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Cloning of Rat Aorta Lysyl Oxidase cDNA: Complete Codons and Predicted Amino Acid Sequence†,‡

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ABSTRACT: Lysyl oxidase cDNA clones were identified by their reactivity with anti-bovine lysyl oxidase in a neonatal rat aorta cDNA λgt11 expression library. A 500-bp cDNA sequence encoding four of six peptides derived from proteolytic digests of bovine aorta lysyl oxidase was found from the overlapping cDNA sequences of two positive clones. The library was rescreened with a radiolabeled cDNA probe made from one of these clones, thus identifying an additional 13 positive clones. Sequencing of the largest two of these overlapping clones resulted in 2672 bp of cDNA sequence containing partial 5′- and 3′-untranslated sequences of 286 and 1159 nucleotides, respectively, and a complete open reading frame of 1227 bp encoding a polypeptide of 409 amino acids (46 kDa), consistent with the 48 ± 3 kDa cell-free translation product of rat smooth muscle cell RNA that was immunoprecipitated by anti-bovine lysyl oxidase. The rat aorta cDNA-derived amino acid sequence contains the sequence of each of the six peptides isolated and sequenced from the 32-kDa bovine aorta enzyme, including the C-terminal peptide with sequence identity of 96%. Northern blots screened with lysyl oxidase cDNA probes identified hybridizing species of 5.8 and 4.5 kb in mRNA of rat aorta and lung, while dot blot analyses were negative for lysyl oxidase mRNA in preparations of rat brain, liver, kidney, and heart. A 258-bp segment of the 3′-untranslated region of lysyl oxidase cDNA is 93% identical with a highly conserved region of the 3′-untranslated sequence of rat elastin cDNA. Southern blotting of rat genomic DNA with lysyl oxidase cDNA probes indicated that the lysyl oxidase gene is located at a single locus and does not appear to be a member of a multigene family. A potential stem–loop structure was found in the 3′-untranslated region of the cDNA. The deduced amino acid sequence contains a putative signal peptide, in addition to sequences that are similar to those of other known copper proteins.

Lysyl oxidase is the copper-dependent amine oxidase responsible for the posttranslational oxidative deamination of peptidyl lysine residues in precursors to fibrous collagen and elastin. The resulting α-aminoadipic δ-semialdehyde residues can undergo nonenzymatic condensation to form the lysine-derived cross-links found in mature collagen and elastin (Pinnell & Martin, 1968). Increased lysyl oxidase activity has been associated with fibrotic diseases including atherosclerosis, hypertension, and pulmonary fibrosis (Kagan, 1986).

Little is known about the nature or processing of biosynthetic precursors of lysyl oxidase or how the biosynthesis of the enzyme may be coordinated with that of its elastin and collagen substrates. Morever, much remains to be learned about its structure, including the sequence of residues that covalently link the PQQ[1] cofactor to the protein (Williamson et al., 1986; van der Meer & Duine, 1986), and the physical–chemical basis of the ionic variants commonly found in preparations of lysyl oxidase (Sullivan & Kagan, 1982; Williams & Kagan, 1985). It is likely that many of these aspects of the structure, function, and biosynthesis of lysyl oxidase may be elucidated by analysis of the genetic apparatus coding for this enzyme.

Accordingly, we describe in this report the identification and sequence analysis of lysyl oxidase cDNA clones, noting evidence by cDNA sequencing and cell-free translation for a 46-kDa polypeptide that contains the sequence of the mature 32-kDa enzymatically functional protein normally isolated from connective tissues. Moreover, a sequence has been identified in the 3′-noncoding region of lysyl oxidase cDNA that is nearly identical with a highly conserved 3′-untranslated region of elastin cDNA.

Materials and Methods

Enzyme Purification and Assay. Lysyl oxidase was purified from bovine ligamentum nuchae as previously described for the isolation of the bovine aorta enzyme (Williams & Kagan, 1985). Bovine aorta lysyl oxidase was also purified by this method as well as by a modification of this procedure in which Bio-Gel HTP hydroxylapatite (Bio-Rad) was substituted for the Cibacron-Blue Sepharose column (manuscript in preparation).

Peptide Isolation and Sequencing. Bovine aorta lysyl oxidase (3.4–7.8 μM) was precipitated in 10% TCA and then digested with TLCK–chymotrypsin or TPCK–trypsin (1:25 w/w) as described (Williams et al., 1984). Peptides were purified by HPLC, initially resolving digests on a Dynamax C-8 reverse-phase column (0.46 × 25 cm, 5 μm) and subsequently purifying to apparent homogeneity on a C-18 column (Rainin) using 0.05% TFA/acetonitrile gradients (Williams et al., 1984). Purified peptides were sequenced on an Applied Biosystems Model 470A gas-phase protein sequencer.

Preparation of Antibodies. Anti-lysyl oxidase was prepared in rabbits by intradermal injection of 40-μg aliquots of purified bovine ligament lysyl oxidase suspended in complete Freund's

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‡ The nucleic acid sequence in this paper has been submitted to GenBank under Accession Number J02903.

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[1] Abbreviations: PFU, plaque-forming units; PTH, phenylthiohydantoin; 20× SSPE, 3 M NaCl, 0.2 M NaH_2PO_4, and 0.02 M EDTA, pH 7.4; 20× SSC, 3 M NaCl and 0.3 M sodium citrate, pH 7.0; SDS, sodium dodecyl sulfate; PQQ, pyrroloquinoline quinone; PAGE, polyacrylamide gel electrophoresis; SMCC, 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

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adjuvant. *Bortadella pertussis* vaccine (0.5 mL) was injected subcutaneously. Further injections of lysyl oxidase (40 μg) mixed with incomplete Freund's adjuvant were given at 2, 4, 6, and 8 weeks after the first injection. The antibody did not recognize bovine plasma fibronectin, calf skin collagen, or α-elastin but did recognize the 32-kDa lysyl oxidase band in 4 M urea extracts of bovine aorta, rat lung, rat aorta, and chick aorta as well as purified rat lung and bovine aorta lysyl oxidase, as indicated by Western blotting with these protein preparations.

