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Transcriptional and Post-Transcriptional Control of Lysyl Oxidase Expression in Vascular Smooth Muscle Cells: Effects of TGF-β1 and Serum Deprivation

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Abstract Transforming growth factor-β1 (TGF-β1) markedly reduced cell proliferation and elevated steady state lysyl oxidase (LO) mRNA 3-fold in neonatal rat aorta smooth muscle cells cultured in medium containing 10% fetal bovine serum. The increase in LO mRNA was prevented by the presence of cycloheximide, indicative of controlling events at the level of protein synthesis. The basal level of mRNA in cells proliferating in 10% fetal bovine serum in the absence of TGF-β1 was enhanced 7-fold upon decreasing growth by shifting to medium containing 0.5% serum. Changes in LO activity paralleled those in LO mRNA. Nuclear run-on assays revealed that the stimulation of expression in 0.5% serum involved increased gene transcription whereas that caused by TGF-β1 was mostly post-transcriptional in origin. LO mRNA was quite labile ($t_{1/2}$ approximately 3 h) in 10% serum but was markedly stabilized ($t_{1/2} > 12$ h) by the presence of TGF-β1 in the 10% serum medium. LO mRNA was also considerably more stable under retarded growth conditions (0.5% serum) in the absence of TGF-β1. LO promoter activity in luciferase reporter constructs transfected into these cells was low and not significantly affected by the addition of TGF-β1 to the 10% serum medium but was markedly elevated by shifting from 10 to 0.5% serum in the absence of TGF-β1. Thus, LO expression is inversely correlated with cell proliferation, and is subject to control at transcriptional and post-transcriptional levels. TGF-β1 enhances LO expression in these cells by dramatically stabilizing LO mRNA. J. Cell. Biochem. 65:395–407.

Key words: lysyl oxidase; vascular smooth muscle cells; mRNA stability; collagen; elastin

The normal architecture of the arterial wall consists of concentric endothelial, intimal, medial, and adventitial layers. The arterial smooth muscle cell, the principal cellular constituent of the media, plays critical roles in the normal and diseased vessel, existing in the contractile or synthetic phenotypes. In the synthetic phenotype, these cells produce the collagen, elastin, and other matrix components of the arterial media, thus providing key elements underlying the mechanical properties of arterial tissue. In the development of atherosclerosis, medial smooth muscle cells become activated, migrate to and proliferate within the intimal space, while changing from a contractile to a synthetic phenotype. The activated smooth muscle cells can then produce abundant quantities of extracellular matrix macromolecules, which are deposited in the intima, contributing to the progressive narrowing of the vessel lumen characteristic of atherosclerotic disease [Ross, 1993]. The excess production and accumulation of insoluble matrix macromolecules is thus an issue of major concern in the pathogenesis of atherosclerosis.

Among the several posttranslational modifications involved in the production of elastin and collagen, that catalyzed by lysyl oxidase (protein-lysine 6-oxidase, EC 1.4.3.13) is pivotal to the insolubilization of these molecules in the matrix. This catalyst oxidatively deaminates peptidyl lysine in these proteins to residues of α-aminoadipic-δ-semialdehyde. The al-

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; EDTA, disodium ethylenediaminetetraacetic acid; FBS, fetal bovine serum; LO, lysyl oxidase; NRASMC, neonatal rat aorta smooth muscle cells; TGF-β1, transforming growth factor-β1.

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dehyde undergoes spontaneous condensation with adjacent aldehydes of ϵ-amino groups of unreacted lysine residues to form covalent crosslinks connecting collagen (or elastin) molecules to each other, thus insolubilizing these molecules within fibrous networks [Kagan, 1986]. Notably, the inhibition of lysyl oxidase maintains collagen in a soluble form, which is more susceptible to proteolytic degradation than crosslinked fibers of this protein [Vater et al., 1979]. Thus, lysyl oxidase is uniquely positioned to exert a key regulatory role in the formation and accumulation of proteins of the arterial matrix. Notably, levels of lysyl oxidase activity are significantly increased within early arterial lesions in an animal model of atherosclerosis [Kagan et al., 1981]. Analysis of the biological mechanisms regulating the production of this catalyst in arterial smooth muscle cells should contribute to the elucidation of potential approaches for the control of the fibrotic process in this and other fibrotic disease states.

TGF-β1 is a 25-kDa homodimeric protein member of the TGF-β superfamily of multifunctional cytokines. TGF-β1 is a potent fibrogenic stimulus and has been shown to increase the production of types I, III, V, and XI collagens [Massague et al., 1994; Lawrence et al., 1994], as well as elastin [Davidson et al., 1993], fibronectin, and proteoglycans [Lawrence et al., 1994; Merrilees and Scott, 1994]. The involvement of this cytokine in the repair response to arterial injury is indicated by findings that its administration to rabbits following balloon catheter injury significantly magnified the resulting increase in the intimal layer of carotid arteries [Kanzaki et al., 1995]. Moreover, the endogenous expression of TGF-β1 mRNA and protein in arteries increased after balloon catheter injury and was associated with overproduction of fibronectin and collagen [Majesky et al., 1991]. The fibrogenic, collagen-producing state of smooth muscle cells appears to be predominantly [Stepp et al., 1986; Rekhter and Gordon, 1994] although possibly not exclusively [Majors and Erhart, 1992; Ang et al., 1990] associated with non-proliferating cells. It is of interest in this regard that TGF-β1 inhibits smooth muscle cell proliferation with evidence that it can induce late G1 cell cycle arrest [Reddy and Howe, 1993] and extend the G2 phase of the cell cycle [Grainger et al., 1994]. Recent observations implicate alterations in levels of specific, macromolecular inhibitors of cyclin dependent kinases [Peter and Herskovitz, 1994; Mazars et al., 1995] in TGF-β1-mediated cell cycle arrest.

In the present study, we report that TGF-β1 strongly stimulates the production of lysyl oxidase by neonatal arterial smooth muscle cells in culture primarily at a post-transcriptional level, contrasting with the transcriptional activation of lysyl oxidase expression accompanying quiescence induced by serum deprivation. The constitutive production of this catalyst and its regulation by TGF-β1 appear to be related to the proliferative state of these cells.

