GTCAGAGCTCTCTT-TGGCCTGCATCTTTAGTAA-3′ (−3,979); forward 2, 5′-GTCAGAGCTCTTTTGGAGAAATGAAAGG-GAC-3′ (−3,237); forward 3, 5′-GTCAGAGGCTGAATGC-ACTAGGAAAGTCTGGAGGA-3′ (−1,865); forward 4, 5′-GTCAAGCTCAGCTCACAAGCTCCTCCCATCCACAG-3′ (−709); forward 5, 5′-GTCAGAGCTCTTGGCGTCGCCCTGC-CCCATGGCCTG-3′ (−598); forward 6, 5′-GTCAGAGCTCAGCTCGAGGCGCCCTTGCGTGC-3′ (−410); and forward 8, 5′-GTCAGAGGCTCAGACACTG-TGCCCTCTCCGGGAC-3′ (−277). LO gene-specific sequences for the primer design were based on the Rattus norvegicus chromosome 18 WGS supercontig NW_047513 (GenBank™ accession number) (16,31). LO promoter fragments were restricted with ScaI and XmaI and ligated into similarly restricted plasmid pGL3-Basic (Promega, Madison, WI) upstream of the firefly luciferase gene as described (16). The resulting LO promoter constructs are referred to as the Prom with a number(s) corresponding to the distance in nucleotides from the 5′ end of the promoter sequence to the translation start codon ATG, including Prom−3,979, Prom−3,237, Prom−1,865, Prom−709, Prom−598, Prom−517, Prom−410, and Prom−277. In addition, the deletion constructs Prom−1,336, Prom−804, Prom−1,865/−1,335 (containing the sequence from −1,865 to −1,335), Prom−1,865/−804 (containing the sequence from −1,865 to −804), and Prom−1,865/−911 (containing the sequence from −1,856 to −911) were derived from the construct Prom−1,865, which was digested with restriction enzymes KpnI/PvuII, KpnI/EcoRV, SmaI/PvuII, NotI/XhoI, and HindIII alone, respectively, followed by self-ligation or treatment with mungbean nuclease and then self-ligation. Thus, a total of 13 LO promoter constructs were prepared (Fig. 4A). Each construct was sequenced from both ends to ensure the correct orientation and fidelity.

To evaluate functionalities of the initiator (Inr), the downstream core promoter element (DPE) and the NFI-binding sites in the LO gene activation, we also isolated two other LO promoter 5′ deletions such as −80/−1 and −160/−1 containing Inr and DPE core promoter elements and NFI-binding sites, respectively. The reverse primer used for PCR amplification was the same as described above corresponding to the region from −23 to +2 of the rat LO gene sequence. The forward primers for the LO promoter −80/−1 and −160/−1 were 5′-GATCGAGCTCTCTTGGCCTGCTCGAGGTACGTGTC-3′ and 5′-GATCGAGCTCAGCTCACAAGCTCCTCCCATCCACAG-3′, respectively. LO promoter-luciferase reporter construct vectors were created with the pGL3-Basic vector. Using these newly and previously prepared LO promoter-reporter gene constructs encompassing sequences −80/−1, −160/−1, and −804/−1 (see Fig. 3A and Fig. 8C) as DNA templates, mutations of the Inr, the DPE, or the NFI-binding sites were performed according to the QuikChange mutagenesis protocol (Strategene, La Jolla, CA). Mutagenic primer pairs used for the PCR amplification were 5′-GATCGAGCTCCTTGGCCTGCTCGAGGTACGTGTC-3′ for the Inr mutagenesis, 5′-CGTCCCTCCCGAGGAGGAGGCATC-3′ for the DPE mutagenesis, 5′-CTGCCGCTCGCCCTGAACCCAGTGCCCTCGACC-3′ for the NFI-binding site 1 mutagenesis, and 5′-CCTCATGCTTTGGGCGCTGCGCTGCGCTG-3′ for the NFI-binding site 2 mutagenesis (complementary reverse primers not shown, mutated nucleotides labeled with underlines). Each construct was verified by direct sequencing.

To further characterize the properties of specific regions of the LO promoter, synthetic oligonucleotides (Integrated DNA Technologies, Coralville, IA) of LO promoter fragments −709/−676, −682/−641, −654/−609, and −612/−580, as shown in Fig. 5A, and the fragment −609/−573 containing the NFI-binding motif or various mutants as well as the NFI-binding consensus sequences, as shown in Fig. 6B, were annealed each to its complementary sequence, phosphorylated with T4 polynucleotide kinase, and ligated into the Smal-treated pGL3-Promoter reporter vector (Promega, Madison, WI) upstream of the SV40 promoter sequence.

Each resulting recombinant construct and the pSV-β-galactosidase plasmid (Promega, Madison, WI) or pRL-TK, an internal control for monitoring the transfection efficiency, were transiently cotransfected into RFL6 cells by Lipofectamine reagents (32). After a 24-h post-transfection, cells were growth-arrested by incubation with 0.3% FBS/DMEM for an additional 24 h. In some experiments, cells were growth-arrested for 6 h and then exposed for 24 h to agents such as CSC at various doses in the FBS-free medium. Note that cells cotransfected with pGL3-Basic or pGL3-Promoter containing the luciferase gene without the LO promoter and β-galactosidase vectors were always included in any experiment to evaluate the background. Luciferase and β-galactosidase activities in cell lysates were measured by luminometry and spectrophotometry, respectively, as described by manufacturers. LO promoter-luciferase activities in transfected cells were normalized to the transfection efficiency as revealed by the β-galactosidase assay. Results are expressed as cpm/optical density of β-galactosidase. In experiments as shown in Fig. 3B, Fig. 8D, and Fig. 9B using the plasmid pRL-TK as an internal control, the LO promoter-directed expression of the firefly luciferase gene in the LO promoter-pGL3-Basic construct was normalized to Renilla luciferase activities derived from the pRL-TK vector and expressed as relative luciferase activities according to the instructions from the supplier (Promega, Madison, WI).

Nuclear Extract Preparation and EMSA—Nuclear extracts were prepared from rat RFL6 cells using nuclear and cytoplasmic extraction kit (Pierce). Protein concentrations were determined by the BCA protein assay reagents (Pierce). For the EMSA (33), synthetic oligonucleotides as shown in Fig. 6B and Fig. 8A were end-labeled with [γ-32P]ATP (PerkinElmer Life Sciences) by T4 polynucleotide kinase (New England Biolabs) and annealed to their complements. A total volume of 20 μl of reaction mixture containing 20 μg of nuclear protein or bovine serum albumin (BSA), a negative control, 1 μg of poly(dI-dC)·poly(dI-dC) (Sigma), and 10,000–20,000 cpm of labeled probes was incubated for 20 min at room temperature. For competition experiments, unlabeled cold oligonucleotides such as the NFI-binding site wild type and various mutants, and NFI-binding site consensus sequences as shown in Fig. 6B at 100-
fold molecular excess were added 10 min prior to addition of the radiolabeled probe. After the reaction, samples were subjected to native 4% PAGE and visualized by exposure of the dried gel to Kodak film. Supershift reactions were run as competition assays as described above with the exception that 2 μg of a corresponding specific antibody against Oct1 (Biovision, Mountain View, CA), CdxA (Cemines, Golden, CO), Pit-1A, or all NFI isoforms (Santa Cruz Biotechnology, Santa Cruz, CA) instead of cold probes was added, as shown in Fig. 6A and Fig. 8B. In the supershift combined with competition assay (Fig. 6D), competitors were added into the reaction mixture before addition of the antibody.