Antibodies were prepared according to established methods (Rothbard et al., 1984; Gariepy et al., 1986) against the purified synthetic peptide Cys-Val-Ser-Val-Asn-Pro-Ser-Tyr-Leu-Val-Pro-Glu-Ser-Asp-Tyr-Ser-Asn-Asn-Val-Val-Arg synthesized by *tert*-butyloxycarbonyl chemistry (Stewart & Young, 1984) on a Sam II peptide synthesizer (Biosearch) to correspond to the sequence determined for a peptide isolated from a tryptic digest of bovine aorta lysyl oxidase.

Identification of cDNA Clones. (A) Anti-Lysyl Oxidase Probe. A cDNA library in vector λgt11 made from neonatal rat aorta RNA was purchased from Clontech Laboratories, Inc., Palo Alto, CA. Primary screening with anti-lysyl oxidase raised against the purified bovine enzyme was performed by using *Escherichia coli* Y1090 as host cells and plating 5×10^4 PFU/90-mm plate (Mierendorf et al., 1987). A commercially available neonatal rat aorta cDNA library was chosen for screening in part because aorta and lung lysyl oxidase activity is highest in neonatal animals, decreasing significantly after 10 weeks of age (Brody et al., 1979). Moreover, as noted, the anti-lysyl oxidase antibody cross-reacts with purified 32-kDa rat lung lysyl oxidase and with lysyl oxidase in a crude extract of rat aorta as analyzed on Western blots. The appropriate probe was available, therefore, to screen a cDNA library likely to contain a significant number of lysyl oxidase cDNA clones. Attempts to clone lysyl oxidase from a smooth muscle cell library derived from 2-week-old calf aorta using synthetic oligonucleotide probes were unsuccessful, presumably because of a low amount of lysyl oxidase mRNA. These results are consistent with our experience indicating that there is considerably less lysyl oxidase activity in cultures of 2-week-old calf aorta smooth muscle cells compared to neonatal rat aorta smooth muscle cells cultured under comparable conditions.

(B) Radiolabeled cDNA Probe. cDNA from clone IIB was isolated from λgt11 DNA (Maniatis et al., 1982a) and then labeled to a specific activity of 1×10^9 cpm/μg (Feinberg & Vogelstein, 1983). Screening of the neonatal rat aorta cDNA library was carried out by using *E. coli* Y1090 host cells and plating 5000 PFU/90-mm plate (Benton & Davis, 1977). The concentration of probe used in the hybridization solution was 0.2–0.5 ng/mL. Positive clones were identified by autoradiography and were replated until pure. cDNA inserts from the two largest clones were subcloned into pUC18 and grown in *E. coli* JM83 host bacteria.

Isolation of RNA. Calf aorta smooth muscle cells were grown in Dulbecco's Vogt medium containing 50 μg/mL sodium ascorbate and were harvested 13 days after second passage (Beldekas et al., 1981). RNA was isolated from these cells (Chirgwin et al., 1979) and from the lungs and aortas of 200-g Sprague–Dawley rats, as described (Chomczynski et al., 1987). RNA isolated from normal rat brain, heart, liver, kidney, and aorta (Chirgwin et al., 1979) used in the dot blot analysis was the gift of Dr. Kevin P. Claffey and Dr. Peter Brecher of Boston University School of Medicine. Poly(A)+ RNA was isolated as described (Maniatis et al., 1982b). Total cellular RNA was purified from neonatal rat aorta smooth muscle cells that had been cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal calf serum and 1 nM dexamethasone and was the gift of Dr. B. Leslie Wolfe and Dr. Carl Franzblau of Boston University School of Medicine. Cells were harvested after 2 weeks in culture following first passage.

Dot Blot and Northern Blot Analysis. Northern blots (Maniatis et al., 1982c) and dot blots (Davis et al., 1986a) were performed as described with nitrocellulose filters (Bio-Rad). Prehybridization (3 h) and hybridization (overnight) of the labeled probe to dot blots and Northern blots were performed at 42 °C in a solution of 50% formamide, 4× SSPE, 5× Denhardt's solution, 0.2% SDS, 50 μg of sheared and denatured salmon sperm DNA/mL, and 0.05% disodium pyrophosphate. The concentration of the labeled cDNA insert (Feinberg & Vogelstein, 1983) was 1 ng/mL in the hybridization solution. Blots were washed for 1 h with 200 mL of each of the following solutions: 2× SSC and 0.1% SDS, 37 °C (twice); 2× SSC and 0.1% SDS, 42 °C (once); 0.2× SSC and 0.1% SDS, 50 °C (once); 0.1× SSC and 0.1% SDS at 60 °C (once). Blots were dried and placed on Kodak XAR 5 film with an intensifying screen at −80 °C for 5 days for autoradiographic analysis.

Western Blotting. SDS–PAGE was performed by using a separating gel of 12.5% acrylamide (Laemmli, 1970), and Western blotting was performed by using goat anti-rabbit IgG coupled with horseradish peroxidase as the second antibody (Davis et al., 1986b). Primary antibodies were diluted 1:500.

Cell-Free Translation and Immunoprecipitation. Six micrograms of total RNA isolated from neonatal rat smooth muscle cells cultured as described was translated in vitro in reticulocyte lysate (Promega) containing 120 μCi of [^{35}S]-cysteine (1118 Ci/mmol) in a final volume of 100 μL, incubating at 30 °C for 1.5 h. Unlabeled cysteine was then added to a final concentration of 2 mM. Mixtures were then boiled in SDS and immunoprecipitated with anti-lysyl oxidase or preimmune serum, with protein A–Sepharose (Bio-Rad) as the immunoadsorbent (Anderson & Blobel, 1983). Samples were subjected to 10% SDS–PAGE and then analyzed by autoradiography (Anderson & Blobel, 1983).