MATERIALS AND METHODS

Neoantal Rat Aortic Smooth Muscle Cell Cultures

Smooth muscle cells were explanted from isolated medial layers of aortas of neonatal (2–3-day-old) Sprague-Dawley rats as described [Oakes et al., 1982] and as modified [Barone et al., 1985]. Briefly, bacterial collagenase (10 mg; Sigma type I, Sigma, St. Louis, MO) and porcine pancreatic elastase (2.5 mg; Sigma type III) in 20 ml of serum-free DMEM containing 3.7 g liter^{-1} sodium bicarbonate, penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) was added to minced medial layers of 20 aortas. Incubation was continued for 30 min at 37°C with stirring and the resulting cell suspension centrifuged at 400*g* for 5 min. The cell pellet was washed twice with the DMEM medium supplemented with 20% fetal bovine serum and then resuspended in 2 to 4 ml of fresh medium. The cells were then subcultivated into first passage following dispersion by incubation with 0.05% trypsin-0.02% EDTA (Gibco, Grand Island, NY) for 5 min at 37°C. The cells were subsequently seeded at 1.2×10^6 cells/100 mm dish and maintained with 10 ml of DMEM containing 10% fetal bovine serum. The cultures were routinely monitored for smooth muscle cell morphology by phase contrast microscopy. Cell numbers were determined by counting with the aid of a hemocytometer.

Lysyl Oxidase Activity Assay

Cell cultures were assayed for LO activity against a recombinant human tropoelastin substrate prepared and biosynthetically labeled with [4,5-^{3}H]-L-lysine (100 mCi mmol^{-1}; NEN, Boston, MA) as described [Bedell-Hogan et al., 1993]. Briefly, the medium was decanted from cell cultures, the cell layer rinsed in 16 mM potassium phosphate, 150 mM NaCl, pH 7.8,

and then extracted in 4 M urea, 16 mM potassium phosphate, pH 7.8, with a Dounce homogenizer. The urea extracts were dialyzed for 24 h to remove urea against 16 mM potassium phosphate, 150 mM NaCl, pH 7.8. Volume recoveries of dialyzed samples were carefully determined. Aliquots (300 µl) of the dialyzed extracts were incubated in triplicate for 2 h at 37°C against 125,000 cpm of the tritium-labeled tropoelastin substrate in assays brought to a final volume of 800 µl with 100 mM sodium borate, 150 mM NaCl, pH 8.0. Tritiated water released during the incubation was isolated by distillation in vacuo and quantified by liquid scintillation spectroscopy. All activities are presented as β-aminopropionitrile-inhibitable counts of tritium, using this irreversible inhibitor of lysyl oxidase to insure specificity of the measured enzyme activities [Tang et al., 1983]. Enzyme activities were expressed per µg of DNA, the latter determined by a modified diphenylamine procedure [Richards, 1974]. Each result documented in this report is typical of those obtained in at least three independent experiments. Results were analyzed for statistical significance by the Student's *t*-test.

DNA Synthesis

[^{3}H]Thymidine incorporation was used as a measure of DNA synthesis using a modification of the technique described [Schreiber et al., 1988]. The cells were distributed into 96 well plates (2×10^4 cells cm^{-2}), cultured for 3 days and then subcultured into the various conditions as defined in 200 µl of fresh medium containing 20 µCi ml^{-1} [^{3}H]thymidine (1 µCi/well). The cultures were then incubated for 4 h, washed with Puck's saline and cell layers treated with 0.05% trypsin in 0.02% EDTA, and hypotonically lysed with distilled water. DNA was collected directly onto Mash II glass fiber filters (grade 934AH, Bioproducts, Walkersville, MA) using a Mash II harvester (Bioproducts) and tritium incorporation was determined by liquid scintillation spectrometry.

RNA Isolation and Hybridization

Total RNA was obtained from cultured smooth muscle cells by guanidinium isothiocynate extraction [Chomczynski and Sacchi, 1987]. The RNA was fractionated on a formaldehyde-agarose gel, transferred to nitrocellulose filters and then immobilized on the filters by UV irradiation. The filters were hybridized in Quikhyb buffer (Stratagene, La Jolla, CA) according to the manufacturer's protocol, with a randomly primed ^{32}P-labeled LO rat cDNA probe corresponding to the 1.6 kb fragment released from plasmid pBSCOD with SalI restriction nuclease, as described [Trackman et al., 1992]. The hybridized filters were washed twice in 0.325 M NaCl, 0.03 M sodium citrate, 0.1% sodium dodecyl sulfate, pH 7.0, buffer at room temperature, and then twice with 16 mM NaCl, 1.5 mM sodium citrate, 0.1% sodium dodecyl sulfate, pH 7.0, at 50°C. The filters were autoradiographed with Kodak (Rochester, NY) XAR film at −80°C for 24 h. To correct for differences in loading of RNA samples to the gels, the filters were hybridized with a probe for rat glyceraldehyde 3-phosphate dehydrogenase, a transcript not expected to change significantly under the conditions of these experiments [Tso et al., 1985]. Films were developed and the image density of the bands was quantified using a Molecular Dynamics (Sunnyvale, CA) Image Quant gel scanner.

Nuclear Run-On Analysis

Following incubation for 24 h in DMEM supplemented with 0.5 or 10% serum in the presence or absence of TGF-β1 (2 ng ml^{-1}), NRSMC were lysed and nuclei isolated as described [Greenberg and Ziff, 1984]. Nuclei were suspended and incubated at 30°C for 20 min in reaction buffer (200 µl final volume) containing 10 mM Tris-HCl, pH 8.0, 5 mM magnesium chloride, 300 mM potassium chloride, 0.5 mM each of CTP, ATP, and GTP, and 150 µCi of [α-^{32}P]UTP (3,000 Ci mmol^{-1}; Dupont, NEN). The incubated reaction mixtures were extracted with phenol/chloroform (1:1), and the transcripts precipitated from the aqueous phase with isopropanol. The pellet was washed with cold 70% ethanol, resuspended in reaction buffer, and RNA precipitated by adding ammonium acetate to 2.5 M and 70% ethanol. The radioactive RNA pellets were isolated by centrifugation, washed with 70% ethanol, and pellets isolated from each culture condition were finally resuspended at 6.5×10^6 cpm ml^{-1} in 10 ml of Quickhyb hybridization buffer (Stratagene). Denatured cDNA probes (10 µg total) previously slot-blotted onto nitrocellulose filters were hybridized with equal quantities of radioactivity of the ^{32}P-labeled RNA products at 50°C for 3 days. The filters were washed as

described for Northern blot preparation and exposed to Kodak XAR film at −80°C.

