Characterization and Developmental Expression of Chick Aortic Lysyl Oxidase*

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The complete primary structure of chick lysyl oxidase was determined by recombinant DNA techniques. The nucleotide sequence of contiguous chick lysyl oxidase cDNA clones contained an open reading frame of 1260 bases which encodes a predicted protein of 420 amino acid residues (48,150 Da). In comparison to the deduced primary structure of rat lysyl oxidase, the chick enzyme is larger in size and exhibits a strong conservation of sequence within the latter two thirds of the molecule (92% identity) and a high degree of divergence in the first 150 amino acid residues (60% identity allowing for several insertions in both sequences).

The developmental steady-state levels of lysyl oxidase mRNA together with the mRNAs encoding two of the enzyme's substrates (tropoelastin and type I collagen) increased between 8 and 16 days of embryonic development. Although levels of lysyl oxidase mRNA increased during aortic embryogenesis, the specific activity of the enzyme remained fairly constant suggesting that lysyl oxidase activity increases in direct proportion to total protein synthesis and cell number. In situ hybridization showed that the spatial expressions of lysyl oxidase and tropoelastin transcripts differ suggesting that the enzyme and substrate genes are differentially regulated within the cells of the arterial wall.

Lysyl oxidase is the key enzyme involved in the cross-linking of elastin and collagen. Although elastin and collagen differ significantly in their primary and secondary structures, both proteins serve as substrates of lysyl oxidase during the extracellular formation of insoluble, elastic, and collagenous fibers (reviewed by Kagan (1986)). The specific reaction catalyzed by lysyl oxidase involves the oxidation of the ε-amino group of peptide lysine or hydroxylysyl residues (Pinnell and Martin, 1968). The resultant semialdehydes can then condense with either an unmodified lysine residue to form a Schiff base or with another aldehyde to form an aldol condensation. The ultimate covalent cross-links formed in both elastin and collagen, i.e. desmosine and pyridinoline, appear to arise from the action of lysyl oxidase alone implying that the generation of specific aldehyde residues within both substrates is sufficient to generate both intra- and intermolecular cross-links (Franzblau and Faris, 1981; Gallop and Paz, 1975). The critical role that lysyl oxidase plays in the formation of biologically functional connective tissues has been demonstrated experimentally in animals through inhibition of the enzyme by α-aminopropionitrile or copper deficiency (Siegel, 1979; Dubick et al., 1985; Harris, 1986) and clinically in Menkes' disease and X-linked cutis laxa (reviewed by Kagan, 1986).

Recently the complete primary structures of rat (Trackman et al., 1989, 1991) and human (Hamalainen et al., 1991; Mariani et al., 1992) lysyl oxidases have been deduced through recombinant DNA techniques. The amino acid sequences determined have provided insight into putative cleavage sites for post-translational processing as well as identifying potential N-glycosylation and metal binding sites. These studies have afforded the accessibility of characterized cDNA clones and have provided information to allow generation of synthetic probes to quantitate gene copy number and chromsome localization (Trackman et al., 1990; Hamalainen et al., 1991; Mariani et al., 1992).

The objective of this study was to determine the structure of chick lysyl oxidase and to use this information to examine the developmental expression of the enzyme together with its major substrates during chick aortic embryogenesis. To achieve these goals we have used rat lysyl oxidase cDNA to identify chick cDNAs within a chick whole embryo cDNA library. We find that: 1) the deduced chick lysyl oxidase primary structure is very similar to that reported for rat lysyl oxidase in the domain of the enzyme thought to contain the active site; 2) the major developmental increase in chick aortic lysyl oxidase expression temporally precedes that of its collagen and elastin substrates; 3) the spatial distribution of tropoelastin and lysyl oxidase transcripts differ within the developing aortic wall; and 4) the chick lysyl oxidase gene exists as one copy per chick haploid genome and encodes a major mRNA of 6.0 kb.1

EXPERIMENTAL PROCEDURES

Screening of cDNA Library—A 10-day chick whole embryo λgt11 cDNA library (Clontech) was infected into bacteria host RY1088 by the method of Huyng et al. (1985). Rat lysyl oxidase cDNA (Trackman et al., 1990) was radiolabeled with 50 μCi of [α-32P]dATP (800 Ci/mol; Du Pont-New England Nuclear) by nick translation to a specific activity of 2.5 x 10⁷ cpm/μg and used to screen the library by plaque hybridization (Poster et al., 1988). Plaque DNA was isolated from single plaques, purified by CsCl density gradient centrifugation, and sequenced.