DNA Sequencing. cDNA to be sequenced was subcloned into pUC18 or pUC19 and was sequenced according to the chain termination principle (Sanger et al., 1977) as applied to plasmid DNA (Chen & Seeburg, 1985; Kraft et al., 1988). Nested deletions were made as described (Henikoff, 1984). Components of the pUC sequencing kit (Boehringer Mannheim) and the M13 sequencing kit (BRL) were used. Sequences exhibiting band compressions were resolved by using the T7 sequencing kit and 7-deaza-dGTP (Pharmacia–LKB) (Tabor & Richardson, 1987). Primers used were 5′-GTAAAACGACGGCAGT-3′ (universal primer) and 5′-CAGGAAACAGCTATGAC-3′ (reverse sequencing primer). The cDNA sequence-specific oligonucleotide primer 5′-GTA-ATCAGTTCAAATTGTGG-3′ was purchased from Midland Certified Reagent Co., Midland, TX. It was synthesized by phosphoramidite chemistry and purified by anion-exchange HPLC. Clone 8-1 was sequenced after being subcloned into pUC18 and was also sequenced without subcloning by using the forward λgt11 sequencing primer (Clontech) 5′-GACTCCTGGAGCCCG-3′ according to the manufacturer's instructions.

Results

Peptide Sequences. Sequences determined for peptides purified from proteolytic digests of bovine aorta lysyl oxidase are found in Table I. The amino terminus of bovine aortic

Table I: Sequences of Peptides Derived from Bovine Aorta Lysyl Oxidase[a]

A. Tryptic peptides

Peptide		
1	Amino Acid:	Phe-Pro-Gln-Arg
	pmol:	485 517 459 132
2	Amino Acid:	Asn-Gln-Gly-Thr-Ser-Asp-Phe-Leu-Pro-Ser-Arg-Pro-Arg
	pmol:	517 474 411 276 69 270 251 192 204 35 36 81 48
3	Amino Acid:	Val-Ser-Val-Asn-Pro-Ser-Tyr-Leu-Val-Pro-Glu-Ser-Asp-
	pmol:	216 92 159 160 168 48 120 103 91 99 76 29 79
	Amino Acid:	-Tyr-Ser-Asn-Asn-Val-Val-Arg
	pmol:	48 16 31 38 18 20 7
4	Amino Acid:	Tyr-Thr-Gly-His-His-Ala-Tyr-Ala-Ser-Gly-Gln-Ser-Ile-
	pmol:	583 274 560 288 384 400 400 468 222 200 128 66 90
	Amino Acid:	-Ser-Pro-Tyr
	pmol:	67 65 29
5	Amino Acid:	Tyr-Arg-Pro-Gly-Tyr-Gly-Thr-Gly-Tyr-Phe-Gln-Tyr-Gly-
	pmol:	1020 466 926 653 756 517 480 486 594 555 336 546 410
	Amino Acid:	-Leu-Pro-Asp-Leu-Val-Pro-Asp-Pro-Tyr-Tyr-Ile-Gln-Ala-Ser-
	pmol:	480 420 456 405 306 354 396 318 360 450 322 235 79 31
	Amino Acid:	-Thr-Tyr-Val-Gln-Lys
	pmol:	40 51 31 31 6

B. Chymotryptic Peptide

6	Amino Acid:	His-Ser-Met-Asp-Glu-Phe-Ser-His-Tyr
	pmol:	148 96 74 80 77 59 55 56 36

[a] The yield of each PTH amino acid from the Applied Biosystems gas-phase protein sequencer is indicated.

lysyl oxidase appears to be blocked, as intact lysyl oxidase could not be sequenced. Antibody raised against synthetic peptide 3 (Table I) recognized the bovine aorta lysyl oxidase band at 32 kDa on Western blots (Figure 1A), consistent with the derivation of this antigenic sequence from lysyl oxidase. This blot also demonstrates that lysyl oxidase is immunologically distinct from bovine plasma amine oxidase and porcine kidney diamine oxidase, as neither of these reacted with the anti-peptide antibody nor with the anti-lysyl oxidase antibody.

Isolation and Sequencing of cDNA Clones. Approximately 1.2×10^6 recombinant phage were screened, from which six positive clones containing cDNA inserts ranging from 300 to 600 bp were identified by reaction with rabbit anti-bovine lysyl oxidase. Southern blot analyses revealed that five of the six clones cross-hybridized with each oher. Two of these overlapping cDNA species were sequenced (Figure 2A, clones 8-1 and IIB) yielding 502 bp of a unique cDNA sequence (Figure 2B, nucleotides 506–1008) that encoded peptides 1, 2, 5, and 6 from Table I, with a single amino acid substitution found in peptide 5. It seems likely that this substitution reflects a species difference since, as noted under Materials and Methods, the cDNA library was derived from rat aorta RNA, while sequenced peptides were derived from bovine aorta lysyl oxidase.

The rat aorta library was rescreened with radiolabeled clone IIB cDNA, thus identifying an additional 13 positive clones among 75 000 recombinants. The cDNA inserts from the two largest clones (clones 7 and 13) were subcloned into pUC18 for restriction mapping and sequence determination (Figure 2). The sequences of all six peptides isolated from digests of

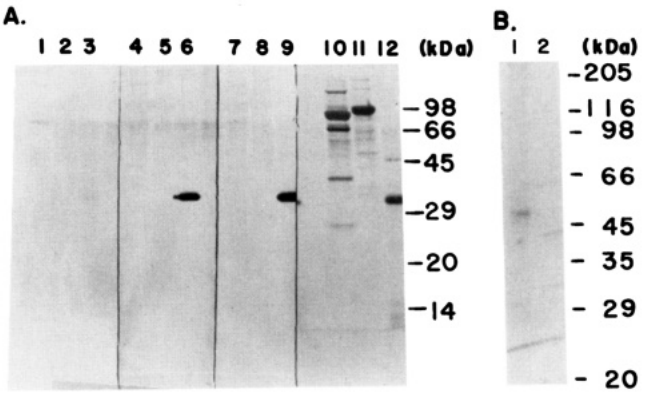
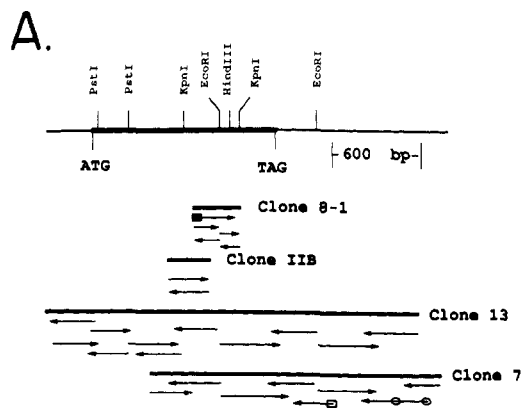
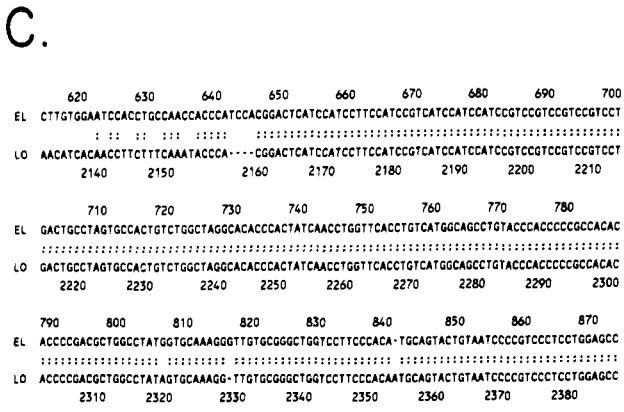