Lysyl Oxidase Promoter Constructs

A human genomic lung fibroblast library [WI38 cell line-lambda Fix library (Stratagene)] was screened with an α-^{32}P random primed cDNA probed derived from the 5′ Pst fragment of the human lysyl oxidase cDNA. One clone containing an 8 kb insert was digested with XhoI/SacI to yield two fragments of 5,450 and 2,500 bp, which were subcloned into the luciferase reporter vector PGL2-basic (Promega, Madison, WI). Sequencing revealed that the 5,450 bp clone contained 245 bp of the 5′ UTR of human lysyl oxidase as well as 5,205 bp 5′ of the putative start of transcription as described previously [Hamalainen et al., 1993]. Neonatal rat aorta smooth muscle cells transiently transfected with this PGL2-lysyl oxidase promoter construct exhibited significant luciferase activity in comparison to cells transfected only with the PGL2-basic vector or the PGL2 basic containing the 2,500 bp fragment, the latter containing partial lysyl oxidase coding region and intron sequences. The −639, −520, and −308 deletion constructs of the lysyl oxidase promoter were created using convenient restriction sites, as shown (Fig. 1). Following incubation under the specified conditions, the cells were extracted and supernatants assayed for luciferase activity according to the commercially supplied protocol (Promega). Luciferase activity was normalized for transfection efficiency, determined by assays for β-galactosidase activity deriving from a co-transfected galactosidase expression vector.

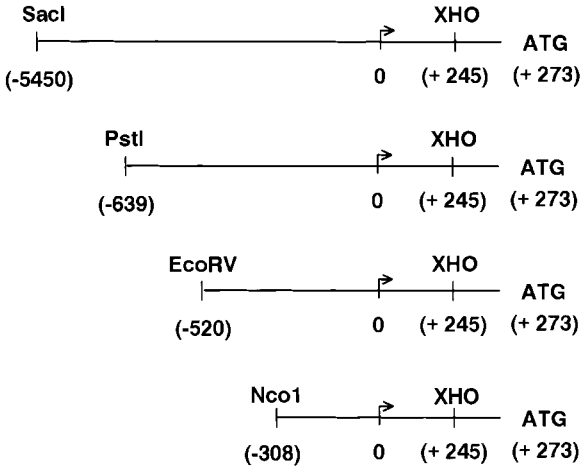

Fig. 1. Deletion constructs of lysyl oxidase promoter region.

RESULTS

Effect of TGF-β1 in Cultures of Proliferating Neonatal Rat Aorta Smooth Muscle Cells

The effect of TGF-β1 on the steady-state levels of LO mRNA was initially investigated in cultures of proliferating neonatal rat aorta smooth muscle cells. Cells were plated at a density of 1.2×10^6/100 mm dish and incubated in 10% FBS/DMEM for 3 days at 37°C. The medium was then changed to fresh 10% FBS/DMEM in the presence or absence of varied concentrations of TGF-β1 and incubation was continued for 24 h. As shown (Fig. 2), LO mRNA increased with increasing dosage of TGF-β1 with a maximum response approximated at 2 ng ml^{-1}. Concomitant with the increase in LO mRNA, the degree of cell proliferation, indexed by the uptake of [^{3}H]thymidine into DNA, decreased in the presence of TGF-β1 (Fig. 2).

The effects of TGF-β1 on the increase in cell numbers of the NRASMC was determined by plating cells as described in 10% FBS/DMEM containing TGF-β1 at 2 ng ml^{-1}. This cytokine suppressed the increase in cell numbers over a 12-day period of culture essentially completely (Fig. 3). Trypan blue exclusion studies verified the viable state of ≥98% of the cells accumulated at each time of sampling. Assessing the time dependency of the response of steady-state levels of LO mRNA to the introduction of TGF-β1 to proliferating cells in 10% FBS/DMEM, revealed that the message levels increased approximately 3-fold over the 24-h period of incubation (Fig. 4). Densities of mRNA bands for GAPDH were employed as controls for loading efficiency since the GAPDH transcript did not appear to change significantly between treatments of the cells. LO mRNA levels were elevated to the same degree at confluency independent of starting cell density, although the rate at which this maximum level was reached increased with increasing cell plating density (not shown).

The possibility that the induction of lysyl oxidase message by TGF-β1 involved events at the level of protein synthesis was assessed by determining the effect of cycloheximide on the response to this cytokine. Proliferating smooth muscle cells were cultured for 3 days in 10% FBS/DMEM. The cells were then refed with fresh aliquots of this medium supplemented

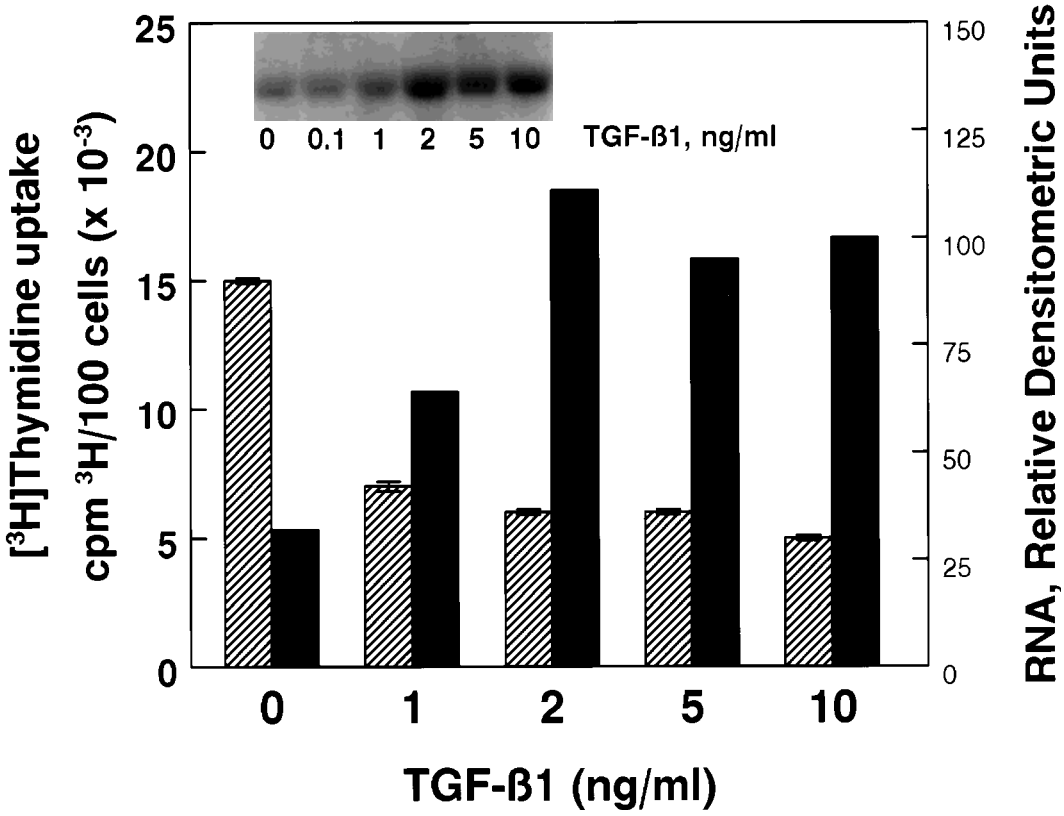

Fig. 2. Effect of varied concentrations of TGF-β1 on lysyl oxidase mRNA and thymidine uptake. Hatched bars, [^{3}H]thymidine uptake; solid bars, steady-state LO mRNA, quantified by scanning densitometry. **Inset:** Northern blot showing LO mRNA bands. Proliferating neonatal rat aorta smooth muscle cells were grown for 3 days in 10% FBS/DMEM and the medium was then changed to fresh 10% FBS/DMEM lacking (control) or containing the supplemented concentrations of TGF-β1, as indicated. Incubation was continued for 24 h before sampling for thymidine uptake and lysyl oxidase mRNA levels.