1 The abbreviations used are: kb, kilobase; bp, base pairs; PBS, phosphate-buffered saline; SMC, smooth muscle cell; SSC, standard saline citrate (0.3 M NaCl, 30 mM sodium citrate, pH 7.0).
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and phage DNA-extracted (Maniatis et al., 1982). The cDNA inserts were released by digestion with EcoRI and isolated by preparative polyacrylamide gel electrophoresis (Maniatis et al., 1982).

Nucleotide Sequence Analysis—For nucleotide sequence analysis, cDNA inserts were subcloned into pbLueScript SK+ (Stratagene) as we described previously (Pollock et al., 1987). Single- and double-stranded sequencing was performed by the dideoxy nucleotide chain termination method (Sanger et al., 1980) using Sequenase (United States Biochemical) according to the manufacturer's directions. Because of the difficulties encountered in high GC regions of the cDNAs, 7-deaza-7GTP was used in some reactions. Sequence reactions were analyzed on denaturing polyacrylamide gels after exposure of the fixed gel to Kodak XRP x-ray film at room temperature (Sanger et al., 1980).

Determination of Chick Lysyl Oxidase Gene Copy Number—Chick genomic DNA was isolated from the liver of one adult chicken by the procedure of Maniatis (1982). The concentration of DNA was determined by the fluorometric method of Hinegardner (1971) using calf thymus DNA as a standard. Aliquots (10 µg) of DNA were individually digested with BamHI, PstI, and BglII to completion, and the digestion fragments were separated by electrophoresis on a 0.7% agarose gel and transferred to Nytran (Foster et al., 1984) and hybridized with 32P-labeled chick lysyl oxidase cDNA clone pbSCL03K according to the procedure of Maniatis (1982) (see “Results” for origin and sequence of pbSCL03K clone).

Lysyl oxidase gene copy number was determined by measurement of haploid genomic equivalents according to the procedure of Oliver et al. (1987). Calculation of genomic copy number was based on the fact that the amount of DNA per chick haploid genome is 1.2 pg (Britten and Davidson, 1969) and the report of Maniatis et al. (1982) that provided a quantitative relationship between DNA amount and base pair content. Serially diluted samples of pbSCL03K equivalent to 0.25, 0.5, 1.0, and 2.0 gene copies were applied to a slot blot with aliquots of chick genomic DNA. The slot blot was hybridized with 32P-labeled pbSCL03K under conditions described by Oliver et al. (1987) and exposed to x-ray film. The resultant films were quantified by densitometry, and the results were subjected to simple regression analysis to ensure linearity and to determine the gene copy number at a confidence level of 95% (Statview, Brainpower, Inc.).

Northern Blot Analysis—Total RNA was extracted from 8- through 16-day chick embryonic aortae and second generation neonatal rat aortic smooth muscle cell cultures (Barone et al., 1988) with 4 M guanidinium thiocyanate as described by Chirgwin et al. (1979). Poly(A)+RNA was isolated by affinity chromatography on an oligo(dT)-cellulose column (Maniatis et al., 1982). For Northern blot analysis, duplicate 20-µg samples of total RNA or single samples of 2-µg poly(A)+RNA were fractionated on a 1.0% agarose gel and blotted onto nylon filters (Nytran, Schleicher and Schuell) by the method of Foster et al. (1987). The filters were heated at 80 °C in a vacuum oven and then hybridized with 32P-labeled chick lysyl oxidase cDNA clone pbSCL03K according to the procedure of Maniatis (1982) (see “Results” for origin and sequence of pbSCL03K clone).