FIGURE 1: (A) Western blot and (B) in vitro translation/immunoprecipitation of rat aorta smooth muscle cell RNA. (A) Lanes 1, 4, 7, and 10, 5 μg of bovine serum amine oxidase; lanes 2, 5, 8, and 11, 5 μg of porcine kidney diamine oxidase; lanes 3, 6, 9, and 12, 2 μg of bovine aorta lysyl oxidase. Lanes 1–3 were stained with preimmune rabbit serum, lanes 4–6 with rabbit anti-lysyl oxidase, and lanes 7–9 with anti-peptide antibody (see Materials and Methods). Lanes 10–12 were stained with amido black protein stain. (B) Immunoprecipitation of translated protein using anti-lysyl oxidase (lane 1) or preimmune serum (lane 2).

bovine aorta lysyl oxidase were found in the resulting cDNA sequence with 96% agreement, indicating homology between rat and bovine aorta lysyl oxidase (Figure 2B). Note that the only tryptic peptide sequenced which ends in an amino acid other than arginine or lysine (peptide 4, Table I) is immediately followed by a stop codon in the cDNA sequence (Figure 2B). This establishes peptide 4 as the carboxyl-terminal se-





B.

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                                                                                                  CTTCTACTTCAGACACTGTGCGCTCTCCCGGACCGTCGTGCGCCGCTC -239

CCCGTCGCCTTCCAGGACTGGGAAAGGGGAGAGGAGGACGGTGCCACGTCCGACGGCCTCCTGGGCTGGGGGCAGGGTCTGCCGCTCGCCCTTGGCACCAGTCCCTGCGACCCATCCCC -120

GAGCCTCGCCCTCCTCCTCCCTGCTCGAAGAGGTCTCCCTCCTTCGCGGGATCTGAGTCCCTGTCTTCATTTTTCTCCTAGCCACGTCCCTCCCCGAGAAGGGACGAGCCGGGAGCATC   -1

ATG CGT TTC GCC TGG ACC GTG CTC TTT CTG GGA CAG CTG CAG TTC TGT CCC CTT CTC CGC TGC GCC CCG CAG GCC CCG CGC GAG CCT CCC    90
 1 Met Arg Phe Ala Trp Thr Val Leu Phe Leu Gly Gln Leu Gln Phe Cys Pro Leu Leu Arg Cys|Ala Pro Gln Ala Pro Arg Glu Pro Pro

GCC GCC CCC GGT GCC TGG CGC CAG ACA ATC CAA TGG GAG AAC AAC GGG CAG GTG TTC AGT CTG TTG AGC CTG GGG GCG CAG TAC CAG CCT   180
31 Ala Ala Pro Gly Ala Trp Arg Gln Thr Ile Gln Trp Glu Asn Asn Gly Gln Val Phe Ser Leu Leu Ser Leu Gly Ala Gln Tyr Gln Pro

CAG CGA CGC CGC GAC TCC AGC GCC ACT GCC CCG AGA GCC GAC GCA ACG CTG CAG CAC AGC CAC GCA CGC CCA TTC TGC TGC TGC GTG ACA   270
61 Gln Arg Arg Arg Asp Ser Ser Ala Thr Ala Pro Arg Ala Asp Ala Thr Leu Gln His Ser His Ala Arg Pro Phe Cys Cys Cys Val Thr

ACC GCA CTG CCT CTG CCC GTG CGA GGA CTC CAA GCC CAT CTG GGG TCG CCG CGG GTC GTC CCC GGC CCG CAG CCC GCC ACT GGT TCC AAG   360
91 Thr Ala Leu Pro Leu Pro Val Arg Gly Leu Gln Ala His Leu Gly Ser Pro Arg Val Val Pro Gly Pro Gln Pro Ala Thr Gly Ser Lys

TTG GTT TCT CGC CGT CGG GGG CCG GCG ATG GAG CCT CAA GGC GCG CAG AAC CGG ACT GCG TCG CCA CAG CCT CCG CAG CTC AGT AAT CTG   450
121 Leu Val Ser Arg Arg Arg Gly Pro Ala Met Glu Pro Gln Gly Ala Gln Asn Arg Thr Ala Ser Pro Gln Pro Pro Gln Leu Ser Asn Leu

AGG CCA CCC AGC CAC GTA GAT CGC ATG GTG GCG ACG ACC CCT ACA ATC CCT ACA AGT ACT CCG ACG ACA ACC CCT ATT ATA ACT ACT ATG   540
151 Arg Pro Pro Ser His Val Asp Arg Met Val Ala Thr Thr Pro Thr Ile Pro Thr Ser Thr Pro Thr Thr Thr Pro Ile Ile Thr Thr Met

ACA CTT ATG AGA GAC CGG TCC GGG AGC AGG CAC CGA CCT GGA TAT GGC ACC GGT TAC TTC CAG TAC GGT CTC CCG GAC CTG GTA CCC GAT   630
181 Thr Leu Met Arg Asp Arg Ser Gly Ser Arg His Arg Pro Gly Tyr Gly Thr Gly Tyr Phe Gln Tyr Gly Leu Pro Asp Leu Val Pro Asp
                                          Tyr