with TGF-β1 in the presence or absence of 10 μg ml^{-1} of cycloheximide, and then incubation was continued for 18 h. The results of triplicate experiments, summarized in Figure 5, indicate that the basal, steady-state levels of LO mRNA were minimally altered by the treatment with cycloheximide, whereas cycloheximide treatment blocked the increase in LO mRNA induced by TGF-β1. These results were qualitatively reproducible in other experiments in which cells were incubated under these conditions for 8 h or by using a lower concentration of cycloheximide (1 μg ml^{-1}) for 18 h, assessing these varied conditions to limit potential toxicity of cycloheximide. In each case, the induction by TGF-β1 was prevented in the presence of cycloheximide. These results point to the conclusion that the synthesis of protein(s) whose cellular concentrations are influenced by TGF-β1 are required for the stabilization of the increased levels of the lysyl oxidase transcript induced by this cytokine.

Expression of LO mRNA Accompanying Quiescence Induced by Serum Deprivation

The apparent correlation between elevated LO expression and the reduction in cell proliferation was further assessed by inducing quiescence by serum deprivation. Smooth muscle cells were plated at low density (1×10^6/100 mm dish) and allowed to grow in 10% FBS/DMEM for 3 days. The medium was then replaced with 0.5% FBS/DMEM and incubation continued. Lysyl oxidase mRNA and relative levels of DNA synthesis were determined at 24-h intervals after changing the medium to the low serum condition. As shown (Fig. 6), this change in serum concentration elicited a time-dependent increase in LO mRNA corresponding with a decrease in tritiated thymidine incorporation into DNA. Tritiated thymidine uptake was decreased 9-fold between 0 and 96 h while LO mRNA levels increased 7-fold during this time period. To determine whether the increase

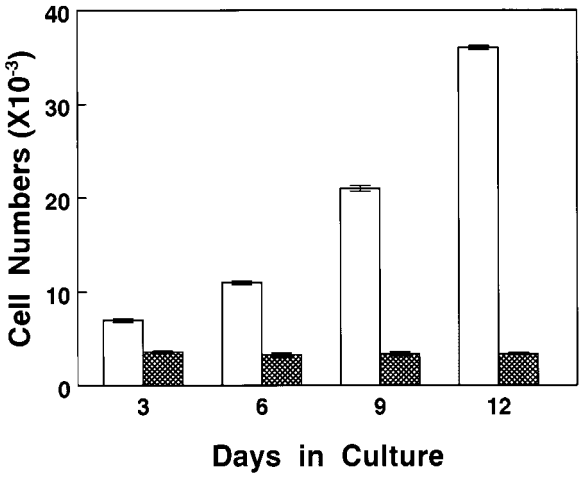

Fig. 3. Effect of TGF-β1 (2 ng ml^{-1}) on the growth of neonatal rat aorta smooth muscle cells. Open bars, control cultures; cross-hatched bars, + TGF-β1. Proliferating cells were incubated in 10% FBS/DMEM for 2 days and the medium was then replaced with fresh 10% FBS/DMEM containing or lacking (control) TGF-β1. The control and TGF-β1-supplemented conditioned medium was replaced with fresh medium every 3 days. Cells were harvested for determination of cell numbers for each of three cultures at each time point using a hemocytometer. The averages of the triplicate values are plotted.

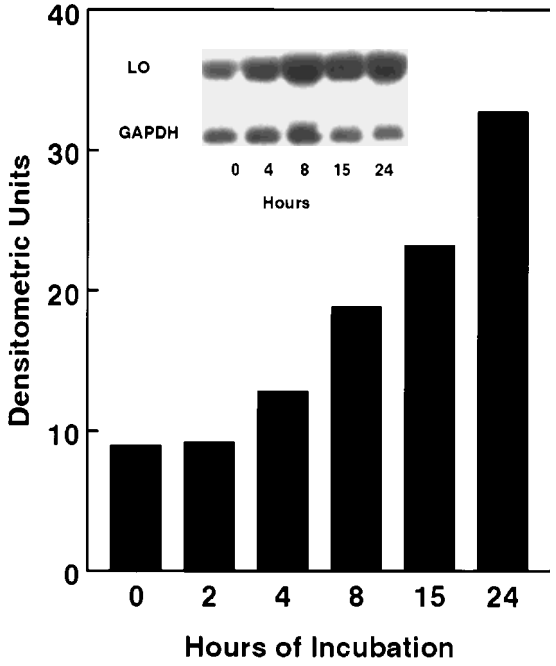

Fig. 4. Time-dependent changes in LO mRNA in response to the presence of TGF-β1 (2 ng ml^{-1}) in cells cultured in 10% FBS/DMEM. Cells were harvested at the indicated time points and RNA was isolated and analyzed as described in Materials and Methods. The solid bars represent the integrated densities of LO mRNA bands seen on Northern blots corrected for loading variations by comparison to the corresponding densities of the glyceraldehyde 3′-phosphate dehydrogenase transcript (GAPDH). A Northern blot representative of repeated experiments is shown in the **inset** of this figure. The increase in density of the GAPDH band at 8 h is due to variation in loading, established by reference to the relative fluorescence of bands in the ethidium bromide–stained gel.

accompanying the approach to quiescence was reversible, cultures of exponentially growing cells in 10% FBS/DMEM were placed under serum deprivation conditions (0.5% FBS/DMEM) for 72 h followed by incubation once again in fresh 10% FBS/DMEM for 12 and 24 h to re-initiate progression into the S-phase of the cell cycle. As shown (Fig. 6, inset), LO mRNA increased over a 96-h period following the change to the low serum growth medium. The subsequent change of the low serum to the high serum condition resulted in a decrease of LO mRNA to the decreased levels of proliferating cells. Thus, LO mRNA expression is reversibly coordinated with the state of proliferation of the arterial smooth muscle cells, becoming elevated in the approach to quiescence and markedly decreased in the proliferative state. The addition of TGF-β1 to smooth muscle cells, which had been incubated for 72 h under growth-limiting conditions in 0.5% FBS/DMEM, did not alter the elevated levels of LO mRNA significantly (not shown).