RESULTS

Isolation and Characterization of Chick Lysyl Oxidase cDNA—The entire chick embryo agt11 cDNA library (1.5 × 106 plaques) was screened with a 257-bp restriction fragment of rat lysyl oxidase (pIIb) that included only translated sequences (Trackman et al., 1990). Eight plaques exhibiting the strongest hybridization signals were prephonically isolated and subcloned into pbLueScript for characterization. A partial restriction map together with the nucleotide sequencing strategy for chick lysyl cDNAs is given in Fig. 1. Seven of the eight phage cDNAs initially detected by hybridization with the rat lysyl oxidase cDNA revealed continuous overlapping sequences. Four of these cDNAs are not shown in Fig. 1 since they represented either shorter or slightly larger versions of the presence or absence of β-aminopropionitrile (50 µg/ml). Analyses were performed on three sets of aortae, and each set was analyzed in triplicate. Total protein within the crude urea extract was determined by the bicinchoninic acid protein assay (Pierce Chemical Co.) according to the manufacturer's instructions. Deoxynibonucleic acid was extracted from each set of aortae by the procedure of Rowe et al. (1978). The amount of DNA was determined by the fluorometric method of Hinegardner (1971) using calf thymus DNA as a standard. All analyses were performed on two separate batches of eggs.

In Situ Hybridization—Fertilized White Leghorn chicken eggs were incubated at 37 °C in 60% humidity and staged according to Hamburger and Hamilton (1951). Eight- and 12-day embryos were dissected in PBS, perfused by cardiac puncture with 4% paraformaldehyde in PBS and fixed with 4% paraformaldehyde/PBS in 4°C for 2 h at 4°C. Dehydration and paraffin embedding were performed according to the protocol of Sassoon et al. (1988). Antisense chick lysyl oxidase and tropoelastin riboprobes were generated using chloroformic plasmids pbBSCL03 and pbSCLEL2 (Baule and Foster, 1988) respectively. The plasmids were linearized with Sall and cDNA transcribed using T3 RNA polymerase in the presence of [35S]UTP. Purification and limited base hydrolysis of the radiolabeled transcript were performed according to the procedures of Sassoon et al. (1988). Sense controls were generated using the same procedure, except that the plasmids were labeled with Sall and transcribed with T7 RNA polymerase.

Northern sections (5 µm) were individually floated onto “subbed” slides (Gall and Pardue, 1971), dried overnight, and stored at 4°C. Hybridization and washing were performed as described by Sassoon et al. (1988). Autoradiography was carried out by dipping the slides in Kodak NTB-2 followed by a 20-h exposure for both tropoelastin and lysyl oxidase riboprobes (sense and antisense). Slides were developed in Kodak D19, fixed in Kodak Rapid Fix, and cell nuclei were stained with 0.2% methylene blue.

FIG. 1. Partial restriction map and sequencing strategy of lysyl oxidase cDNA clones. The arrows indicate the direction and extent of sequence determination. The open reading frame which begins at ATG and terminates at TAG is indicated.
clone pBSCLO1. An exception to the complete overlap of sequence was the cDNA contained within pBSCLO5 which possessed a restriction map identical to the other cDNAs within most of its length, but differed in the 5' region. Nucleotide sequence analysis of the 5' area of pBSCLO5 revealed a string of over 30 adenosine bases suggesting the presence of a poly(A) tail and implying that this cDNA acid sequence.