DNase I Footprinting—For the DNase I footprinting assay (34), a synthetic oligonucleotide of the LO promoter fragment −612/−580 was end-labeled with [γ-32P]ATP (PerkinElsmer Life Sciences) by T4 polynucleotide kinase. Twenty nmoles of DNA was incubated for 30 min with 20 μg of nuclear extract or BSA, a negative control. Samples were then treated with different concentrations of DNase I (1.0–0.25 units; Sigma) for 5 min. Reactions were stopped by addition of DNase I stop solution (1% SDS, 200 mM NaCl, 20 mM EDTA, pH 8.0, and 40 μg/ml tRNA). Reaction mixtures were phenol/chloroform-extracted and followed by ethanol precipitation. Resulting DNA samples were mixed with 5 μl of loading buffer (95% (v/v) formamide, 10 mM EDTA, pH 8.0, 0.1% (w/v) bromphenol blue, 0.1% (w/v) xylene cyanol) and analyzed on 6% acrylamide gel in 1× TBE buffer with a Sequi-Gen Sequencing Cell (Bio-Rad). Single-strand DNA ladder (10/60) (Integrated DNA Technologies, Coralville, IA) labeled with [γ-32P]ATP was electrophoresed alongside the digestion products. The gel was dried and exposed to Kodak film.

Reverse Transcription (RT)-PCR and Preparation of NFI Expression Vectors—To assess the expression of NFI isoforms in rat lung fibroblasts, RT-PCR was carried out as described (28). Briefly, approximately 500 ng of total RNA extracted from RFL6 cells by TRIzol were converted to cDNA, which was then amplified using the SuperScript One-Step RT-PCR with Platinum Taq kit (Invitrogen) under the following conditions: reverse transcription at 50 °C for 30 min and predenaturation at 94 °C for 2 min, denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 90 s in a total of 35 repetitive cycles. The final extension was performed at 72 °C for 6 min. The forward (F) and reverse (R) primers used were as follows: 5′-GACCTCAGCTGATGTATTCTCGCTGTGCT-TCA-3′ (F) and 5′-GACCTCAGGTATCTCCAGGTACCAGGACTTGTG-3′ (R) for amplification of nfi-A cDNA; 5′-GACCTCAGCTGATGTATTCTCGCTGTGCT-TCA-3′ (F) and 5′-GACCTCAGGTATCTCCAGGTACCAGGACTTGTG-3′ (R) for amplification of nfi-B cDNA; 5′-GACCTCAGCTGATGTATTCTCGCTGTGCT-TCA-3′ (F) and 5′-GACCTCAGGTATCTCCAGGTACCAGGACTTGTG-3′ (R) for amplification of nfi-C cDNA; and 5′-GACCTCAGCTGATGTATTCTCGCTGTGCT-TCA-3′ (F) and 5′-GACCTCAGGTATCTCCAGGTACCAGGACTTGTG-3′ (R) for amplification of nfi-X cDNA (restriction sites in primer pairs are labeled with underlines). PCR products were separated on agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. The mammalian NFI expression vectors pcDNA3.1-nfi-A and pcDNA3.1-nfi-B were, respectively, constructed by ligating the rat nfi-A and nfi-B coding sequences with the pcDNA3.1/V5-His-TOPO vector (Invitrogen) in their PvuII/XhoI sites for nfi-A and HindIII/XhoI sites for nfi-B. All expression vectors were sequenced to ensure fidelity and subjected to cotransfection into RFL6 cells with the LO promoter-reporter gene vector for assaying their capacities for the gene activation.

Chromatin Immunoprecipitation (ChIP) Assay—To determine cellular NFI binding to the LO promoter region, the ChIP assay was performed with the EpiQuik chromatin immunoprecipi-tation kit based on the protocol provided by the supplier (Epigentek Group Inc., Brooklyn, NY). Cellular components were cross-linked by incubation of control and CSC-treated cells at the same number (2 × 10^6) with 1% formaldehyde at room temperature for 10 min. The cross-linking reaction was stopped by addition of glycine to a final concentration of 125 mM. Nuclei were extracted with a nuclear isolation buffer, resuspended in a nuclear lysis buffer with protease inhibitor mixture, and then sonicated to shear DNA to lengths between 200 and 1000 bp. After centrifugation, cell debris was discarded, and DNA-containing supernatants were diluted with the ChIP dilution buffer, and aliquots of samples were removed out as “input” DNA. Diluted DNA samples were transferred into the strip wells that were precoated with the antibody against rat NFI (Santa Cruz Biotechnology), RNA polymerase II (a positive control provided by the kit supplier), or nonspecific rat IgG (a negative control from Santa Cruz Biotechnology) and incubated at room temperature for 90 min with shaking. After successively washing wells with the washing buffer and finally with the TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA), precipitated DNA-protein complexes as well as the input samples were treated with proteinase K (250 μg/ml) in the DNA release buffer for 15 min and then incubated in the reverse buffer for 90 min at 65 °C. The DNA samples were collected by the P-spin columns, washed with 70 and 90% ethanol successively, and then eluted with the elution buffer. Using purified DNA as a template, PCR was conducted under the following conditions: initial denaturation at 94 °C for 2 min, 30 cycles each with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min, and final extension at 72 °C for 5 min. Three forward (F) and reverse (R) primer pairs were used in PCR to characterize the NFI binding to the LO gene and the RNA polymerase II binding to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene including the following: pair 1, 5′-GGAGTTACAGAGGTGAGGTGAAC-3′ (F) and 5′-AGGCGGACCTCCTGAG3′ (R) for amplification of the LO NFI-binding site 1 fragment; pair 2, 5′-CCAGCAATCTCAGACATGACCA3′ (F) and 5′-GATTTGAATCCAGGATCCAGGAC3′ (R) for amplification of the LO NFI-binding site 2 fragment; and pair 3, 5′-GATTTGAATCCAGGATCCAGGAC3′ (F) and 5′-GCTTACGAGCAGCAGGAC3′ (R) for amplification of the RNA polymerase II binding region in the GAPDH promoter. PCR products were analyzed on a 2.2% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator. Densities of PCR-amplified gene fragments on the gel were measured with the 1D Scan software as described (28).
Regulation of the Rat Lysyl Oxidase Gene Promoter

Statistical Analysis—Data were expressed as mean ± S.D. of at least three independent experiments. Statistical differences between means were determined using one-way analysis of variance followed by Bonferroni’s post hoc test or two-tailed Student’s t-test when appropriate. A p value < 0.05 was considered significant.