CCC TAC TAC ATC CAG GCA TCC ACG TAC GTA CAA AAG ATG TCT ATG TAC AAC CTG AGA TGC GCT GCG GAA GAA AAC TGC CTG GCC AGT TCA   720
211 Pro Tyr Tyr Ile Gln Ala Ser Thr Tyr Val Gln Lys Met Ser Met Tyr Asn Leu Arg Cys Ala Ala Glu Glu Asn Cys Leu Ala Ser Ser

GCA TAT AGG GCG GAT GTC AGA GAC TAT GAC CAC AGG GTA CTG CTA CGA TTT CCT CAG AGA GTG AAA AAC CAA GGG ACG TCT GAC TTC TTA   810
241 Ala Tyr Arg Ala Asp Val Arg Asp Tyr Asp His Arg Val Leu Leu Arg Phe Pro Gln Arg Val Lys Asn Gln Gly Thr Ser Asp Phe Leu

CCA AGC CGC CCC CGC TAC TCC TGG GAG TGG CAC AGC TGC CAC CAA CAT TAC CAC AGC ATG GAT GAA TTC AGC CAC TAC GAC CTG CTG GAT   900
271 Pro Ser Arg Pro Arg Tyr Ser Trp Glu Trp His Ser Cys His Gln His Tyr His Ser Met Asp Glu Phe Ser His Tyr Asp *Leu Leu Asp

GCC AGC ACA CAG AGG AGA GTG GCC GAG GGC CAC AAA GCA AGC TTC TGT CTG GAG GAC ACT TCC TGT GAT TAT GGG TAC CAC AGA CGA TTT   990
301 Ala Ser Thr Gln Arg Arg Val Ala Glu Gly His Lys Ala Ser Phe Cys Leu Glu Asp Thr Ser Cys Asp Tyr Gly Tyr His Arg Arg Phe

GCC TGT ACT GCA CAC ACA CAG GGG TTG AGT CCC GGA TGT TAT GAT ACT TAT GCA GCA GAC ATA GAC TGC CAG TGG ATT GAT ATT ACA GAT  1080
331 Ala Cys Thr Ala His Thr Gln Gly Leu Ser Pro Gly Cys Tyr Asp Thr Tyr Ala Ala Asp Ile Asp Cys Gln Trp Ile Asp Ile Thr Asp

GTA CAA CCC GGA AAT TAC ATT CTA AAG GTC AGT GTA AAC CCC AGC TAC CTG GTG CCT GAA TCA GAC TAC AGT AAC AAT GTC GTA CGC TGT  1170
361 Val Gln Pro Gly Asn Tyr Ile Leu Lys Val Ser Val Asn Pro Ser Tyr Leu Val Pro Glu Ser Asp Tyr Ser Asn Asn Val Val Arg Cys

GAA ATT CGC TAC ACA GGA CAT CAC GCC TAT GCC TCA GGC TGC ACC ATT TCA CCG TAT TAGAAAGAAGCTCACCTTCCCAAAGGATGAAGCAGTACCTGGT 1270
391 Glu Ile Arg Tyr Thr Gly His His Ala Tyr Ala Ser Gly Cys Thr Ile Ser Pro Tyr END
                                                Gln Ser

GTTTGGACCTATGAAAACCGTAGATTAGCTTAAGTAGGAAGACTTAGATATTTTAAAAGGCAAACGGAAAAACAACAAAGAAGGTTTTGTTTGGACTCTTTCACAACAAATCACATAAC 1389

TGGATTTTGAGTGTTTAAATCAGCATTAGATTGGCACATTTTAAATACTTATTCATGTTGCTTTATGAAGTAATGGTGTTTCAATTCTGTGGGTGCATAGTGGGCTCTTTCAAAGAATT 1508

CTGAATTTCTTACCTTCTTTTGAAATTATAGTGCAAAAAGAAGAGGATATTTTAATGAATGAGCCACAATTTGAACTGATTACTTTCTAAATTGCCAGACCCATGAGACAATGATGATG 1627

GGTTTGTATTTGCCTCAACATAGATTCGCTTTTTAAAAAGGGTGTTCCTATTGTATAGGCAAAAATGGATACACTTGGTGCTGAGGAAGGGTCAAATACTAACTATTGTTGTCACGAAA 1746

TATAGGTCTACAGCAGAGAGATGGTGAGTATATATTCAGATAGTTACATCCCTATATAAACTATGTTTACATTTTAGATGCTTTTCTTTCTGTTAATTGCTTAATCTCACTCTGACTTG 1865

AGGTACAACTTCTGTTTTGGAATGAATTAGATAATTCCAGATTCTGGTTTGATAATTGTTGACATTCCCCCATGCTACTTTTTCTGAGGGCAGAAACGTCTAATGTGACGACTCTTCAC 1984

ATTACCATTACGAGGATACACAGCACAGCGAAATCATTCCGATGACAGGTGTGATAGATGGAGAGCTAACATGCAACTGCCGAGTGTTTCACTGTTAGCCAGAACTAAGTCACTTGCCC 2103

CACACAGCAATTACACCATGAATCTCTAACATCACAACCTTCTTTCAAATACCCACGGACTCATCCATCCTTCCATCCGTCATCCATCCATCCGTCCGTCCGTCCGTCCTGACTGCCTA 2222

GTGCCACTGTCTGGCTAGGCACACCCACTATCAACCTGGTTCACCTGTCATGGCAGCCTGTACCCACCCCCGCCACACACCCCGACGCTGGCCTATAGTGCAAAGGTTGTGCGGGCTGG 2341

TCCTTCCCACAATGCAGTACTGTAATCCCCGTCCCTCCTGGAGCC                                                                          2386
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FIGURE 2: (A) Partial restriction map and sequencing strategy. (B) cDNA and predicted amino acid sequence. (C) Comparison of cDNA sequences of 3′-untranslated regions of rat lysyl oxidase and rat elastin cDNA (Deak et al., 1988). In (A), circles indicate subclones made by nested deletion (Henikoff, 1984), the open box indicates sequencing done with the cDNA-specific primer, and the closed box indicates sequencing performed without subcloning using the λgt11 forward sequencing primer (see Materials and Methods). In (B), underlined peptides are those sequenced from bovine aorta lysyl oxidase protein, and the vertical line indicates the putative signal peptidase cleavage site.