The nucleotide sequence and the amino acid sequence encoded by the open reading frame of chick lysyl oxidase cDNAs are provided in Fig. 2. The length of the contiguous cDNA sequence is 2,229 nucleotides. A single, long open reading frame of 1,260 bases initiates at an ATG and terminates at a TAG. In addition, the contiguous sequence contains 972 bases coding for the open reading frame of the 3' untranslated region. The open reading frame of the composite cDNA encodes a protein of 420 amino acids, giving a calculated molecular weight of 48,150 for the initial translation product. The deduced primary sequence contains a grouping of amino acids in the amino-terminal part that corresponds to a signal peptide sequence for vectorial transport across the rough endoplasmic membrane (von Heijne et al., 1985). A comparison of the deduced amino acid sequence of chick lysyl oxidase to that deduced for rat lysyl oxidase is given in Fig. 3. One of the most striking features of this comparison is the strong conservation of sequences (92% identity) among the latter two thirds of primary structure encompassing amino acid residues from position 153 to 420. On the other hand, sequences encompassing the putative signal peptide (amino acid residues 1–21) are only 38% identical, amino acid residues 30–62 are 81% identical, whereas residues 63–152 are 60% identical allowing for several insertions in both the chick and rat sequences. Hamalainen et al. (1991) have compared the primary structures of rat and human lysyl oxidases and have also found an uneven distribution of identity between the two proteins. Their comparative analyses show that the putative signal peptide regions are 71% identical, and the region between 153 and 417 residues shares a 95% identity. In contrast,

![Fig. 2. Nucleotide and deduced amino acid sequences of the chick lysyl oxidase cDNA. Numbers to the left refer to the nucleotide sequence, and numbers to the right refer to the amino acid sequence.](image-url)
FIG. 3. Comparison of the deduced primary structures of chick and rat lysyl oxidases. The upper row of each set is the amino acid sequence of chick lysyl oxidase which is numbered on the left side of the figure. The lower row of each set is the amino acid sequence of rat lysyl oxidase which is not numbered. The contiguous chick lysyl oxidase sequence is given, whereas the rat lysyl oxidase sequence is depicted to provide optimum alignment. – denotes identical amino acid residues in the rat sequence; . denotes deletions in the rat sequence; and " denotes the insertion of an amino acid residue or residues in the rat sequence.

the region between 22 and 152 residues exhibits only a 67% identity and also differs in size. These data, in combination with the sequence analyses within this study, suggest that lysyl oxidase is comprised of several domains. One of these domains, i.e. residues 153 to the carboxyl-terminal amino acid residue, is highly conserved among species and appears to contain the elements necessary for catalysis and co-factor binding (Trackman et al., 1990). A second domain consists of a region extending from the putative cleavage point of the signal peptide (between residues 21 and 22) to residue 153. This second domain exhibits a high sequence variance and represents the area of the enzyme that is post-translationally cleaved and whose function other than as a pre-form of the enzyme is unknown (Trackman et al., 1992). Nevertheless, it is clear from the primary structures derived from three different species that this area tolerates significant alteration in sequence and size. Despite the overall variation within the second domain, there is a segment of 32 amino acids (residues 30 to 62) showing an 81% identity between the chick and rat enzymes and the conservation of the only N-glycosylation site.

It has previously been shown that chick cartilage lysyl oxidase has a molecular weight of about 28,000 (Stassen, 1976). We have confirmed this molecular weight for the chick aortic enzyme by Western blot analysis using rat lysyl oxidase antisera (data not shown). In order to achieve this polypeptide size, a cleavage site would have to exist somewhere within the area of amino acid residues 140 to 160 in the chick lysyl oxidase sequence. As seen by the primary structure in Fig. 2, this region contains three arginine triplet sequences which can potentially serve as polybasic consensus cleavage sites (Hosaka et al., 1991).

Determination of Chick Lysyl Oxidase Gene Copy Number—Chick genomic DNA was digested to completion with several restriction enzymes and the digestion fragments analyzed by a Southern blot analysis (Fig. 4) using radiolabeled chick lysyl oxidase cDNA, pBSCL03K (see Fig. 1 for origin of pBSCL03K). The results show that only one fragment hybridized with the chick lysyl oxidase cDNA in each of the restriction enzyme digests suggesting that the lysyl oxidase gene exists as one copy in the chick genome.