RESULTS

Isolation of the 5'-Flanking Region of the Rat LO Gene—A genomic DNA fragment containing 3,979 bp compassing the 5'-flanking region of the rat LO gene upstream of the translation start codon ATG was isolated by PCR from RFL6 cells and restricted into the pGL3-Basic vector. This DNA fragment was shown below to drive the expression of the luciferase reporter gene indicating its promoter property. Sequence analysis revealed that the rat LO promoter region lacks canonical TATA box and CAAT boxes. It contains putative binding sites for the following transcription factors: NFI, GR, SP1, Oct-1, TAF-1, C/EBPγ, etc. In addition, the cloned rat LO promoter also contains the metal-response element, the hypoxia-response element, the antioxidant-response element, etc. (Fig. 1). Comparative sequence alignment across species of rat, human, and mouse showed that the proximal promoter region was highly conserved, whereas the distal promoter region was more divergent among three species (Fig. 1). For example, regions −410/−1 (as shown here and below indicating the fragment between two nucleotide numbers using the first nucleotide preceding the ATG codon as −1), −804/−411, and −1,336/−805 displayed 75.4, 38.6, and 24.8% homology, respectively, in sequences of rat, human, and mouse (Fig. 1). These analyses suggest that the proximal promoter region might play a fundamental role in the regulation of LO transcription in vivo.

The Transcription Start Sites of the Rat LO Gene—To identify start sites of the LO transcription, we first carried out a primer extension assay (29). Total cellular RNA isolated from RFL6 cells was incubated with the γ-32P-labeled LO antisense primer corresponding to positions from −23 to +2 in the rat LO sequence using the first nucleotide preceding the ATG codon as −1 in the presence of reverse transcriptase. Following analysis of the reaction product (Fig. 2A, lanes 5 and 6) on the sequencing gel, the transcription start sites were identified by the sequencing ladders (Fig. 2A, lanes 1–4) directly parallel to the run-off reverse transcripts. As shown (Fig. 2A), there was a conspicuous extension product parallel to the position of cytosine (labeled with underline) at the sequence CCCTG (arrow) in addition to several minor extension products (arrowheads) on the gel. Thus, the cytosine at the sequence CCCTG is a major transcription start site which was then mapped at −61 bp upstream of ATG in the LO promoter region.

Furthermore, the transcription start sites of the LO gene were confirmed by 5’-RLM-RACE analysis (Fig. 2B) (30), which has proven to be a very sensitive and accurate method to identify full-length 5’ ends of cDNAs by eliminating truncated messages from the amplification reactions. The advantage of this method over others is that only authentic capped 5’ ends of mRNA are detected. After reverse transcription and nested PCR, a band of about 450 bp was revealed by the agarose gel (Fig. 2B). Notably, using identical procedures, non-tobacco acid pyrophosphatase-treated RNAs, a negative control, did not induce PCR products (data not shown). Resulting RT-PCR product was cloned into pBluescript II SK(−) vector. Sequencing of 16 clones showed that the initiations of LO transcripts were clustered at the region from −78 to −51 (Fig. 2C). In addition to two major start sites at the −51 adenine and the −61 cytosine (31.3% of tested clones each, i.e. 5/16), other potential start sites for the LO transcription included −54 (6.3%), −55 (12.5%), −57 (6.3%), −60 (6.3%), and −78 (6.3%) upstream of the ATG. Notably, the transcription start site at the −51 adenine overlaps with the sequence TCATTTT (labeled with underline) identical with the Inr element consensus sequence YYAN(T/A)YY (Y is pyrimidine; N is any nucleotide) (36, 37). Furthermore, a sequence 5’-GGACG-3’ is located at the region from −18 to −14 upstream of ATG consistent with the consensus sequence of the DPE, (A/G)(A/T)(C/T)(G/A/C) (37). Thus, a potential Inr core promoter element combined with the DPE was mapped in the LO promoter region (Fig. 1). As indicated above, the −61 cytosine as another major transcription start site has also been demonstrated by the primer extension assay. Thus, multiple transcription start sites exist in the rat LO gene.

Roles of the Inr and the DPE in the LO Promoter Activation—To evaluate roles of the Inr and the DPE in the LO gene activation, we introduced site-directed mutations at the Inr, the DPE, or both in the LO promoter-reporter gene construct spanning from −80 to −1 (Fig. 3A). LO core promoter activities were tested by transfection assays in RFL6 cells. As shown in Fig. 3B, mutations of the Inr consensus TCATTTT (−53/−47) to TCGGTGTT and the DPE consensus GGACG (−18/−14) to CCCCG (mutations are labeled with underlines) reduced the luciferase gene expression to 58 and 1.4% of the wild-type control, respectively, whereas double mutations decreased the promoter activity to 1.8% of the wild-type control. These results suggest that the Inr and the DPE did not work as one combined unit initiating the LO gene transcription because mutation of the Inr only partially inhibited the reporter gene expression. Furthermore, the DPE may represent a more important core promoter to activate the LO promoter in comparison to the Inr because its mutation essentially abolished the luciferase activity. Thus, the LO promoter contains functionally active Inr and DPE sequences, which act as independent core promoter modules for the LO gene transcription.

Deletion Analysis of the LO Promoter—To characterize the 5’-regulatory region of the LO gene, we further prepared a set of luciferase reporter gene constructs containing successive 5’ deletions of the LO promoter (Fig. 4A). After transient transfection into RFL6 cells, the transcription activities derived from these constructs were tested. The background luciferase expression levels were evaluated by transfection of equal amounts of the pGL3-Basic reporter vector without the LO promoter insert. A series of systematic luciferase assays as shown in Fig. 4B indicated that a high level of the LO promoter activity was found in the construct Prom−804 reaching 168% of the full-length control (Prom−3,979). When the sequence length extended from −804 to −3,237, no significant alterations of luciferase activities were observed. Thus, critical control elements that regulate LO gene expression may not be pres-
Comparison of the promoter sequences of rat (R), human (H), and mouse (M) LO genes. Sequences were aligned with the ClustalW program (35). Gaps introduced to maximize similarity are indicated by dashes. Asterisks represent identical nucleotides. Translation start sites are labeled as +1. Putative transcription factor-binding sites and cis-elements are labeled above the sequence. Core promoters Inr and DPE are labeled with boxes.
Regulation of the Rat Lysyl Oxidase Gene Promoter

FIGURE 2. Determination of LO transcription start sites. A, primer extension analysis. Oligonucleotide Rev−23/+/2 was end-labeled with [γ-32P]ATP and used in a primer extension experiment with 20 μg of total cellular RNA isolated from RFL6 cells. Lanes 5 and 6 showed the major (arrow) and minor (arrowheads) extension products. LO sequencing ladders (GATC, lanes 1–4) were produced from Prom−804 with the same oligonucleotide primer (Rev−23/+/2). Notably, experiments shown here and below were repeated at least three times with reproducible results, and a representative one is presented. B, mapping of LO transcription start sites by RLM-RACE. Agarose gel electrophoresis of nested PCR products from the RLM-RACE procedure using decapped rat RFL6 total RNA ligated with an RNA oligonucleotide adaptor as a template. Molecular weight (MW) markers (base pairs) are indicated on the left. The major PCR product was marked by two arrows on the right. C, multiple transcription start sites clustered in the rat LO promoter region. Line A, numbers indicate the relative nucleotide positions upstream of ATG. Line B, asterisks show transcription start sites identified by cloning and sequencing the RLM-RACE products. Line C, indicates the number of clones initiated at the corresponding site labeled with an asterisk in a total of 16 sequenced clones, such that −61 and −51 have 5 clones each.