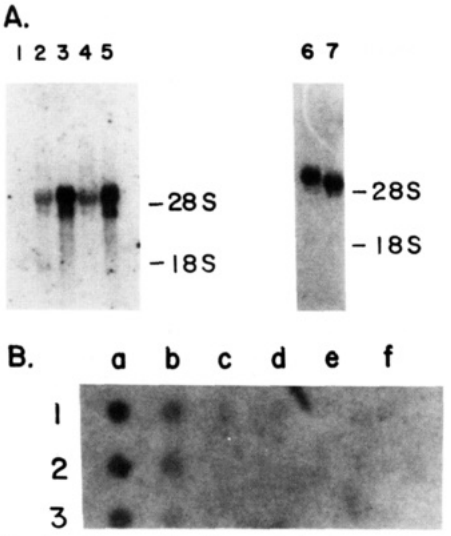

FIGURE 3: Northern blot and dot blot analysis of rat and bovine RNAs. (A) Northern blots: lane 1, 20 μg of total RNA from rat kidney; lane 2, 20 μg of total RNA from rat aorta; lane 3, 8 μg of poly(A)+ RNA from rat aorta; lane 4, 20 μg of total RNA from rat lung; lane 5, 8 μg of poly(A)+ RNA from rat lung; lane 6, 20 μg of total RNA from bovine aorta smooth muscle cells; lane 7, 20 μg of total RNA from rat lung. (B) Dot blot: column a, total RNA from rat lung; column b, total RNA from rat aorta; column c, total RNA from rat kidney; column d, total RNA from rat liver; column e, total RNA from rat brain; column f, total RNA from rat heart. Row 1, 20 μg of RNA; row 2, 10 μg of RNA; row 3, 5 μg of RNA. The probe used was radiolabeled lysyl oxidase cDNA (nucleotides −282 to +875).

quence of lysyl oxidase, as trypsin specifically hydrolyzes on the carboxyl-terminal side of endopeptidyl lysine or arginine residues. These data also indicate that the 32-kDa lysyl oxidase species as isolated from connective tissues is encoded by the 3′-end of the open reading frame and that cleavage is likely to occur in the N-terminal region of a larger enzyme form to generate the 32-kDa species.

The open reading frame of 1227 nucleotides (Figure 2) encodes a polypeptide product of 409 amino acids with a calculated molecular mass of 45 979 Da. Consistent with the possibility that this is the size of a lysyl oxidase precursor, immunoprecipitation with anti-bovine lysyl oxidase of the products of in vitro translation of neonatal rat aorta smooth muscle cell mRNA resulted in the appearance of a single band at 48 ± 3 kDa by SDS–PAGE that is absent in the control immunoprecipitated with preimmune serum (Figure 1B). Furthermore, the cDNA sequence surrounding the putative translation initiation codon (Figure 2B) conforms to the rules and consensus sequence described by Kozak (1987). There is a putative signal peptidase cleavage site between amino acids 20 and 21 that conforms to the "(−3, −1) rule" (von Heijne, 1986). There is a 5′-untranslated sequence of 286 nucleotides and 1159 nucleotides of 3′-untranslated sequence in addition to the 1227 nucleotides in the open reading frame, accounting for the 2672-base length of this lysyl oxidase cDNA sequence. It is unlikely that additional upstream translation initiation codons exist which would result in a putative lysyl oxidase precursor larger than 46 kDa, since there is a stop codon in the 5′-untranslated sequence (nucleotides −66 to −64) in frame with the putative translation–initiation codon, and proteins of molecular mass higher than 48 ± 3 kDa were not immunoprecipitated from in vitro translations. Neither a poly(A) tail nor a polyadenylation consensus sequence was found in the sequence determined for the 3′-untranslated region.

Northern Blot and Dot Blot Analysis of RNA. Northern blots and dot blots of various preparations of RNA were generated and hybridized with a 1.1-kb probe taken from the 5′-end of lysyl oxidase cDNA. Both total and poly(A)+ selected RNA of rat aorta and rat lung contained a prominent hybridizing species of 5.8 kb and a minor hybridizing band at 4.5 kb (Figure 3A). Similarly, a 6.3-kb hybridizing band was seen with the RNA derived from calf aorta smooth muscle cells, while there was no hybridization of lysyl oxidase cDNA to RNA of rat kidney (Figure 3A). These RNA species are larger than the 2672-nucleotide sequence obtained from the cDNA clones. Thus, there may be additional 3′- and 5′-untranslated sequences of at least 1800 nucleotides. This is consistent with the noted absence of a 3′-poly(A) tract and lack of a polyadenylation consensus sequence in the cDNA sequence documented here. Similarly, unusually long 3′-untranslated sequences have been documented for other proteins, such as interstitial binding protein (Fong & Bridges, 1988), adenylyl cyclase (Krupinski et al., 1989), and elastin (Deak et al., 1988; Indik et al., 1987b). Long 5′-untranslated regions have been found in rat collagen (Kohno et al., 1984) and many other regulated genes (Kozak, 1984).

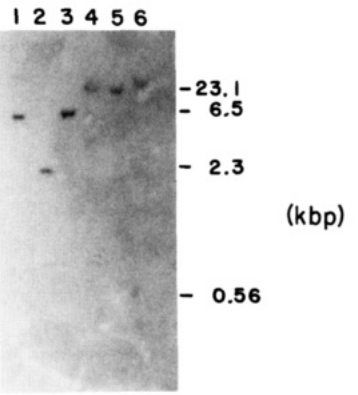

FIGURE 4: Southern blot of rat genomic DNA restriction digests. Ten-microgram samples of rat genomic DNA (Blin & Stafford, 1976) were subjected to 0.8% agarose gel electrophoresis and Southern blotting after digestion with *Bam*HI (lane 1), *Pst*I (lane 2), *Hin*dIII (lane 3), *Bgl*II (lane 4), *Eco*RV (lane 5), or *Apa*I (lane 6) (Maniatis et al., 1982d). The probe used was radiolabeled clone IIB cDNA. Hybridizations and washes were carried out as described for Northern blots and dot blots (see Materials and Methods).