Lysyl oxidase gene dosage was quantitated by slot blot analysis, where the hybridization intensities of gene copy equivalents of chick lysyl oxidase sequences within
Oxyribonucleic acid was isolated from the liver of one adult chicken and digested with BglII (lane A), BamHI (lane B), PstI (lane C), and PvuII/BamHI (lane D). The resulting fragments were separated by electrophoresis on 0.7% agarose gels, transferred to Nytran by capillary diffusion, and then hybridized with chick lysyl oxidase clone pBSCL03K.

**Fig. 4. Southern blot analysis of chick genomic DNA.** Deoxyribonucleic acid was isolated from the liver of one adult chicken and digested with BglII, BamHI, PstI, and PvuII/BamHI. The resulting fragments were separated by electrophoresis on 0.7% agarose gels, transferred to Nytran by capillary diffusion, and then hybridized with chick lysyl oxidase clone pBSCL03K.

Triplicate samples of chick genomic DNA and triplicate samples of pBSCL03K corresponding to 0.25, 0.5, 1.0, and 2.0 gene copies were slotted on Nytran and hybridized with 32P-labeled lysyl oxidase and tropoelastin cDNAs. Lane A, 10-day chick aortic total RNA hybridized with chick lysyl oxidase clone pBSCL03K; lane B, rat aortic SMC total RNA hybridized with chick lysyl oxidase clone pBSCL03K; lane C, 10-day chick aortic poly(A)+ RNA hybridized with chick lysyl oxidase clone pBSCL03K; lane D, rat aortic SMC poly(A)+ RNA hybridized with chick lysyl oxidase clone pBSCL03K; lane E, 10-day chick aortic total RNA hybridized with rat lysyl oxidase clone pIII; lane F, rat aortic SMC total RNA hybridized with rat lysyl oxidase clone pIII; lane G, 10-day chick aortic total RNA hybridized with the 5'-PvuII/SmaI restriction fragment of pBSCL03; lane H, 10-day chick aortic poly(A)+ RNA hybridized with chick tropoelastin cDNA; lane I, rat aortic SMC poly(A)+ RNA hybridized with chick tropoelastin cDNA.

**Fig. 5. Quantitation of chick lysyl oxidase gene copy number.** Lysyl oxidase gene dosage was quantitated by slot blot analysis comparing the hybridization intensity of chick lysyl oxidase cDNA gene copy equivalents to that contained within total chick genomic DNA. Triplicate samples of chick genomic DNA and triplicate samples of pBSCL03K were compared to the intensity contained within total chick genomic DNA. The results of these analyses are plotted in Fig. 5, where the standard curve relating gene equivalents to integrated absorbance values (calculated by laser densitometry) was generated using linear regression analysis. Exposure times of the slot blot were varied in order to ensure that the RNA samples analyzed were not degraded (especially poly(A)+), where rRNA could not be used as a criteria chick tropoelastin cDNA was hybridized with chick aortic total RNA (lane E), 20 µg of total RNA or 2-µg aliquots of poly(A)+ RNA were separated by 1% formaldehyde-agarose gel electrophoresis, and the RNA was electrophoretically transferred to Nytran and then hybridized with 32P-labeled lysyl oxidase and tropoelastin cDNAs. Lane A, 10-day chick aortic total RNA hybridized with chick lysyl oxidase clone pBSCL03K; lane B, rat aortic SMC total RNA hybridized with chick lysyl oxidase clone pBSCL03K; lane C, 10-day chick aortic poly(A)+ RNA hybridized with chick lysyl oxidase clone pBSCL03K; lane D, rat aortic SMC poly(A)+ RNA hybridized with chick lysyl oxidase clone pBSCL03K; lane E, 10-day chick aortic total RNA hybridized with rat lysyl oxidase clone pIII; lane F, rat aortic SMC total RNA hybridized with rat lysyl oxidase clone pIII; lane G, 10-day chick aortic total RNA hybridized with the 5'-PvuII/SmaI restriction fragment of pBSCL03; lane H, 10-day chick aortic poly(A)+ RNA hybridized with chick tropoelastin cDNA; lane I, rat aortic SMC poly(A)+ RNA hybridized with chick tropoelastin cDNA.