ent in the region from −3,237 to −804. Inclusion of more nucleotides up to −3,979 from −3,237 resulted in a 60% decrease in luciferase activities relative to the full-length control, suggesting the presence of inhibitory cis-elements in this distal 5′-flanking region. More 5′ deletions of nucleotides from the Prom−804 induced reduction of luciferase activities such that the Prom−709, −598, −410, and −277 decreased LO promoter activities to 109, 36, 22, and 10% of the full-length control, respectively. Note that there was one exception, i.e. the Prom−517, which restored 17% promoter activity relative to that of the Prom−598. Furthermore, deletions of the 5′-proximal regions upstream of ATG from the Prom−1,865 totally abolished luciferase activities as shown by those in constructs Prom−1,865/−804, Prom−1,865/−911, and Prom−1,865/−1,335. It should be noted that the construct Prom−804 significantly enhanced the expression of the luciferase gene reaching 75-fold of the pGL3-Basic control, whereas the construct Prom−277 displayed only 2-fold of the basal level of luciferase activity in comparison to the pGL3-Basic. Thus, it appears that the major role of the fragment between −804 and −277 is transactivation of the LO gene. Taken together, these results suggest that there are at least four positive regulatory segments (i.e. −804/−709, −709/−598, −598/−410, and −410/−277) and two negative regulatory segments (i.e. −3,979/−3,237 and −598/−517) identified in the full-length, cloned rat LO promoter and that the sequence from −1 to −277 as a basic region and the sequence from −277 to −804 as a regulatory region both are essential for LO promoter functions.

Screening cis-Elements in the Region from −709 to −598 bp of the Rat LO Promoter—As shown in deletion analysis (Fig. 4B), the upstream extension of the LO promoter from −598 to −709 markedly increased the pGL3-Basic luciferase activity reaching 3.2-fold of the former level, suggesting the critical role of this region in the regulation of the LO gene expression. To characterize cis-elements and their corresponding transcription factors in this region, we synthesized four oligonucleotides with overlap in their sequences, i.e. −709/−676, −682/−641, −654/−609, and −612/−580 (Fig. 5A), and we examined their functional activities. Each oligonucleotide was annealed to its complementary sequence and inserted into the pGL3-Promoter reporter vector upstream of the SV40 promoter sequence. Resulting constructs were transiently transfected into RFL6 cells and the expression of the reporter gene was determined. As shown (Fig. 5B), pSV−709/−676, pSV−682/−641, pSV−654/−609, and pSV−612/−580 constructs all...
Regulation of the Rat Lysyl Oxidase Gene Promoter

A

![Diagram A](image1)

B

![Diagram B](image2)

FIGURE 4. Luciferase activities in RFL6 cells transfected with LO promoter-deletion constructs. A, schematic representation of LO promoter-deletion constructs. B, relative luciferase activities of LO promoter-deletion constructs. LO promoter-deletion constructs were transiently transfected into RFL6 cells. Twenty-four hours after transfection, cells were growth-arrested in 0.3% FBS/DMEM for an additional 24 h and then harvested for assaying luciferase activities. The plasmid pSV-β-galactosidase was cotransfected with LO deletion constructs for data normalization. Data were expressed as % of the full-length promoter control (100% luciferase activity in cells transfected with Prom-3979). All values represent the mean ± S.D. of three experiments, each determined with triplicate dishes.

exhibited enhanced levels of luciferase activities in transfected cells amounting to 186, 216, 168, and 179%, respectively, of the pGL3-Promoter basal level. These results suggest the presence of enhancer cis-elements in these LO promoter fragments, and thus the oligonucleotide −612/−580 as a subregion of the LO promoter was first screened by this laboratory for its cis-element property.

The DNase I footprinting assay was performed as described (34) in an effort to explore the binding sites of transcription factors in the subregion −612/−580. As shown (Fig. 5C), RFL6 nuclear extracts (lanes 5–8), but not BSA (lanes 1–4), an internal control, induced a protection pattern in this LO promoter subregion. Precise positioning of the protected area from −598 to −588 was achieved by comparison of the footprints with 32P-labeled single strand DNA molecular weight ladders (M in Fig. 5C). Computational analysis (TESS, TF Search and Ali-baba 2.1) showed that the protected region may be a potential binding site for several transcription factors such as Oct1, CdxA, Pit-1A, NFI, etc.

Identification of NFI Binding to the Subregion −612/−580 of the LO Promoter—To identify transcription factors that bind to the putative cis-element −612/−580 as described above, we conducted EMSA and supershift assays (33) with antibodies against Oct1, CdxA, Pit-1A, and NFI. As shown (Fig. 6A), only the NFI antibody, rather than other antibodies tested, yielded a supershifted band, indicating that the putative cis-element in this subregion is a NFI-binding site. Because the putative NFI-binding site (TTG-GCTTGGGCCCCAT, −594/−580) locates at the 3' terminus of the fragment −612/−580, we further synthesized the oligonucleotides encompassing the sequence from −609 to −573 of the LO promoter containing the putative NFI-binding site or its mutants as shown (Fig. 6B). These oligonucleotides were used as competitors as described (38) in gel mobility shift (Fig. 6C) and supershift assays (Fig. 6D) in the presence of the RFL6 nuclear extract using 32P-labeled NFI-binding site wild type (NFI-BS-WT) as a probe. As expected (Fig. 6C), a 100-fold molar excess of cold NFI-BS-WT (lane 7) as well as NFI-binding site-high affinity consensus (NFI-BS-High) (lane 3) and NFI-binding site-consensus (NFI-BS-Con1 and NFI-BS-Con2) (lanes 3 and 4) (38) oligonucleotides competed successfully with labeled probes to bind to the NFI protein. In contrast, NFI-binding site mutant oligonucleotides such as NFI-BS-Mut (Fig. 6C, lane 6) (38), NFI-BS-Mut-1, NFI-BS-Mut-2, NFI-BS-Mut-3, and NFI-BS-Mut-4 (lanes 8–11) that were derived from the LO promoter sequence lost, at least in part, their competitive capacities to block the formation of the labeled DNA-protein complex. Moreover, similarly competitive effects elicited by NFI-BS-WT (lane 4) or mutant oligonucleotides (lanes 5–9) were also observed in the gel supershift assay (Fig. 6D). Together, these results strongly support the hypothesis that the NFI-binding site exists in the −612/−580 fragment of the rat LO promoter, and the transcription factor, NFI, specifically binds to this cis-element.
Regulation of the Rat Lysyl Oxidase Gene Promoter

Functionalities of the LO Promoter-NFI-binding Site −594/−580 and Its Response to CSC in Transfected RFL6 Cells—To verify the biological role of the NFI cis-element in the gene expression, we cloned the subregion −609/−573 of the LO promoter containing the NFI-binding motif −594/−580 or various mutants as described above (Fig. 6B) into the pGL3-Promoter vector upstream of the SV40 promoter. The constructs were then transiently transfected into RFL6 cells. Twenty hours after transfection, cells were growth-arrested in 0.3% FBS/DMEM for an additional 24 h and then harvested for assaying luciferase activities. The plasmid pSV-β-galactosidase was cotransfected into cells for data normalization. Data were expressed as % of the pGL3-Promoter control vector. All values represent the mean ± S.D. of three experiments, each determined with triplicate dishes.

