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CHARACTERIZATION OF ELASTIN PROTEIN AND mRNA FROM SALMONID FISH (*ONCORHYNCHUS KISUTCH*)

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Abstract—1. Elastin was isolated from the bulbus arteriosus of a salmonid fish. Monoclonal and polyclonal antibodies, elicited against a CNBr digest of this protein, immunoprecipitated a polypeptide of M_r 43,000 from fish cell culture medium.

2. Cell-free translation of salmon poly A$^+$ RNA produced a protein of approximately 43 kD that was immunoprecipitated with anti-elastin antibodies. The corresponding mRNA had an approximate M_r of 2 kb.

3. Despite similarities in amino acid composition, the differences in M_r between mammalian and salmon mRNA and protein suggest a divergence of fish and higher vertebrate elastins from an earlier ancestral gene.

INTRODUCTION

The problem of tissue resilience has been satisfied by several genetically unrelated proteins throughout the animal kingdom. In vertebrates, elastin confers the property of rubber elasticity to the tissues in which it is most abundant (aorta, ligament, skin) (for recent reviews, see Sandberg and Davidson, 1984 and Rosenbloom, 1984). The protein is secreted as tropoelastin into the connective tissue extracellular space and assembled into elastin-containing fibers.

Elastin was one of the first "modular" proteins to be characterized. With an M_r of 68,000–72,000, both the mammalian and avian proteins contain large numbers of unusually hydrophobic peptide repeats (e.g. PGVG, GGVP, PGVGVA) that form β-spirals, and Ala-rich, α-helical regions that contain Lys and/or the Des/Ide crosslinks (Sandberg and Davidson, 1984; Bressan *et al.*, 1987). Recent studies on the structure of human, sheep, and bovine elastin genes have revealed that the hydrophobic and crosslinking domains are encoded in separate exons of disparate size and are separated by large intervening sequences (Rosenbloom, 1984). The levels of elastin in developing systems appear to be under transcriptional control, and Northern blotting has revealed mRNA species of approximately 3.5 kb in mammals and birds (Rosenbloom, 1984; Sandberg and Davidson, 1984).

From earlier studies on the elastin protein in blood vessels, Sage and Gray (1979, 1980, 1981) made several observations regarding its evolutionary history: (a) elastin occurred only in vertebrates and appeared first in cartilagenous fish; it was absent from cyclostomes, the present-day descendants of jawless fishes, (b) the morphology of aortic elastin fibers was variable; thicker lamellae were generally associated with higher blood pressures, (c) the hydrophobicity of elastin increased with the evolution of the more complex circulatory systems associated with homeothermic animals, (d) coacervation (fiber formation) and elasticity were related to the hydrophobicity of the protein, and (e) elastin and other structural connective tissue proteins did not have a common genetic origin, although there could be distinct genetic types of elastin (for reviews see Sage, 1982 and 1983).

These conclusions were based on a definition of the elastin protein that included several criteria (a characteristic amino acid composition, the presence of Des and Ide, histological appearance, and physicochemical properties). Biochemical analysis revealed increases in both the degree of crosslinking and hydrophobicity in elastins from higher vertebrates (mammals and birds) as compared to those from bony fish (teleosts). It was of particular interest that selection for an increasingly hydrophobic elastin appeared to parallel the development of a highly pressurized, closed circulatory system in homeothermic animals. Moreover, the increase in hydrophobicity was found to be related to the thermodynamic properties of the elastin polymer.

The rather significant changes in the levels of certain amino acids in elastins from different verte-

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Abbreviations used—Des, desmosine; DMEM, Dulbecco Modified Eagle's Medium; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; HPLC, high pressure liqud chromatography; Ide, isodesmosine; kD, kilodaltons; kb, kilobases; PBS, phosphate-buffered saline; Tris–saline–0.15 M NaCl, 50 mM Tris–HCl, pH 7.5; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

brate classes, as well as the presence of discrete domains in elastin, suggest an evolutionary assembly from smaller units which later underwent a multifold duplication (Sage and Gray, 1981). One of the consequences of this mechanism is the creation of gene products of different molecular size. If any of these putative elastin isoforms were retained in present day organisms, one would predict that such differences would be apparent both in the mRNA and its translation product(s).

In this study we have initiated a comparison between mammalian and teleost elastin genes by characterizing elastin mRNA and protein from salmonid fish. We show that salmon elastin mRNA and its translated protein are considerably smaller than those of mammals (approximately 2.0 kb and 43 kD in the fish *versus* 3.5 kb and 70 kD in mammals). Moreover, monoclonal and polyclonal antibodies raised against salmon elastin peptides reacted with a soluble protein of M_r 43,000 secreted by fish cells in culture. Although the amino acid compositions of the salmon elastin peptides were similar to those of elastins found in mammals, there were distinct differences with respect to several of the most abundant residues. These data support an evolutionary divergence of the genes encoding elastin in bony fish and higher vertebrates from a common ancestor.