The resulting fragments were separated by electrophoresis on 0.7% agarose gels, transferred to Nytran by capillary diffusion, and then hybridized with chick lysyl oxidase clone pBSCL03K.

Identification of Chick Lysyl Oxidase mRNA—In order to establish the size of the chick lysyl oxidase mRNA, Northern blot analyses were performed on chick aortic and rat aortic SMC total and poly(A)+ RNA preparations using both chick and rat lysyl oxidase cDNA probes. Northern blot analyses displaying a composite of RNA samples and cDNA probes is provided in Fig. 6. Hybridization of chick lysyl oxidase cDNA clone pBSCL03K with total chick aortic RNA (lane A) and poly(A)+ RNA (lane C) revealed one major transcript of 6.0 kb, whereas total rat aortic SMC RNA (lane B) exhibited no major hybridization and poly(A)+ RNA (lane D) showed a transcript of 5.3 kb. When rat lysyl oxidase cDNA was used to probe total chick aortic RNA (lane E), a single 6.0-kb transcript was seen, whereas total rat aortic SMC RNA contained two transcripts of 5.3 and 4.6 kb which are comparable to previously reported values (Trackman et al., 1990). These results demonstrate that chick aortic tissue possesses one major lysyl oxidase transcript of 6.0 kb. Furthermore, chick lysyl oxidase cDNA hybridization to rat lysyl oxidase mRNA is weaker under these hybridization conditions.

Since the 5'-region of the chick lysyl oxidase sequence differs from that of the rat lysyl oxidase (see Fig. 3, a 5'-PvuII/SmaI restriction fragment of pBSCL03 (pBSCL03F/S, see Fig. 1) was used to probe chick aorta RNA (lane G). The mRNA detected was the same size as that selected by both chick (lanes A) and rat (lane E) lysyl oxidase cDNAs. In order to ensure that the RNA samples analyzed were not degraded (especially poly(A)+), where rRNA could not be used as a criteria chick tropoelastin cDNA was hybridized with chick aortic (lanes H) and rat aortic SMC (lanes I) RNA samples. The results demonstrate that the poly(A)+ RNA samples examined were not degraded since they contain intact tropoelastin mRNAs comparable in size to those reported for rat (Rich and Foster, 1989) and chick (Foster et al., 1988) transcripts.

**Developmental Expression of Lysyl Oxidase during Chick Aortic Embryogenesis**—In order to examine the expression of lysyl oxidase during development of the chick aorta, the specific activity of lysyl oxidase and the steady-state levels of its encoding mRNA were determined in 8- through 16-day embryos. Fig. 7 provides the specific activity of the enzyme expressed as activity per µg of protein assayed (A), the specific activity per ng of DNA (B), and the number of cells and micrograms of extractable protein per aorta (C) as a function of embryonic age. These results show that the specific activity of lysyl oxidase remains relatively constant during a period of development.
rapid growth of the aortic tissue.

The developmental steady-state levels of lysyl oxidase mRNA and mRNAs encoding its major substrates, i.e. tropoelastin and type I collagen, were examined by Northern blot analyses (Fig. 8). Actin transcripts were examined to provide information regarding the SMC phenotypic state. Lysyl oxidase and tropoelastin mRNAs were examined on the same filter and a2(I) collagen and actin mRNAs hybridizations were performed on a separate filter. Although not shown, the blot hybridized with actin and collagen cDNAs was reprobed with tropoelastin cDNA to ensure that the two separately hybridized blots were equivalent relative to tropoelastin mRNA levels. It should be noted that different exposure times were used for each of the cDNA probes (see Fig. 8, legend) so that hybridization intensity among the various transcripts is not indicative of relative abundances, e.g. lysyl oxidase mRNA (36-h exposure) is not more abundant than tropoelastin mRNA (3-h exposure).