Because CSC is known to modulate LO transcription (16), we further examined the NFI-binding element in response to CSC. RFL6 cells were transfected with pSV-NFI-BS-WT chimeric constructs and exposed to CSC at indicated doses for 24 h. CSC effects on the SV40 promoter activated by the NFI-binding element was determined by assaying levels of luciferase activities and expressed as fold of the pGL3-Promoter control. As shown (Fig. 7B), 40, 60, and 80 μg/ml of CSC inhibited NFI-binding site-directed luciferase activities by 34, 50, and 58%, respectively. These results indicated that the NFI interaction with its binding site in the LO promoter region is a critical molecular target for CSC insult.

FIGURE 5. Screening cis-elements in the region from −709 to −598 bp of the rat LO gene promoter. A, schematic representation of synthetic LO promoter fragments. Each synthetic oligonucleotide is represented as a solid line below the sequence. Number pairs to the right of the solid lines indicate positions of the corresponding oligonucleotide in the LO gene promoter upstream of ATG. B, relative luciferase activities of synthetic LO promoter-oligonucleotide constructs. Synthesized LO promoter oligonucleotides were inserted into pGL3-Promoter vector upstream of the SV40 promoter. The constructs were then transiently transfected into RFL6 cells. Twenty four hours after transfection, cells were growth-arrested in 0.3% FBS/DMEM for an additional 24 h and then harvested for assaying luciferase activities. Each synthetic oligonucleotide is represented as a solid line below the sequence. Number pairs to the right of the solid lines indicate positions of the corresponding oligonucleotide in the LO gene promoter upstream of ATG. C, in vitro DNase I footprinting of the LO promoter subregion −612/−580. A synthesized fragment of the LO promoter from −612 to −580 upstream of ATG was end-labeled with [γ-32P]ATP using T4 polynucleotide kinase. The labeled fragment was then allowed to bind to BSA, an internal control, or RFL6 nuclear extract (NE) and digested with 2, 1, 0.5, or 0.25 units of DNase I for 2 min at 25 °C. The material was then electrophoresed and visualized by autoradiography. 32P-Labeled single strand DNA molecular weight ladders were used as a size marker (M). Lanes 1–4, partial digestion of the probe in the presence of BSA. Lanes 5–8, partial digestion of the probe in the presence of RFL6 nuclear extract.
Evaluation of Biological Activities of All Putative NFI-binding Sites in the LO Promoter Region −804/−1—Computational analysis (Alibaba2.1) (39) showed that there are three putative NFI-binding sites located at −147/−133, −594/−580, and −676/−662 in the LO promoter region from −804 to −1, which yielded the maximal reporter gene expression (Fig. 4B).

FIGURE 6. Identification of the NFI-binding site in the subregion −612/−580 of the LO promoter. A, electrophoretic mobility supershift assay of oligonucleotide −612/−580-transcription factor complexes in the presence of different antisera. 1st lane shows the binding reaction of the 32P-end-labeled −612/−580 probe with BSA, an internal control. 2nd to 6th lanes show the binding reactions of the end-labeled −612/−580 probe with RFL6 nuclear extract (NE) in the absence (2nd lane) or presence of antibodies against Oct1 (3rd lane), CdxA (4th lane), Pit-1A (5th lane), or NFI (6th lane). Reaction products were analyzed on the native 6% polyacrylamide gel followed by autoradiography. B, sequences of LO promoter probes. Sequences of LO promoter probes containing the NFI-binding site wild type (NFI-BS-WT) and mutants (NFI-BS-Mut1, NFI-BS-Mut2, NFI-BS-Mut3, and NFI-BS-Mut4), and three positive controls, i.e., one NFI-binding site high affinity probe (NFI-BS-High) and two NFI-binding site consensus probes (NFI-BS-Con1 and NFI-BS-Con2), and a negative control probe (NFI-BS-Mut) (36). The LO promoter fragment −599/−573 is labeled with uppercase letters. The NFI-binding site −594/−580 is underlined, and the mutated nucleotides in the NFI-binding site are marked with a boldface letter. C, NFI binding to the subregion −609/−573 of the LO promoter determined by EMSA competition assays. 32P-Labeled synthetic LO promoter oligonucleotide probe −609/−573 containing NFI-BS-WT and 5 µg of nuclear extracts (NE) prepared from RFL6 cells or BSA, an internal control, were incubated in the reaction mixture in the absence or presence of 100-fold molecular excess of competitors (Comp) as indicated. After the reaction, samples were analyzed on the native 6% polyacrylamide gel followed by autoradiography. D, NFI binding to the subregion −609/−573 of the LO promoter determined by EMSA supershift competition assays. Experiments were performed as described in C, and competitors were added into the reaction mixture before addition of the antibody against NFI (Ab-NFI).
Regulation of the Rat Lysyl Oxidase Gene Promoter

The NFI-binding site (−594/−580) containing LO promoter fragment (−609/−573) was demonstrated to enhance the SV40 promoter activity (Fig. 7A). To examine whether other putative NFI-binding sites are also capable of incorporation with NFI and regulation of the LO gene expression, we carried out electrophoretic mobility shift and supershift assays with synthetic oligonucleotides spanning LO promoter sequences −158/−133, −604/−570, and −686/−652, respectively, as shown in Fig. 8A. After incubation with nuclear extracts from RFL6 cells, labeled oligonucleotides containing −147/−133, −594/−580, but not −676/−662, induced mobility shift bands in the absence of and supershift bands in the presence of the antibody against NFI in gel electrophoresis (Fig. 8B). These results indicated that in addition to the region −594/−580, the region of −147/−133 is another one of the functional NFI-binding sites in the LO promoter −804/−1.

To further confirm biological activities of these two NFI-binding sites, we used two LO promoter-reporter constructs containing NFI-binding sites −147/−133 and −594/−580 as templates. Mutations of these NFI-binding sites individually or in their combination were performed by PCR (Fig. 8C). The LO promoter-directed reporter gene expression was monitored by transfection assays in RFL6 cells. As shown in Fig. 8D, extension of the LO promoter in length from −160 to −804 enhanced the expression of the reporter gene by 18-fold. Mutations of NFI-binding sites −147/−133, −594/−580, and −147/−133 plus −594/−580 reduced LO promoter activities to 20, 58, and 42% of corresponding controls, respectively. These results provide strong evidence that there are at least two biologically active NFI-binding sites in the LO promoter region −804/−1, and the NFI binds to this cis-element enhancing the LO gene expression.