To confirm that the changes seen in lysyl oxidase mRNA were accompanied by changes in levels of the enzyme produced by these cells, the specific enzyme activity of lysyl oxidase was determined against a tropoelastin substrate in cultures that had been incubated for 3 days in 10% FBS/DMEM followed by subculturing for 3 days either in fresh 10% FBS/DMEM in the presence and absence of 2 ng ml^{-1} of TGF-β1 or in 0.5% FBS/DMEM. Since the bulk of the lysyl oxidase activity occurs in these cultures as a urea-extractable form bound to the cell layer-matrix fraction, relative levels of enzyme activity were determined by assays of dialyzed aliquots of urea extracts of the cell layers, as described. As shown (Fig. 7), cultures maintained in a proliferative state by continuous incubation in 10% FBS exhibit low levels of lysyl oxidase activity and, as expected, high levels of [^{3}H]thymidine uptake. The presence of TGF-β1 in cultures maintained in 10% FBS markedly elevated levels of lysyl oxidase activity (5-fold) and markedly decreased thymidine uptake (10-fold). Similarly, changing from the 10% FBS (proliferative) to the 0.5% FBS conditions, as described, resulted in essentially the

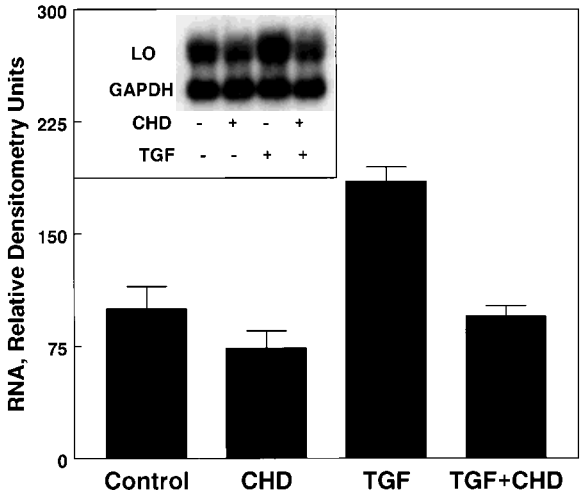

Fig. 5. Effects of cycloheximide on steady-state levels of LO mRNA. The heights of the bars, expressed as percentages of the control incubation lacking TGF and CHD, represent the averages of band densities determined in triplicate Northern blot assays, with standard deviations as shown. A typical Northern blot result is shown in the **inset.** Cultures were incubated with 10% FBS/DMEM supplemented with 2 ng ml^{-1} TGF-β1 (TGF) and/or cycloheximide (CHD; 10 μg ml^{-1}), as indicated [(+) = present; (−) = absent]. The cells were incubated under these conditions for 18 h and harvested for mRNA analysis.

same changes in lysyl oxidase activity and DNA synthesis seen in the cultures incubated with TGF-β1 in 10% FBS. Thus, the proliferation-associated and cytokine-dependent changes in LO mRNA seen in this study are accompanied by corresponding changes in the functional levels of the mature protein product of lysyl oxidase gene expression.

Relative Transcription Rates

Transcription run-on experiments revealed that the rate of accumulation of newly synthesized LO transcript synthesized by nuclei isolated from NRASMC, which had been incubated in 10% FBS/DMEM supplemented with TGF-β1, approximated that of cells grown in this medium in the absence of added cytokine (Fig. 8). Transcript levels produced by nuclei obtained from cells incubated for 72 h in 0.5% FBS/DMEM were elevated over those seen in the medium supplemented with 10% FBS in the presence or absence of TGF-β1. Controls in this experiment included the hybridization of equal quantities of radioactivity of newly labeled transcripts to slots blotted with the pUC19 plasmid lacking LO cDNA as well as slots blotted with cDNA for GAPDH. There appears to be a slight increase in the density of the GAPDH band in 0.5% FBS, apparently reflecting a modest increase in transcription of this gene in the lower serum condition. Nevertheless, the increase in the LO transcript of the cells grown in 0.5% FBS remains elevated over that of the 10% FBS control regardless of whether the data are normalized for the GAPDH band or not. Thus, relating the absolute densities of the LO transcript bands in 10 and 0.5% FBS yields a value of 3.1-fold increase in the lower serum concentration; when the ratios of the densities of the LO bands relative to those of the GAPDH band are compared, this value is 1.8-fold. The pUC19 controls exhibited uniformly negligible levels of hybridization (not shown). These data indicate that the increase in steady-state levels of LO mRNA induced by the cytokine in 10% serum is not primarily due to stimulation of LO transcription. In contrast, the increase in LO mRNA seen in response to serum deprivation significantly reflects transcriptional activation of the lysyl oxidase gene.

mRNA Stability

The possibility was assessed that TGF-β1 and/or serum deprivation may alter lysyl oxidase mRNA stability under the varied growth conditions. NRASMC initially cultured in 10% FBS/DMEM were then incubated in 0.5% FBS/DMEM for 72 h to induce LO mRNA. The cultures were then refed with fresh 0.5% FBS/DMEM or 10% FBS/DMEM, the latter in the presence and absence of TGF-β1 (2 ng ml^{-1}) and incubated for 24 h. Actinomycin D was then added to each refed culture to a final concentration of 2 μg ml^{-1} and incubation was continued for 24 h with sampling of cultures at intervals for LO mRNA levels, as described. As shown (Fig. 9), the $t_{1/2}$ of LO mRNA stimulated with TGF-β1 in 10% FBS/DMEM exceeded 12 h while that of cultures refed with 10% FBS without added TGF-β1 was decreased markedly to ~3 h. While the steady-state mRNA levels remained unchanged during the 24 h sampling period in cultures continuously incubated with 0.5% FBS/DMEM in the absence of actinomycin D (not shown), some decay of message was seen in cultures incubated under these conditions but with the addition of actinomycin D at the start of the sampling period ($t_{1/2} \sim 22$ h). Corresponding experiments in which de novo transcript synthesis was prevented by 60 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole rather than actinomycin D also revealed the