Dot blot analysis confirmed that total RNA of rat aorta or lung hybridized to the lysyl oxidase cDNA probe, while such hybridization was not evident in dot blots of total RNA of rat kidney, heart, brain, and liver (Figure 3B), consistent with the expected tissue distribution of lysyl oxidase in normal, nonfibrotic tissues (Kagan, 1986).

Southern Blot of Rat Genomic DNA. Samples of rat genomic DNA were each digested with restriction endonucleases and the digests were analyzed by Southern blotting, probing with ^{32}P-labeled clone IIB cDNA that does not contain the 3′-untranslated sequence of lysyl oxidase cDNA. A single band was hybridized in each digest (Figure 4). The same results were obtained when the labeled probe used was 1.1 kb of cDNA derived from the 5′-end of clone 13 (not shown). Thus, as the lysyl oxidase gene appears to be unique and does not appear to be a member of a multigene family, this result provides further evidence supporting the conclusion that the cloned DNA is that of lysyl oxidase and is not cDNA which is merely related to lysyl oxidase.

Sequence Comparisons. Significant similarities were not found between the deduced amino acid sequence of lysyl oxidase and known sequences of other proteins in the NBRF/PIR data base (IntelliGenetics/BIONET) (Pearson & Lipman, 1988). However, comparison of the full lysyl oxidase cDNA sequence shown in Figure 2B with the NIH GenBank data base (IntelliGenetics/BIONET) (Pearson & Lipman, 1988)

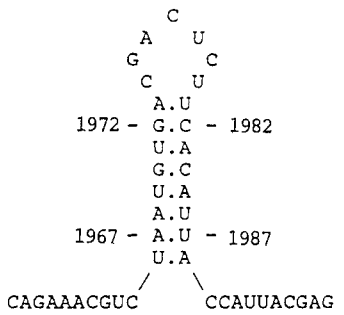

FIGURE 5: Structure of a potential stem–loop in the 3′-untranslated sequence of lysyl oxidase mRNA. This structure was found by using the HAIRPIN program of PC/GENE (IntelliGenetics) according to Tinoco et al. (1973). The numbers refer to nucleotide residues derived from the cDNA sequence in Figure 2.

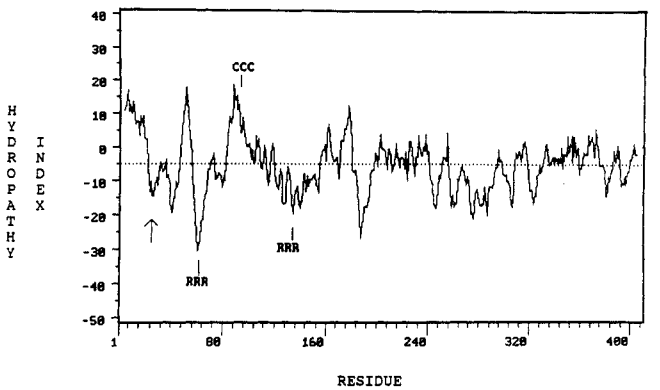

FIGURE 6: Hydropathy plot of the deduced amino acid sequence of the putative lysyl oxidase precursor. Analysis was performed by using the SOAP program in PC/GENE (IntelliGenetics) according to Kyte and Doolittle (1982) with an interval of nine amino acids. The arrow marks the position of the putative signal peptidase cleavage site. The positions of triplet arginine and cysteine residues in the deduced amino acid sequence are also indicated.

revealed a 70% degree of identity between a 196-bp sequence of the 3′-untranslated region of lysyl oxidase and the 3′-untranslated region of human and bovine elastin (Kaliannan & Anwar, 1987; Indik et al., 1987b). Similar comparison revealed an even greater sequence correspondence of 93% with the 3′-untranslated sequence of rat elastin (Deak et al., 1988) over a 258-nucleotide sequence extending to the 3′-end of the lysyl oxidase cDNA sequence obtained (Figure 2C). It has been established previously that there is homology between the 3′-untranslated sequences of bovine, human, rat, and chicken elastin cDNA (Deak et al., 1988), although the functional significance of this sequence conservation between species is not known.

Secondary Structure Analyses. Analysis for secondary structure in the lysyl oxidase cDNA sequence as described (Tinoco et al., 1973) revealed a potential stem–loop structure in the 3′-untranslated region (Figure 5) but none in the 5′-untranslated region. The free energy of this potential stem–loop structure at 25.5 °C was calculated to be −7.6 kcal (Tinoco et al., 1973). The potential stem–loop (nucleotides 1966–1988) is not contained within the region with near identity to elastin cDNA but begins about 240 nucleotides upstream of this sequence region (Figures 5 and 2).

The hydropathic index for the deduced amino acid sequence of the putative lysyl oxidase precursor was determined as described (Kyte & Doolittle, 1982) by using the SOAP computer program in PC/GENE (IntelliGenetics). The amino-terminal end has sharply alternating hydrophobic/hydrophilic regions, whereas fluctuations are less intense toward the carboxyl terminus (Figure 6). No evidence for integral membrane domains in lysyl oxidase was found (Klein et al., 1985).

Discussion

Evidence has been presented that the cDNAs described in this paper are derived from lysyl oxidase mRNA. We have presented cDNA and protein sequence data and results of immunoprecipitation studies that are consistent with a putative lysyl oxidase polypeptide precursor of 46 kDa. Biosynthetic studies in progress in this laboratory have also resulted in the immunoprecipitation of a 46–48-kDa protein from the intracellular protein fraction of pulse-labeled neonatal rat aorta smooth muscle cell cultures by using affinity-purified chicken anti-lysyl oxidase. Pulse–chase studies also in progress should shed light on a possible precursor–product relationship between the 46-kDa species and the 32-kDa lysyl oxidase normally isolated from connective tissues. Since the C-terminal peptide sequence of the 32-kDa lysyl oxidase was followed by a stop codon in the cDNA sequence, it appears that N-terminal processing is likely during the maturation of lysyl oxidase. An N-glycosylation site was found in the sequence encoding the 32-kDa protein, Asn-Arg-Thr (amino acids 137–139). This potential site for N-glycosylation (Bause, 1983) is immediately followed by a threonine- and serine-rich sequence (amino acids 141–181), thus raising the possibility that N- and O-glycosylation of a pre-lysyl oxidase molecule may occur.