The Expression of NFI Isoforms and Their Effects on LO Promoter Activation in Rat Lung Fibroblasts—RT-PCR was performed to identify the expression of NFI isoforms in rat lung fibroblasts. As shown in Fig. 9A, although four primer pairs were designed specifically and used for amplification of different NFI isoforms, only 1.5-kb nfi-A and 1.3-kb nfi-B cDNA bands were produced following RT-PCRs indicating these two, rather than all four, NFI isoforms expressed in rat lung fibroblasts. To further assess the role of major NFI isoforms in LO promoter activation, the LO promoter (from −804 to −1)-luciferase chimera was cotransfected into RFL6 cells with NFI expression constructs containing the full-length cDNA encoding the major NFI proteins, i.e. NFI-A and NFI-B. As indicated (Fig. 9B), NFI-A and NFI-B both enhanced LO promoter activities in transfected cells reaching 3.5- and 2.7-fold of the pCDNA3.1 control, respectively, for individual transfection, and 5.8-fold of the pCDNA3.1 control, 1.7-fold of the NFI-A

incubation, luciferase and β-galactosidase activities in cell lysates were determined. Luciferase activity for each group was normalized to transfection efficiency. Cis modification of NFI-BS-WT-directed SV40 promoter activity was expressed as fold of the basal level of the pGL3-Promoter activity in cells exposed to CSC at the same dosage. Data were expressed as % of control in cells without CSC treatment (100%) = = the luciferase activity in cells transfected with pSV-NFI-BS-WT chimeric construct ÷ the luciferase activity in cells transfected with the pGL3-Promoter vector = 2.53-fold). All values represent the mean ± S.D. of three experiments, each determined with triplicate dishes. *, p < 0.05; **, p < 0.01 compared with the pSV-NFI-BS-WT basic control.

**TABLE 2. Functional analysis of the NFI-binding site −594/−580 in the LO promoter.** A, effects of NFI-BS-WT and its mutants on the SV40 promoter-directed reporter gene expression. The NFI-BS-WT and various mutants as shown in Fig. 6B were inserted upstream of the SV40 promoter in the pGL3-Promoter reporter vector. Resulting chimeric constructs were cotransfected with the plasmid pSV-β-galactosidase into RFL6 cells. After a 24-h post-transfection, cells were growth-arrested in 0.3% FBS/DMEM and incubated for 24 h in FBS-free media in the absence or presence of CSC at the indicated doses. In parallel, cells cotransfected with the pGL3-Promoter and pSV-β-galactosidase vectors were exposed to CSC under same conditions to evaluate CSC effects on the SV40 promoter basal activity. After
Regulation of the Rat Lysyl Oxidase Gene Promoter

FIGURE 8. Determination of functional NFI-binding sites in the LO promoter region. A, sequences of synthetic LO promoter fragments with putative NFI-binding sites (labeled with underlines). B, evaluation by EMSA. 32P-Labeled synthetic LO promoter fragments containing putative NFI-binding sites as shown in A and 5 μg of nuclear extracts (NE) prepared from RFL6 cells or BSA, an internal control, were incubated in the reaction mixture in the absence or presence of the specific antibody against NFI. After the reaction, samples were analyzed on the native 6% polyacrylamide gel followed by autoradiography. Reaction products are shown in lanes 1–3 with the probe NFI-BS1 (−158/−123), lanes 4–6 with the probe NFI-BS2 (−604/−570), and lanes 7–9 with the probe NFI-BS3 (−686/−652). C, schematic representation of LO promoter-reporter chimeras with or without NFI-binding site mutations. The site-directed mutations of NFI-binding sites were performed with the QuikChange mutagenesis kit. The mutated site is labeled with shaded circle with ×, and the nonmutated site is marked with shaded circle. D, relative luciferase activities of LO promoter-reporter constructs. The LO promoter-reporter constructs each as shown in C, and the pRL-TK vector, an internal control, were transiently cotransfected into RFL6 cells. Twenty four hours after transfection, cells were growth-arrested in 0.3% FBS/DMEM for an additional 24 h and then harvested for assaying the reporter gene expression. Firefly luciferase activities elicited by the LO promoter with or without mutations of NFI-binding sites were normalized to Renilla luciferase activities derived from the pRL-TK vector. Data were expressed as % of the control in cells cotransfected with pcDNA 3.1 without NFI cDNA insert and incubated in the absence of CSC. All values represent the mean ± S.D. of three experiments, each determined with triplicate dishes. ***, p < 0.001 compared with the pcDNA3.1 control without CSC treatment; (***), p < 0.001 compared with the NFI-A control without CSC treatment; (**), p < 0.001 compared with the NFI-B control without CSC treatment; (***), p < 0.001 compared with the NFI-A control without CSC treatment; ****, p < 0.01 compared with the corresponding control without CSC treatment.

FIGURE 9. Identification of the expression of NFI isoforms in rat lung fibroblasts and examination of their roles in LO promoter activation. A, the expression of NFI isoform mRNAs. Total RNA was isolated from growth-arrested RFL6 cells, and the expression of NFI isoform mRNAs was determined by RT-PCR analysis. M in lane 1, 1-kb DNA molecular marker. B, roles of NFI isoforms in LO promoter activation. RFL6 cells were cotransfected with 0.5 μg of NFI isoform expression vectors (note: 0.25 μg of NFI-A plus 0.25 μg of NFI-B in the combination assay) each as shown in B and the LO promoter (−804/−1)-reporter construct as well as the pRL-TK vector, an internal control. After a 24-h post-transfection, cells were growth-arrested for 6 h in 0.3% FBS/DMEM and incubated for 24 h in FBS-free media in the absence or presence of 80 μg/ml CSC. After incubation, firefly luciferase and Renilla luciferase activities in cell lysates were determined. Firefly luciferase activity for each group was normalized to Renilla luciferase activities derived from the pRL-TK vector. Data were expressed as % of the control in cells cotransfected with pcDNA 3.1 without NFI cDNA insert and incubated in the absence of CSC. All values represent the mean ± S.D. of three experiments, each determined with triplicate dishes. ***, p < 0.001 compared with the pcDNA3.1 control without CSC treatment; (***), p < 0.001 compared with the NFI-A control without CSC treatment; (**), p < 0.001 compared with the NFI-B control without CSC treatment; (***), p < 0.001 compared with the NFI-B control without CSC treatment; (****), p < 0.01 compared with the NFI-A control without CSC treatment; ****, p < 0.01 compared with the corresponding control without CSC treatment. These results provide additional evidence for NFI activation of the LO gene.