MATERIALS AND METHODS

Isolation of elastin

Elastin was extracted from the bulbus arteriosus of mature Pacific Northwest Coho Salmon (*Oncorhynchus kisutch*) as previously described (Sage and Gray, 1979). The purified insoluble protein was solubilized with CNBr (Eastman Chemical Co., Rochester, NY) in 70% formic acid, according to the procedure of Rasmussen *et al.* (1975). Soluble peptides were chromatographed at 4°C on Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated with 0.05 M acetic acid. Alternatively, the CNBr digest was fractionated by reverse-phase HPLC on a Vydac C18 column (Separations Group, Hesperia, CA) as described by Kapoor *et al.* (1986).

Amino acid compositions were performed by W. R. Gray (University of Utah, Salt Lake City, UT), according to Sage and Gray (1979).

RNA purification

Total RNA was purified from the bulbus arteriosus of developing coho salmon fingerlings (1 yr) (*Oncorhynchus kisutch*) by a guanidinium isothiocyanate–cesium chloride protocol (Maniatis *et al.*, 1982). Enrichment of mRNA from preparations of total RNA was accomplished by 10–35% sucrose density gradient centrifugation. Poly A^+ RNA was selected by chromatography on oligo(dT)-cellulose (Pharmacia, Uppsala, Sweden) as described by Aviv and Leder (1976).

Cell-free translations were performed on both poly A^+ and total RNA using a nuclease-treated rabbit reticulocyte lysate (Promega Biotec, Madison, WI). Incorporation of radiolabel into trichloroacetic acid-precipitable polypeptides was measured on glass-fiber filters (Wrenn *et al.*, 1987). Radioimmunoprecipitations were carried out as described by Anderson and Blobel (1983) with antisera coupled to Protein A-Sepharose CL-4B beads (Pharmacia, Uppsala, Sweden).

Preparation and characterization of antisera

1 mg of CNBr-digested salmon elastin peptides was emulsified in a 1:1 mixture of PBS and Freund's complete adjuvant (Gibco Laboratories, Grand Island, NY). New Zealand rabbits were immunized by subcutaneous and intramuscular injections and boosted every 21 days thereafter with 1 mg of the immunogen emulsified in Freund's incomplete adjuvant. 5–10 ml of serum were collected prior to each immunization. A polyclonal antibody raised against homogenates of insoluble elastin, as well as elastin solubilized by oxalic acid treatment of rainbow trout (*Salmo gairdneri*) bulbus arteriosus, was a gift from Anna Jacques (University of St. Andrews, Fife, Scotland) (Wright *et al.*, 1988).

Monoclonal antibodies were raised in mice against a CNBr digest of salmon elastin peptides (Wrenn *et al.*, 1986). Tissue culture supernates from several clones reacted positively by ELISA and Western blotting with the immunogen, and one of them (Mab G7) was used in this study.

Solubilized peptides and control proteins (e.g. BSA, collagens, fibronectin) were coated onto polystyrene 96-well costar dishes and incubated with an elastin antibody as previously described (Mecham and Lange, 1982). Competition ELISAs were performed by incubation of increasing amounts of soluble elastin CNBr peptides (0.1–10 μg), dissolved either in PBS or in a 50 mM Tris–HCl buffer, pH 7.5, containing from 1–4 M urea, with a fixed amount of anti-elastin antisera, prior to addition to the elastin-coated wells.

Cell culture and metabolic labeling

Cells from bluegill fry (*Lepomis macrochirus*) (BF-2, American Type Culture Collection, Rockville, MD) and rainbow trout gonadal tissue (*Salmo gairdneri*) (a gift from Kathleen Saybo, University of Washington, Seattle, WA) were grown at room temperature in L-15 medium (Sigma Chemical Co., St Louis, MO) containing antibiotics and 10%, by volume, fetal calf serum (lot #1111593, Hyclone Laboratories, Sterile Systems, Inc., Logan, UT). The embryonic cell line CSE-119, derived from *Oncorhynchus kisutch*, was a gift from Dr C. Lannan (Marine Science Center, Newport, OR) (Lannan *et al.*, 1984). At 80% confluency, cells were metabolically radiolabeled with [^{35}S]-methionine (50 μCi/ml culture medium, approximately 1000 Ci/mmol, ICN Radiochemicals, Irvine, CA) in serum-free DMEM supplemented with Na ascorbate (50 μg/ml) and β-aminopropionitrile fumarate (64 μg/ml) (Gibco Laboratories, Grand Island, NY). After 24 hr, media were removed in the presence of protease inhibitors, and the secreted proteins were precipitated in 10% trichloroacetic acid (Sage *et al.*, 1984).