Visual assessment of the Northern blot reveals that the transcripts encoding lysyl oxidase, tropoelastin, and Type I collagen increase between 8- and 16-day aortic tissue, whereas actin levels exhibit an opposite trend. The actin cDNA probe used in this study reacts with α, β, and γ actins. Under the electrophoretic conditions used in this study, the slower migrating band is comprised of both β and γ actin mRNAs, while the faster band corresponds to α actin mRNA. The developmental pattern of actin mRNAs seen in Fig. 8 shows a distinct change in the relative proportions of isoactin transcripts between days 10 and 12. This change, i.e. α actin mRNA significantly decreases, whereas β and γ actin mRNA levels remain relatively constant, suggests that the aortic smooth muscle cells have undergone a transition to the synthetic phenotype within the 10–12-day period. The synthetic phenotype is characterized by increased cell proliferation and synthesis of matrix proteins concomitant with the down-regulation of the α actin transcript (Glukhova et al., 1990). This interpretation is supported by our finding that cell proliferation (Fig. 7) and levels of collagen and tropoelastin transcripts are increasing during the same time that α actin mRNA levels decrease and nonmuscle actin transcript levels stabilize.

A quantitative assessment of three Northern blots representing RNA extracted from two separate sets of chick embryos is given in Fig. 9. It should be noted that optical densities plotted were obtained from different exposure times of x-ray films as mentioned above and therefore do not reflect abundance of the three transcripts relative to one another.
The results show that mRNAs for lysyl oxidase as well those encoding its major substrates increase during the major phase of aortic development with the enzyme mRNA peaking several days before that of its substrates. These data, although indirect in the sense that only mRNA levels and not protein levels were measured, suggest that the developmental impetus to the growth of aortic tissue involves a concerted increase in both matrix proteins and the enzyme responsible for extracellular cross-linking.

**In Situ Hybridization of Lysyl Oxidase and Tropoelastin Transcripts during Chick Aortic Embryogenesis**—Results from the Northern blot analysis (see Fig. 9) demonstrated that the steady-state levels of lysyl oxidase and tropoelastin transcripts increase during aortic development. In order to determine whether the increases in mRNA levels were spatially related, *in situ* hybridizations were performed on serial sections of aorta. These results are given in Fig. 10, where aortic tissues sections from 8- and 12-day embryos were examined. These particular ages were chosen since they correspond to those ages exhibiting minimal (8-day) and maximal (12-day) steady-state levels of lysyl oxidase transcript. Although the *in situ* analyses were not intended to be quantitative measurements of transcript levels, the relative intensities of lysyl oxidase and tropoelastin transcripts in 12-day aortic sections (D and C) are greater than those seen in the 8-day tissue (B and A). These observations are in agreement with the increases observed in the steady-state levels of lysyl oxidase and tropoelastin mRNAs determined by Northern blot analyses (Fig. 9). However, the patterns of lysyl oxidase and tropoelastin transcript expressions differ spatially. Tropoelastin mRNA exhibits a high level of expression in the outer third of the media and appears to move to inward with age, *i.e.* from 8 to 12 days. Lysyl oxidase mRNA expression appears uniformly distributed throughout the tissue section. These results suggest that the expression of lysyl oxidase and elastin transcripts are differentially regulated within specific cells of the aortic wall. It is interesting to note that Kadar (1979) has reported that chick aortic elastogenesis, as defined by ultrastructural analysis of elastic fibers, proceeds from the outer media inward in a manner similar to what we have found for tropoelastin mRNA expression.

**DISCUSSION**

In this study we have isolated and characterized cDNA clones encoding the complete deduced amino acid sequence of chick lysyl oxidase and have used the recombinant DNA probes to establish gene copy number, transcript size, and developmental expression. Within the region of the lysyl oxidase molecule thought to represent the active form of the enzyme, the deduced primary structure of chick lysyl oxidase is very similar to that characterized from nucleotide sequencing of rat and human lysyl oxidase cDNAs. This region of high sequence identity contains sites potentially active in post-translational processing and metal binding (Kagan and Trackman, 1991; Hamalainen et al., 1991). However, an interesting feature of the comparison among lysyl oxidases from different species is the amount of variation in the amino-terminal region which is thought to be cleaved prior to the formation of the active form of the enzyme (Trackman et al., 1992). The divergence in the amino-terminal region may point to species-specific signals or may indicate amino acid sequences that play no functional role and are therefore under no evolutionary pressure for conservation.