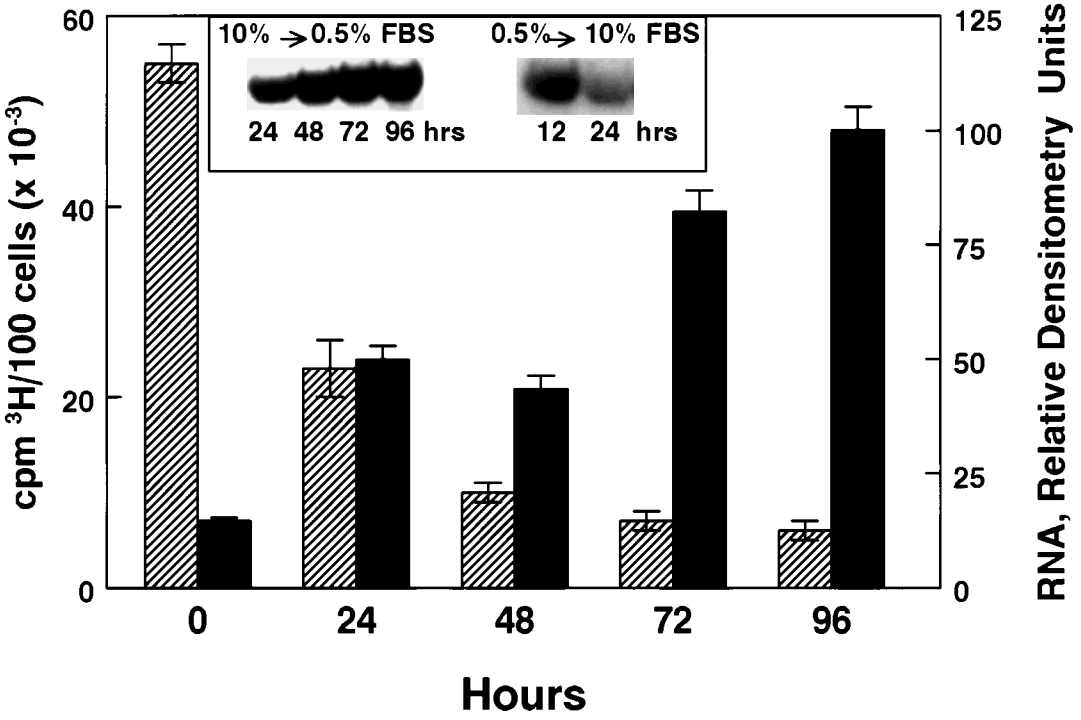

Fig. 6. Effect of serum deprivation on the expression of LO mRNA and on [^{3}H]thymidine uptake by rat aorta smooth muscle cells. Hatched bars, [^{3}H]thymidine uptake; solid bars, steady-state LO mRNA, quantified by scanning densitometry. NRASMC were grown to day 3 in first passage in 10% FBS/DMEM and then the medium was changed to fresh 10% FBS/DMEM. The heights of the solid bars and vertical lines represent the averages and range, respectively, of duplicate analyses. Lysyl oxidase mRNA band densities were determined relative to those of GAPDH and expressed relative to the resulting value for the 96-h samples. **Inset:** Reversibility of the effect of serum concentration on LO mRNA levels. The LO mRNA bands shown on the left portion of the inset were derived from cultures grown to day 3 in first passage in 10% FBS/DMEM. At time zero, the medium was replaced with fresh 0.5% FBS/DMEM. On the right portion of the inset, the LO mRNA bands are those of these cell cultures in which the medium was changed to 10% FBS/DMEM after the 96-h incubation (time zero) to fresh 0.5% FBS/DMEM.

markedly increased lability of the message in cells incubated in 10% FBS/DMEM, while the presence of TGF-β1 in this medium or incubation of cells in 0.5% FBS/DMEM markedly stabilized LO mRNA. Thus, the stimulation of LO expression by the addition of TGF-β1 to cells growing in 10% FBS is predominantly due to the TGF-β1-dependent stabilization of lysyl oxidase mRNA. In view of the significant difference in stability seen in cultures transferred from 10 to 0.5% FBS, the increase in steady-state levels of LO mRNA accompanying this perturbation also involves increased mRNA stabilization, as well as transcriptional stimulation.

Lysyl Oxidase Promoter Activity in Luciferase Reporter Constructs

As shown (Fig. 10), luciferase activity in extracts of cells grown in 0.5% FBS is significantly elevated in cells transfected with the −5450, −639, and −520 constructs, relative to the promoter-free vector (pGL2) control, and relative to that of the cells grown in 10% FBS or 10% FBS + TGF-β1. These results are consistent with the nuclear run-on data, indicating that the effect of TGF-β1 is exerted at a post-transcriptional level while that of serum deprivation involves transcriptional level stimulation of lysyl oxidase expression in addition to post-transcriptional mechanisms. These results also indicate that the 331 bp region between −639 and −308 appears to contain sequence element(s) modulating the stimulation of transcription induced by the withdrawal of serum. Since the −639 construct exhibits the highest activity, this may indicate the presence of suppressor element(s) in the larger construct (−5450).

DISCUSSION

Expression of LO mRNA in cultured NRSMC is strongly correlated with the growth state of the cells. Proliferating cells express low levels