Cellular NFI Binding to the Cognate cis-Element in the LO Promoter—To determine cellular NFI binding to the LO promoter region, the ChIP assay was carried out using an antibody against rat NFI. As demonstrated, there were at least two functionally active NFI-binding sites located at the regions −147/−133 (site 1) and −594/−580 (site 2) in the LO promoter region; −804/−1 that displayed the maximal promoter activity (Fig. 4B and Fig. 8). Thus, these two active NFI-binding sites were tested for their cellular protein binding by using specific primer pairs as described above. Because CSC inhibited LO promoter and NFI-binding site-directed SV40 promoter activity (Fig. 7B) (16), the NFI binding status of LO DNA was examined in cells treated with CSC at various doses, and its specificity was evaluated by comparison with that of RNA polymerase II binding to the GAPDH promoter under the same conditions. According to the designed primer pairs, we anticipated that LO promoter fragments −218/−77 and −635/−516, which control, and 2.1-fold of the NFI-B control, respectively, for both transfections in combination. Significantly, such enhancement of LO promoter activity by NFI isoforms was blocked in transfected cells incubated in the presence of 80 μg/ml CSC (Fig. 9B). These results provide additional evidence for NFI activation of the LO gene.
tait active NFI-binding sites 1 and 2, respectively, would result following immunoprecipitation with the NFI antibody and amplification by PCR. Indeed, gel analysis of PCR products confirmed our expectation. Approximately 140- and 120-bp DNA bands were observed on gels as the PCR amplified using primer pairs that encompass NFI-binding sites 1 and 2, respectively (Fig. 10, A and D). In contrast, no signal was detected in control experiments with a nonspecific antibody (Fig. 10, B and E). These results suggest that the assay conditions were appropriate and can be used to measure the relative levels of NFI binding to the LO gene in response to stimuli. As shown (Fig. 10, A and D), CSC that inhibited LO promoter activity (16) markedly reduced NFI incorporation with the cis-elements at either binding site 1 (Fig. 10A) or binding site 2 (Fig. 10D) within the LO promoter region −804/-1 in a dose-dependent manner. Density measurements indicated that CSC at 40, 80, and 120 μg/ml decreased NFI incorporation with site 1 to 54, 28, and 33% of the control, and with site 2 to 53, 42, and 41% of the control, respectively. These changes apparently were not because of the difference of DNA amounts that were initially used for assays. As noted, the same cell number (2 × 10⁶) for each group was used for DNA extraction, and there was no significant difference in the yields of PCR products among groups using input (before immunoprecipitation) DNA as a template (Fig. 10, C and F). More importantly, reduction of NFI binding to the LO promoter by CSC may be a specific, epigenetic response because identical treatment of cells with CSC did not change RNA polymerase II binding to the promoter region −148/-58 (upstream of ATG) of the GAPDH gene (Fig. 10G). These results reflect the status of cellular NFI binding to the LO gene, which is highly sensitive to modification of exogenous stimuli.

**DISCUSSION**

Regulation of LO transcription is a critical control point for the LO gene expression involved in physiology and pathology of the ECM (1). This study presents the data for the rat LO gene promoter by addressing three issues. First, it identified the transcription start sites of the rat LO gene. Second, it characterized functional Inr and DPE core elements and other active subregions of the rat LO gene promoter. Third, it demonstrated the NFI-binding site as an important cis-element for transactivation of the rat LO gene.

To our knowledge, few previous studies reported structural and functional characteristics of the LO promoter in mouse (18, 19), rat, and human (17). Comparing LO promoter activities in c-Ha-ras-NIH-3T3 fibroblasts (RS485), 3T6-5 myoblast-like cells, and vascular smooth muscle cells indicated the main positive and negative cis-acting regions and binding sites for several putative transcription factors in the mouse LO promoter (40). Transcription factor AP2 binding to the control region of the mouse promoter was identified by the DNase I footprint assay (19). Analysis of over 13 kb of intervening sequence and 5′-flanking region of the human LO gene revealed a concentration of conserved consensus sequence elements within the first intron and 1-kb fragment immediately 5′ of exon 1 (17). Human LO promoter activity in luciferase reporter constructs transfected into rat aortic smooth muscle cells was markedly elevated by serum deprivation (41). However, the detailed transcriptional regulation of the LO gene is still poorly understood. Although the rat LO cDNA was cloned in 1990 (42, 43) and rat cell lines were widely used as a model for studies of LO biology, the rat LO gene promoter has not been well defined. Comparatively little is known about transcriptional control of the rat LO gene. Thus, we cloned and characterized the rat LO gene promoter in an effort to understand mechanisms for the LO transcriptional regulation.

The 5′-flanking region of the rat LO gene appears to contain no typical TATA or CAAT box. The TATA box as the first core promoter element identified in RNA polymerase II-transcribed genes is generally present 25–30 bp upstream of the transcription start site (36). Primer extension and 5′-RLM-RACE assays indicated multiple transcription start sites clustered in the rat LO promoter region from −78 to −51 relative to ATG. One major transcription start site is located at the −51 adenosine residue. The sequence from −53 to −46, *i.e.* 5′-TCATTTTTTTT-3′, overlapping with the transcription start site −51 adenosine (labeled with underline) is fully homologous to the consensus sequence of the Inr element present in many TATA-less promoters (36, 37). Notably, although alternative transcription start sites with predicted TATA boxes have been reported, sites of most putative TATA boxes are located far upstream of the proximal promoter excluding their role in the LO transcription
Regulation of the Rat Lysyl Oxidase Gene Promoter

initiation (18, 19). Moreover, other transcription start sites located at −54, −55, −57, −60, −61, and −78 identified in this study do not have any similarities with those published, indicating complex regulation of the rat LO gene transcription.

Furthermore, we found that in addition to the Inr element mapped at −53/−46 of the LO gene promoter, the sequence from −18 to −14, i.e. 5′-GGAGC-3′, upstream of ATG perfectly matches the consensus sequence of the DPE that is located at −30 bp after the adenosine residue in the Inr motif (37). Apparently, core promoter elements of the Inr in conjunction with the DPE may exist in the rat LO promoter region (Fig. 1). A variety of transcription factors has been shown to interact with the Inr element in a sequence-specific manner, including TFIID, TFII-I, and YY1. RNA polymerase II binds to the Inr element mediating the Inr-dependent transcription (37). The DPE was originally identified as a downstream core promoter binding site for purified Drosophila TFIID (44). It was reported that TFIID bound to the Inr and DPE motifs cooperatively, as mutation of either the Inr or the DPE resulted in loss of TFIID binding to the core promoter (44, 45). Thus, the Inr and the DPE coordinate function as a single core promoter unit for the RNA polymerase II-directed gene transcription (37). However, our studies indicated that the DPE in the rat LO gene promoter worked as an independent core promoter module playing a key role in the LO gene transcription. This conclusion is based on the observation that site-directed mutation of the DPE fully abolished the LO promoter activity, but the same treatment of the Inr only partially inhibited the LO promoter activity (Fig. 3, A and B). Functions of the DPE were well characterized in Drosophila (44, 45) but rarely reported in mammalian genes (44, 46). Therefore, discovery of the predominant and independent property of the DPE in comparison with the Inr in the TATA-less rat LO gene may be an unusual finding in mammalian promoters. As shown in Fig. 1, Inr and DPE core elements in the rat LO promoter are conserved in mouse and human.

To determine whether the 5′-flanking region of the LO gene is functionally active for the regulation of transcription, an ~4-kb genomic fragment spanning nucleotides from −3,979 to −1 was inserted in front of the luciferase gene in the reporter gene vector (Prom −3,979). The promoter activity was assessed by transient transfection of the Prom −3,979 construct into RFL6 cells. As shown in Fig. 4B, the Prom −3,979 induced a 50-fold increase over the pGL3 basal level in reporter gene expression indicating its LO promoter property. A series of 5′ deletions of the Prom −3,979 indicated that the smallest construct Prom −277 in which the core promoter element Inr-DPE is located retained promoter activity amounting to at least 2-fold of the pGL3-Basic control. The region from −3,979 to −278 contains four positive and two negative regulatory segments in our assay system. The maximal luciferase activity was found in the 804-bp region immediately upstream of ATG as shown in the construct Prom −804. Similar results were observed in the mouse and human LO gene promoters. As reported, an 808-bp region and a 924-bp fragment before the ATG were required for the highest reporter gene expression in fibroblasts transfected with the mouse and human LO promoter constructs, respectively (17, 18). Considering the high degree of homology in the proximal LO promoter sequences associated with the lack of the typical TATA box in rat, mouse, and human, these results suggest that the LO transcription control is likely to be regulated by similar elements in these species.