*Northern blot analysis of chick aortic total and poly (A)*^+* RNA revealed only a 6.0-kb transcript which is consistent with the measurement of one gene copy. This situation differs from that found in human and rat tissues and cell cultures where multiple transcripts, ranging in size from 5.5 to 2.0 kb, have been reported. The origin of the multiple transcripts is thought to involve the use of multiple polyadenylation signals (Trackman et al., 1990; Hamalainen et al., 1991). Our data do not exclude the possibility that multiple lysyl oxidase transcripts exist in the chick, but do show that during chick aortic development only one major transcript is present.

Our overall objective in this study was to examine the developmental expression of lysyl oxidase during chick aortic embryogenesis and to relate this information to expression of primary substrates of lysyl oxidase activity. Although the gene expression of collagenous and elastic fiber protein components are commonly studied separately, both types of fibers are very important to the formation of the aortic wall and both necessitate the action of lysyl oxidase to result in functional biopolymers. We have found that during aortic development, lysyl oxidase, collagen, and tropoelastin mRNAs all increase although not equally during a period that temporally coincides with a major change in the phenotypic expression of actin isoforms within cells of the aortic wall. This finding suggests that there may be at least one common developmental signal set in motion to accommodate the functional, *i.e.* cross-linked, formation of extracellular collagenous and elastic fibers. The effectiveness of this common developmental signal may be controlled by the phenotypic state of aortic smooth muscle cells which in turn could modulate the
responsiveness of specific genes to effector molecules. For example, the increase in lysyl oxidase mRNA levels and relatively constant enzyme-specific activities suggest that lysyl oxidase expression during aortic embryogenesis is not selectively controlled but rather is an integral component of the general growth phase of cells within the aortic wall. In contrast, our laboratory (Barrineau et al., 1981; Foster et al., 1989) as well as others (Eichner and Rosenbloom, 1979; Burnett et al., 1980) have found that tropoelastin rates of synthesis and transcript levels are selectively increased during chick embryogenesis.

Data obtained from Northern blots do not address spatial expression of transcripts but rather provide only temporal averages. In situ hybridization of aortic wall sections has provided some insight into the spatial relationships of lysyl oxidase and tropoelastin mRNA expressions. We have found a fairly uniform distribution of lysyl oxidase transcript within tissue sections obtained from 8- and 12-day aortic tissue. Tropoelastin mRNA expression exhibited a different spatial pattern that originated from the outer media and proceeded inward. These data suggest that cells within the aortic wall differentially express lysyl oxidase and one of its major substrates, i.e., tropoelastin, in a manner suggestive of constitutive transcription of enzyme mRNA together with increased, and spatially programmed expression of tropoelastin transcript.

In conclusion, previous studies from our laboratories have independently examined lysyl oxidase structure and developmental expression of a major lysyl oxidase substrate, tropoelastin, in aortic development. Within this study we have combined interests to provide a more holistic approach to understanding the formation of the aortic wall during chick embryogenesis. For this purpose chick lysyl oxidase cDNAs were characterized in order to obtain the complete amino acid sequence of the chick enzyme and to provide the essential cDNA probe for determining developmental gene expression of the enzyme relative to those of its major substrates, collagen and elastin. Our results suggest that lysyl oxidase gene expression increases in direct proportion to increases in cell number, whereas tropoelastin gene expression is differentially regulated by a factor(s) that dictates a selective and spatially concerted pattern of expression. One obvious question that arises from these observations is how a uniformly distributed enzyme can accommodate the need for a spatially concerted mode of substrate expression. However, it should be noted that the spatial distribution of transcripts does not provide information on potential translational or post-translational mechanisms of regulation.