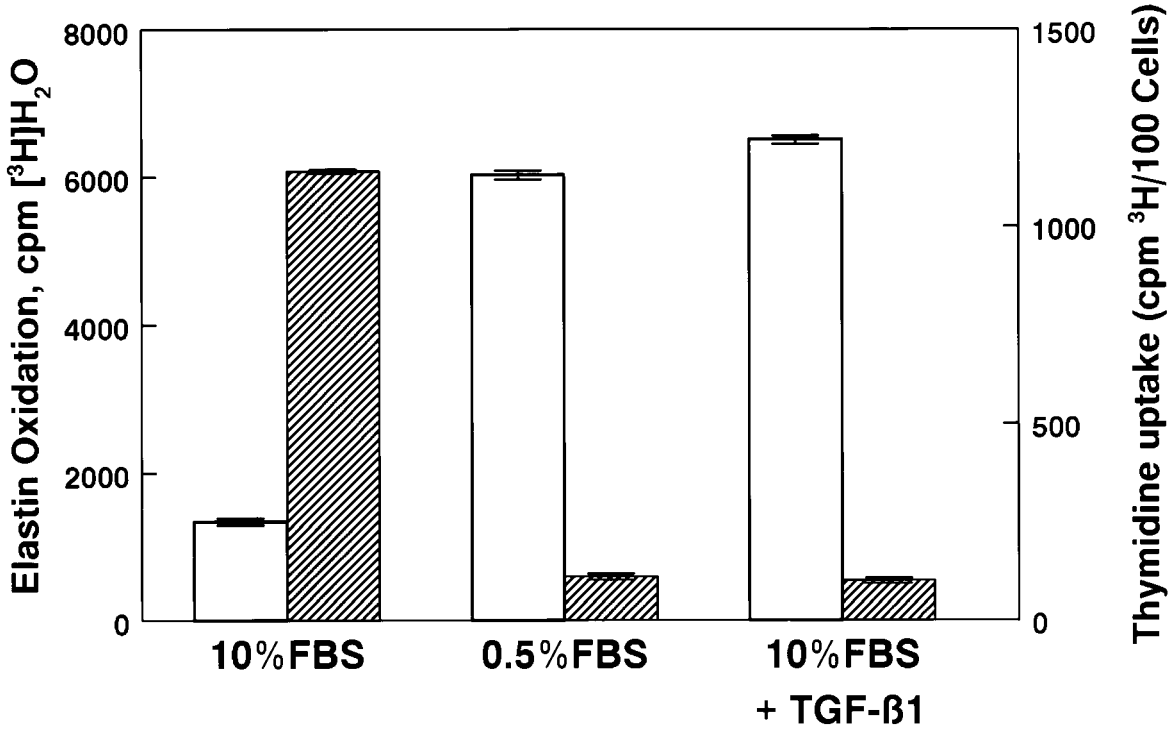

Fig. 7. Effects of TGF-β1 and serum on lysyl oxidase activity. Solid bars, LO activity; hatched bars, [^{3}H]thymidine uptake. Cells were grown in 10% FBS/DMEM in first passage for 3 days and then the medium was changed to fresh 10% FBS/DMEM in the presence or absence of 2 ng ml^{-1} TGF-β1; or the medium was changed to fresh 0.5% FBS/DMEM and incubation continued for 72 h, as shown. Means and standard deviations of triplicate analyses are shown.

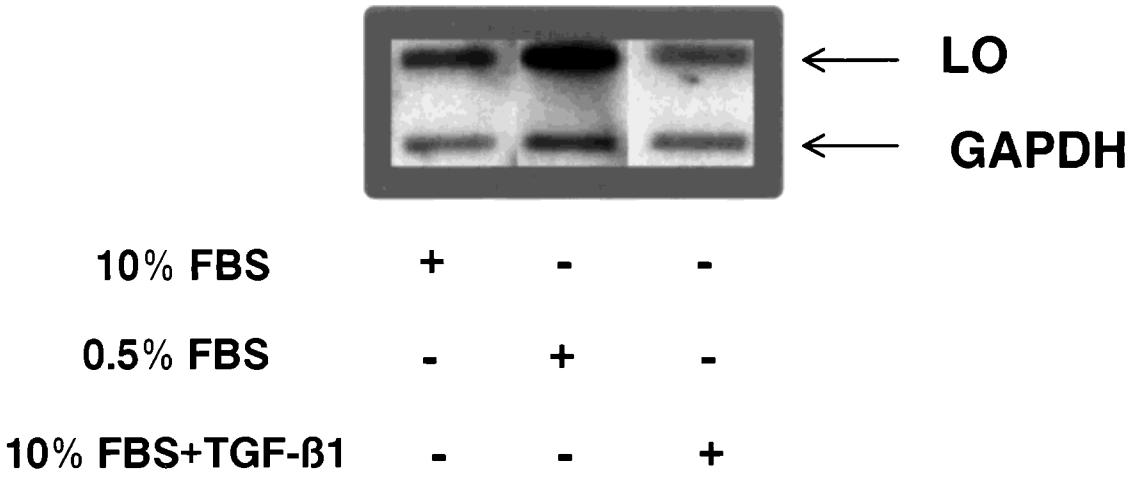

Fig. 8. Nuclear run-on transcription assays. Cells were grown in 10% FBS/DMEM to day 3 in first passage (10% FBS); or, after growth in 10% FBS/DMEM, the medium was replaced with fresh 0.5% FBS/DMEM (0.5% FBS) or fresh 0.5% FBS/DMEM supplemented with 2 ng ml^{-1} TGF-β1 (10% FBS + TGF-β1). Nuclear run-on assays were carried out as described in the text.

of LO mRNA whereas message levels dramatically increase upon the approach to quiescence by deprivation of serum. The reciprocal relationship of lysyl oxidase expression with the state of proliferation was also upheld when the high levels of enzyme expression of cells cultured under serum deprivation conditions markedly decreased when these cells were stimulated to proliferate by increasing the serum concentration. Consistent with the effect of serum depri-

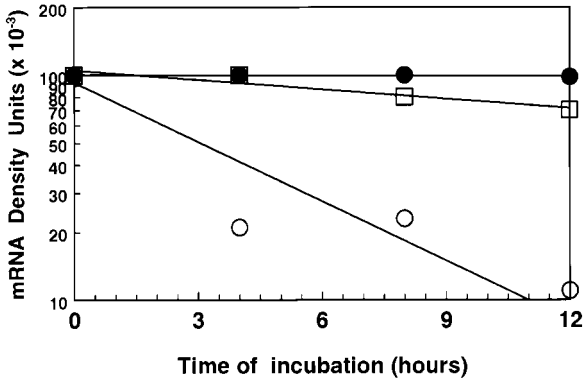

Fig. 9. Effect of TGF-β1 and serum deprivation on stability of LO mRNA. Cultures were incubated in 0.5% FBS/DMEM for 72 h prior to the onset of the experiment. The medium was then changed to medium supplemented with 2 μg ml^{-1} of actinomycin D in the following media: 10% FBS/DMEM (○); 10% FBS/DMEM containing 2 μg ml^{-1} of TGF-β1 (●); fresh 0.5% FBS/DMEM (▲). Northern blots of total RNA isolates were hybridized with the lysyl oxidase-specific cDNA probe and, following autoradiography, the densities of the LO bands were determined as described.

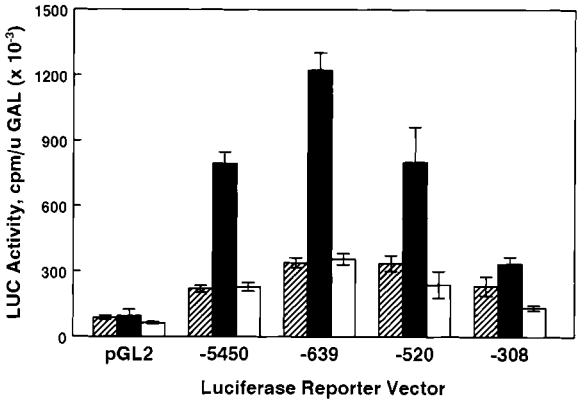

Fig. 10. Lysyl oxidase promoter modulation of luciferase activity in smooth muscle cells. Cells transfected with luciferase reporter constructs were cultured in 10% FBS/DMEM (hatched bars); in 0.5% FBS/DMEM (solid bars); or 10% FBS/DMEM supplemented with 2 ng ml^{-1} TGF-β1 (open bars). pGL2, promoter-free vector; −5450, −639, −520, and −308: luciferase reporter constructs driven by fragments of lysyl oxidase promoter as defined in Materials and Methods.

vation, TGF-β1 suppressed the proliferative state, indexed here by thymidine uptake, as it up-regulated lysyl oxidase expression.