Radioimmunoprecipitations were conducted with polyclonal antibodies bound to CNBr-activated Sepharose beads (Pharmacia, Uppsala, Sweden). Complexes were washed several times with Tris–saline containing 0.05% Tween, and the final wash was 0.5 M Tris–HCl, pH 6.8. Precipitated proteins were analyzed by SDS-PAGE in the presence of 50 mM DTT (Laemmli, 1970). Alternatively, soluble protein and peptides immunoreactive with anti-elastin antibodies (diluted 250-fold) were identified by a Western blotting procedure as described by Kapoor *et al.* (1986).

Immunofluorescence of cell cultures

Cells were cultured on coverslips, rinsed with Hank's solution, and fixed with 3% paraformaldehyde for 30 min at room temperature (Sage *et al.*, 1984). After several rinses with PBS and 0.05 M glycine in PBS, the fixed cells were treated with ethanol at −70°C for 15 sec and rinsed again with PBS for 10–20 min. Cells were incubated with normal rabbit IgG (200 μg/ml) or with the primary antibody for 30 min at room temperature (Mab were used as undiluted tissue culture supernates, and polyclonal antisera were diluted 10-fold). The coverslips were next rinsed with Hank's solution for 10 min and twice with PBS for 15 min, and they were subsequently incubated with an FITC-labeled

secondary antibody (FITC-goat anti-rabbit IgG or FITC-goat-anti-mouse IgG) (Sigma Chemical Co., St Louis, MO). After 1 hr at room temperature, the cells were washed with PBS and were mounted on glass slides with 90% glycerol in PBS.

The preparations were photographed with a Zeiss Photomicroscope II and Kodak Ektachrome film, ASA 400.

RESULTS

Insoluble elastin was isolated from salmon bulbus arteriosus by a procedure that included sequential extraction of lipid in organic solvents, soluble proteins and proteoglycans in guanidinium HCl/mercaptoethanol, and collagen in boiling water (Sage and Gray, 1979). The amino acid composition of this material is shown in Table 1 (Column A). The salmon elastin contained characteristically high levels of Gly and Tyr, and decreased amounts of Val, Ala, and other hydrophobic amino acids, relative to bovine insoluble elastin (Column D). Another noteworthy difference was the presence of Met in the salmon protein; this residue is characteristically absent in avian and mammalian mature elastin (Sage, 1982). The crosslinking amino acids Ide and Des were less abundant in salmon elastin and averaged 0.4 (Ide) and 0.7 (Des) residues/1000 residues (Sage and Gray, 1979, and data not shown).

Our initial attempts to solubilize salmon elastin by digestion with SV8 protease, trypsin, or oxalic acid resulted in a large number of peptide fragments of very low molecular weight. However, the presence of Met in salmon elastin allowed us to use CNBr to solubilize the protein for the preparation of discrete, larger peptides for subsequent immunization. As shown in Table 1 (Column B), this soluble peptide fraction appeared very similar to the insoluble parent preparation shown in Column A and therefore was

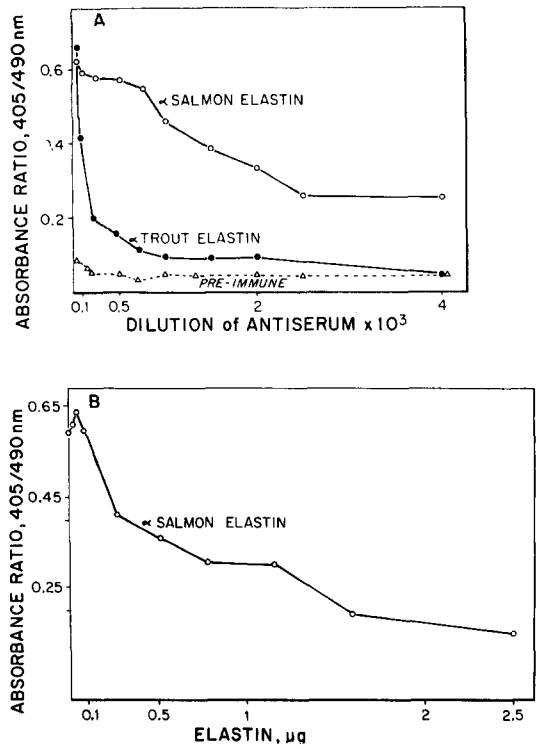

Fig. 1. Characterization of polyclonal antisera generated against salmon elastin. A: Wells were coated with peptides derived from CNBr digestion of insoluble salmon elastin. Dilutions of rabbit antisera were incubated with test peptides and the reaction products monitored by ELISA. Antigens were salmon elastin CNBr peptides (○---○) and oxalic acid-solubilized trout elastin (●---●). Reactivity toward preimmune rabbit serum is shown in triangles. B: Competition ELISA was performed by incubation of increasing amounts of salmon elastin CNBr peptides with anti-salmon elastin antisera.