Progressive 5′ deletion assays indicated the sequence from −1 to −277 as a basic region and the sequence from −278 to −804 as a regulatory region, and both are essential for the maximal expression of the LO promoter activity. Because the expansion of sequences from −598 to −709 greatly increased luciferase activity (Fig. 4B), it was necessary to characterize this region for its transactivation ability. Four synthetic oligonucleotides derived from the sequence −709/−598 were cloned into pGL3-K promoter vector upstream of the SV40 promoter, and the resulting chimeric constructs were transiently transfected into RFL6 cells. Results showed that all four synthetic LO promoter oligonucleotides enhanced levels of the SV40 promoter activity (Fig. 5B) consistent with the data obtained from the 5′ deletion assays (Fig. 4B). Screening this region using footprint, gel shift, supershift, and competition assays demonstrated an NFI-binding site, i.e. 5′-TTGGCCTTGGGCCCAT-3′, at the region of −594/−580, and NFI binding to this sequence enhanced SV40 promoter activity (Fig. 7A). Mutating as few as one nucleotide in the LO promoter-NFI-binding site at the region of −594/−580 successfully reduced its competition for NFI binding (Fig. 6, C and D) and inhibited luciferase activities in chimeric construct-transfected RFL6 cells (Fig. 7A). These results strongly support the hypothesis that NFI is one of the transcription enhancers that binds to the cis-element located at −594/−580, facilitating LO transcription.

The LO promoter region −1 to −804 that yielded the maximal activity contains at least three putative NFI-binding sites at regions of −676/−662, −594/−580, and −147/−133. To gain insight into biological activities of all putative NFI-binding sites in the LO promoter, we performed electrophoretic mobility shift and supershift assays. Results showed that except the oligonucleotides containing the sequence of −676/−662, two oligonucleotides with the sequences −594/−580 and −147/−133 incorporated with NFI proteins probed by the specific antibody. Furthermore, site-directed mutations of the consensus TTGGC to TGAAC at the sequences −594/−580, and −147/−133 individually or in their combination strongly inhibited LO promoter activities. In toto, these results are consistent with the enhancer activity of NFI in the LO promoter. The RT-PCR assays indicated only NFI-A and NFI-B expressed in rat lung fibroblasts. Cellular NFI binding to the LO promoter region was further illustrated by the ChIP assay. More importantly, cells cotransfected with NFI-A and NFI-B expression vectors in combination displayed a greater transactivation activity (Fig. 9B) sug-
suggesting that the heterodimer of NFI-A and NFI-B may play a key role in maximal LO gene expression. NFI regulates the gene expression by multiple mechanisms (20). The C-terminal proline-rich domain is required for transcriptional modulation of an NFI site-containing promoter in Drosophila, yeast, and mammalian cells (20). A motif of the heptapeptide SPTSPSY, existing in the C-terminal proline-rich domain of NFI-C, is homologous to the C-terminal domain repeat YSPTSPS, present in RNA polymerase II, critical for transactivation of a gene. This domain was shown to interact directly with human TFIIIB or yeast TBP, triggering the transcription machinery (47, 48).

NFI-A that was expressed in rat lung fibroblasts as determined in this study contains a similar heptapeptide sequence, SPTSPTY. Thus, NFI-A may participate in transcription initiation of the TATA-less LO gene, particularly as it binds to the cis-acting element –147/–133 near the cluster of transcription start sites (–78/–51). Consistent with this suggestion, NFI-A was shown to have a stronger capacity in comparison with NFI-B to activate the LO promoter (Fig. 9B). Histone H1 inhibited the gene transcription by binding to the consensus NFI-binding sites. NFI proteins were suggested to activate the gene transcription by the direct competition with the repressor histone H1 for the DNA sites (49). In addition, a number of studies have indicated that the C-terminal proline-rich domain of NFI proteins can interact with a variety of transactivators such as TAFII55, Sp1, YY1, etc. (50). Notably, the C-terminal domain of NFI proteins was also involved in the repression of the gene expression possibly by recruitment of corepressor proteins or by interaction with the basal transcriptional apparatus (20).

The NFI regulation of gene expression is sensitive to oxidation because its DNA binding domain contains a cysteine residue susceptible for oxidative damage (21–23). We tested this possibility and showed that the luciferase reporter gene expression in RFL6 cells transfected with LO NFI-binding site (–594/–580)-SV40 promoter chimeric constructs was significantly inhibited by CSC in a dose-dependent manner (Fig. 7B). Luciferase activities in NFI-binding site-SV40 promoter chimeric construct-transfected cells treated with or without CSC were expressed relative to controls obtained from cells transfected with NFI element free pGL3-Promoter vector under the same conditions to ensure the inhibition mediated by the perturbation of the NFI and cis-element interaction by CSC. CSC inhibition of LO promoter activity was further confirmed by the cotransfection assay of NFI expression constructs with the LO promoter-reporter vector (Fig. 9B). Moreover, CSC blockage of cellular NFI binding to the LO gene was demonstrated by the ChIP assay (Fig. 10). These results indicate the high sensitivity of NFI interaction with its cognate cis-element of the LO gene in response to stimuli. As reported, each puff of cigarette smoke produces $10^{14}$–$10^{16}$ free radicals inducing oxidative damage to the lung (51). CSC contains at least 3,500 compounds, including oxidants, heavy metals, and carcinogens. CSC has been shown to inhibit LO transcription at multiple levels such as the LO promoter activity, the transcription initiation rate, and the steady-state RNA level (16). CSC induced elevated levels of cellular metallothionein and glutathione, markers for the oxidative stress, suggesting that oxidative damage may represent a key mechanism for CSC down-regulation of LO (28). In view of the molecular structural feature of the NFI-DNA binding domain, CSC components may oxidize N-terminal cysteine residues of NFI proteins, thus weakening its interaction with DNA inducing decreased cellular NFI binding to the LO gene (Fig. 10) and inhibition of the reporter gene expression (Figs. 7B and 9B).

In sum, this study describes the cloning and characterization of the LO gene promoter in rat lung fibroblasts. The Inr core promoter element encompassed the major one of multiple transcription start sites mapped at –51 upstream of the ATG, whereas the DPE core promoter element worked predominantly and independently for the activation of the TATA-less LO gene. The maximal promoter activity was displayed within an 804-bp 5′-flanking region upstream of the exon 1 composed of a basic domain from −277 to −1 and a regulatory domain from −804 to −278. Two NFI-binding sites at the sequences −594/–580 and −147/–133 were determined, which acted as an enhancer element elevating the LO promoter activity. Two NFI isoforms, NFI-A and NFI-B, were expressed in rat lung fibroblasts, and NFI-A exhibited a stronger transactivation activity than NFI-B. NF1 transactivation of the LO gene was strongly modulated in response to exogenous stimuli such as CSC. By pursuing these findings, we may elucidate mechanisms for controlling transcription of LO, a key enzyme for ECM cross-linking and remodeling.

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Regulation of the Rat Lysyl Oxidase Gene Promoter

AUGUST 31, 2007 • VOLUME 282 • NUMBER 35 • JOURNAL OF BIOLOGICAL CHEMISTRY 25337

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