Nuclear run-on assays indicated that the 7-fold increase in steady-state message levels accompanying serum deprivation derives in part from an approximately 3-fold stimulation of lysyl oxidase gene transcription and in part from the significant stabilization of the lysyl oxidase message accompanying this perturbation. The marked stability of the message seen under low serum conditions is consistent with the considerable stability ($t_{1/2} > 14$ h) previously reported for the lysyl oxidase message in quiescent IMR90 cells [Roy et al., 1996]. In contrast, the stimulation of enzyme expression by TGF-β1 in 10% serum may involve some, although thus far undetectable, increase in transcription of the lysyl oxidase gene but is primarily due to the post-transcriptional stabilization of steady-state mRNA levels, which otherwise decay rapidly in the high serum growth conditions in the absence of TGF-β1. Thus, transcript stabilization appears to be the predominant mode of induction of lysyl oxidase by this cytokine in these cells. It is of interest that this cytokine was reported to enhance lysyl oxidase steady-state mRNA levels and enzyme activity in osteoblastic cells [Feres-Filho et al., 1996]. While that study did not assess whether mRNA stabilization might underlie the increase in steady-state mRNA levels, it was demonstrated that post-translational control, reflecting increased processing of the proenzyme to the mature catalyst, contributed to the increase in lysyl oxidase activity. The correlation between the increase in enzyme activity and mRNA expression noted in the present study suggests that this level of control is not as significant in the arterial smooth muscle cells. It should also be noted that TGF-β1 significantly enhanced lysyl oxidase message levels in quiescent cultures of IMR90 fibroblasts [Roy et al., 1996], in contrast with the negligible effect of this cytokine on the intrinsically enhanced levels of lysyl oxidase message of quiescent arterial smooth muscle cells cultured in 0.5% serum observed in the present study. Thus, specific mechanisms of regulation of lysyl oxidase appear to differ in these different types of fibrogenic cells.

Since the induction by TGF-β1 under proliferating conditions is prevented by the inhibition of protein synthesis by cycloheximide, it is presumed that TGF-β1 induces the increased levels and/or activities of protein(s), which can potentially stabilize lysyl oxidase mRNA. Precedent for this possibility has recently been reported in the case of the TGF-β1-induced expression of ribonucleotide reductase in BALB/c 3T3 fibroblasts. TGF-β1 induces a cytosolic protease-sensitive factor, which forms a 75 kDa complex with a novel 9 nucleotide *cis* element, 5′-

GAGUUUGAG-3′ site, in the 3′-UTR, apparently stabilizing the reductase mRNA [Amara et al., 1995]. Available sequence does not reveal the presence of a corresponding element in the lysyl oxidase 3′ UTR. Nevertheless, the 3′ UTR of rat lysyl oxidase mRNA does contain an AU-rich region similar to those previously shown to mediate high turnover of other mRNA species [Sachs, 1993]. Moreover, this 3′ UTR is large (3.5 kb) and thus may also contain specific elements yet to be defined which may be linked to the TGF-β1-induced stabilization of its message. It is of particular interest, in view of the present results, that TGF-β1 is a potent inducer of elastin in human skin fibroblasts largely as a result of the stabilization of elastin mRNA resulting from exposure of cells to the cytokine [Zhang et al., 1995]. Indeed, the markedly decreased production of elastin by skin fibroblasts derived from a patient with inherited cutis laxa was reversed by the 10-fold stabilization of the elastin message resulting from incubation of the cells with TGF-β1 [Kähäri et al., 1992]. The decrease in rat lung elastin mRNA as rats progress through adulthood has also been shown to be due to post-transcriptional mechanisms [Swee et al., 1995]. In contrast, the up-regulation of α1(I) and α2(I) procollagen expression by TGF-β1 is transcriptional in origin [Ritzenthaler et al., 1993; Chang and Goldberg, 1995], with evidence reported for a TGF-β1 activation element within the α1(I) collagen gene [Ritzenthaler et al., 1993]. Thus, while common mechanisms might exist for the induction of lysyl oxidase and its elastin substrate by this fibrogenic cytokine, this appears to be unlikely in the case of the type I collagen substrate.

The involvement of transcriptional control in the up-regulation of lysyl oxidase message accompanying retardation of growth by serum deprivation and the apparent lack of involvement of transcriptional control in response to TGF-β1 in high serum are consistent with the results of the assays for lysyl oxidase promoter activity in response to these perturbations. Indeed, cells transfected with the lysyl oxidase promoter-luciferase reporter constructs were markedly elevated over controls transfected with constructs lacking lysyl oxidase promoter sequences when cultures were incubated in 0.5% FBS/DMEM, whereas, when added, TGF-β1 did not significantly alter the depressed promoter activity seen in cells incubated in 10% FBS/DMEM, alone. Minimally, the results suggest the presence of activating element(s) within the 331 bp region between the −639 and −308 fragments, which respond to the decreased growth state induced by the withdrawal of serum, the identity of which are under investigation. A recent report describes the functional responses of putative *cis*-acting elements in the lysyl oxidase promoter. Thus, an interferon regulatory factor (IRF-1) stimulates lysyl oxidase transcription and binds to a consensus IRF-1 *cis* element at −886 to −898 of the mouse lysyl oxidase gene promoter [Tan et al., 1996]. This activity of IRF-1 has been suggested to be related to the apparent tumor suppressive activity accompanying lysyl oxidase expression in *ras*-transformed cells [Tan et al., 1996; Kenyon et al., 1991].

The effects of TGF-β1 on the cell cycle and on the expression of extracellular matrix genes are varied and complex. Although the production of matrix macromolecules, including collagens types I, III, V, and XI, elastin and fibronectin, occurs predominantly in the quiescent state in vascular smooth muscle cells [Stepp et al., 1986; Rekhter and Gordon, 1994], interactions between factors associated with the quiescent cellular response to TGF-β1 and the activation of matrix synthesis by this cytokine are not well understood. For example, TGF-β1 appears to promote growth arrest in G_1 by its induction of an inhibitor of cyclin-dependent protein kinase complexes involved in cell cycle control [Li et al., 1995]. These complexes seem essential to phosphorylate and thus inactivate the retinoblastoma gene product, which in turn inhibits cell cycle progression in its underphosphorylated state. Nevertheless, if retinoblastoma gene function is blocked, TGF-β1 can still stimulate α2(I) collagen and plasminogen activator inhibitor-1 gene expression [Chang and Goldberg, 1995]. Thus, TGF-β1-induced factors, whether associated with its effect on the cell cycle or not, may directly or indirectly be involved in the regulation of lysyl oxidase expression noted here to be exerted predominantly at the post-transcriptional level of processing of LO mRNA.

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