The predicted amino acid sequence has an Arg-Arg-Arg sequence occurring at residues 62–64 and again at residues 124–127, while there is a Cys-Cys-Cys sequence at residues 86–88. These sequences are located in areas where the hydropathic index of the putative lysyl oxidase precursor is at or near a local maximum or minimum value (Figure 6). The hydrophilic arginine triplets may be exposed to solvent, whereas the cysteine triplet appears to be in a hydrophobic environment. These sequences may be important with respect to proteolytic processing events or may be involved in interactions of lysyl oxidase with the acidic- and cysteine-rich microfibrillar proteins associated with elastic fibers (Cleary et al., 1981; Serafini-Fracassini et al., 1981; Sakai et al., 1986; Gibson et al., 1989) or with tropoelastin and/or collagen substrates. Indeed, cleavage of the putative lysyl oxidase precursor at arginine-127 would result in a protein with a calculated molecular mass of 32 119 Da, which compares favorably with the ca. 32-kDa molecular mass of the enzyme normally isolated from connective tissues (Kagan, 1986). As noted, amino-terminal sequencing of mature lysyl oxidase has not been successful. Isolation and sequencing of the blocked amino-terminal peptide by mass spectral or other techniques will be required to further assess whether arginines 125–127 may represent a site for proteolytic processing.

Results of Southern blotting suggest that lysyl oxidase is encoded by a single unique gene and not by a family of related genes. Thus, the multiple ionic forms of lysyl oxidase isolated from connective tissues may stem from posttranslational events yet to be defined. There is currently no evidence for alternative splicing of rat aorta lysyl oxidase RNA, as all four sequenced clones overlap each other with no inserted or deleted internal sequences. It is also possible that multiple polyadenylation sites exist in the 3′-untranslated region, accounting for the two bands observed on Northern blots (Yeh et al., 1989; Indik et al., 1987b). Additional, long cDNA clones will have to be characterized to more fully address this issue.

The high degree of identity between the 3′-untranslated region of lysyl oxidase cDNA and a highly conserved 3′-untranslated region of elastin cDNA is remarkable. It is also of interest that a stem–loop structure was found in the 3′-

untranslated region of lysyl oxidase cDNA. Recent evidence indicates that 3′-untranslated regions have importance with respect to mRNA stability (Petersen et al., 1989; Wilson & Treisman, 1988; Brewer & Ross, 1988; Levine et al., 1987; Casey et al., 1988; Shaw & Kamen, 1986) and also may influence the efficiency with which an mRNA is translated independently of mRNA stability (Krays et al., 1989). The active sequence elements, which were initially identified by virtue of sequence homology between 3′-untranslated regions of different cytokines (Shaw & Kamen, 1986) or histones (Busslinger et al., 1979; Hentschel & Birnstiel, 1981), are generally either AU-rich or contain potential stem–loop structures (Levine et al., 1987; Shaw & Kamen, 1986; Casey et al., 1988). The molecular mechanisms by which these sequence elements act are not yet known but are thought to include the binding of protein factors to the RNA. Evidence supporting this hypothesis for the transferrin receptor mRNA and ferritin mRNA has been reviewed (Klausner & Harford, 1989). The authors cite evidence that the conserved, potential stem–loop structures which are present in both the 5′- and 3′-untranslated regions of transferrin receptor mRNA and in the 5′-untranslated region of ferritin mRNA serve as binding sites for the same regulatory protein factor, thereby allowing the coordinate posttranscriptional regulation of these two proteins which has been observed. It seems possible that the apparently conserved 3′-untranslated region could be involved in the coregulation of lysyl oxidase and its elastin substrate while there may also be additional sequence elements which regulate the enzyme independently of elastin. Systematic analysis of these interesting sequences with respect to the modulation of mRNA stability and translation is required to explore these possibilities.

As noted, no significant identity of the amino acid sequence of lysyl oxidase with other sequenced proteins was found. However, a closer examination of the amino acid sequence from residues 280 to 335 reveals that it is rich in histidine and contains a His-X_4-Cys-X_5-Cys-X_4-His sequence (residues 311–327) which is similar to a consensus sequence for metal-binding proteins, i.e., a-X_{2-4}-a-X_{2-15}-Cys-X_{2-4}-Cys where a is Cys or His and X is any amino acid (Berg, 1986). Furthermore, the sequences Tyr-Gly-Tyr-His-Arg-Arg-Phe-Ala-Cys (residues 324–332) and Tyr-Thr-Gly-His-His-Ala-Tyr (residues 394–400) are similar to the conserved copper-binding peptide sequences Leu-Pro-Trp-His-Arg-Arg-Tyr-Leu-Leu and Phe-Trp-Leu-His-His-Ala-Tyr, respectively, both of which are found in *Streptomyces glaucescens* tyrosinase (Huber et al., 1985; Huber & Lerch, 1988). These sequences are conserved among some copper-containing proteins, as homologous sequences have been found and compared in tyrosinases from *Neurospora crassa*, mouse, and human and in *Eurypelma californicum* hemocyanin (Huber et al., 1985; Shibahara et al., 1986; Kwon et al., 1988). A similar sequence is also found in subunit I of human cytochrome *c* oxidase residues 287–292 (Lundeen, 1988). In vitro mutagenesis of expressed and active lysyl oxidase should be valuable as an approach to determine whether these sequences actually serve as copper ligands in lysyl oxidase. In vitro mutagenesis experiments should also allow the evaluation of the importance of individual amino acid residues that may be involved in posttranslational processing pathways, enzyme mechanism, and covalent binding of the organic cofactor to the protein.

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