Table 1. Amino acid composition of salmon elastin*†

	A	B	C	D
Lys	18.4	16.1	23.3	8.7
His	8.4	7.7	8.0	1.4
Arg	31.8	27.6	23.7	8.9
Asx	35.4	34.2	31.3	10.2
Thr	37.2	36.1	43.0	11.3
Ser	35.8	33.9	45.6	11.0
Glx	49.0	46.2	62.5	18.2
Hyp	10.4	ND	ND	9.1
Pro	82.2	85.8	82.2	116.4
Gly	387.0	407.9	394.3	320.0
Ala	126.0	125.3	126.2	228.1
Val	47.6	51.2	48.1	126.6
Ile	14.3	15.6	12.9	25.4
Leu	47.3	47.5	44.2	62.1
Tyr	45.7	42.1	40.2	8.5
Phe	15.6	17.4	13.5	31.5
Met	3.7	2.5	ND	ND
Cys/2	3.0	1.9	ND	ND

*Analyses are shown as residues/1000 residues. ND, not detected (< 1 res/1000); analysis for Trp was not performed.

†Fractions analyzed correspond to: A: insoluble elastin purified from adult salmon bulbus arteriosus. B: soluble peptide fraction after digestion of insoluble elastin (A) with CNBr. C: preparation shown in B, after chromatography on Sephadex G-100. D: insoluble elastin purified from adult bovine thoracic aorta and further treated with CNBr.

felt to be representative of salmon elastin and not other, Met-containing proteins which might have been co-purified in the initial preparation. The presence of Met in Column B indicates that cleavage with CNBr was incomplete. Fractionation of the CNBr-cleavage products on Sephadex G-100 produced 3 peaks (data not shown), the first of which eluted slightly behind the void volume of the column and comprised the majority of the peptide material. The amino acid composition of this fraction, shown in Table 1 (Column C), was also highly similar to the original insoluble elastin (Column A).

Salmon elastin peptides (Table 1, Column B) were used as immunogens in rabbits for the production of polyclonal antibodies. An ELISA with the antiserum from one of these animals showed reactivity with CNBr-derived salmon elastin peptides at dilutions from $0.1–2.5 \times 10^3$ (Fig. 1A). This reactivity was also seen against insoluble salmon elastin (not shown) and could be competed by incubation of the antiserum with increasing concentrations of salmon elastin peptides (Fig. 1B). For comparison, the reactivity of an anti-trout elastin antiserum against the salmon peptides was also tested (Fig. 1A). Although the titer of the anti-trout elastin antibody appeared lower, at

dilutions of less than 1 to 200 there was clearly recognition of salmon elastin epitopes (in both insoluble and CNBr-solubilized preparations) by this antibody. We were, however, unable to demonstrate reactivity of the anti-salmon polyclonal antibody against bovine aortic elastin, and several monoclonal and polyclonal antibodies elicited against mammalian and avian elastins also failed to recognize oxalic acid- or CNBr-solubilized salmon elastin peptides (data not presented). A monoclonal antibody specific for chick tropoelastin (a gift from Dr J. Foster, Syracuse University, Syracuse, NY) similarly did not crossreact on a Western blot with either CNBr-solubilized salmon elastin peptides or products of salmon RNA-directed cell free translation. These data provide strong evidence that the immunoreactive epitopes on salmon elastin are unique.

We next tested the ability of several anti-salmon elastin antibody preparations to recognize native tropoelastin produced by fish cells in culture. Positive immunofluorescence was seen with the anti-salmon elastin polyclonal antiserum on both trout fibroblasts (Fig. 2B) and bluegill fry cells (a teleost fish belonging to a family separate from that of salmonids) (not shown). The anti-trout elastin antisera also reacted positively with both cell types (Fig. 2C shows immunofluorescence throughout the cytoplasm, but not in the nuclei, of bluegill fry cells). Figure 2(A) depicts trout fibroblasts in the presence of one of the monoclonal antibodies (Mab G7) produced after immunization of a mouse with CNBr-derived elastin peptides. This antibody stained the cells in a pattern similar to those seen in Figs 2(B) and 2(C). Incubation of cells with irrelevant Mabs, or polyclonal antisera specific to fibronectin or to different collagen types, produced essentially no immunofluorescence (data not shown but comparable to image in Fig. 2D). Although elastin is a secreted protein, its apparent absence from the extracellular matrix in Fig. 2(A–C) could be due to the short time that the cells were cultured on the glass coverslips prior to the immunofluorescence experiments (generally 24 hr). Either a longer time is needed for the extracellular deposition of elastin by teleost cells in quantities

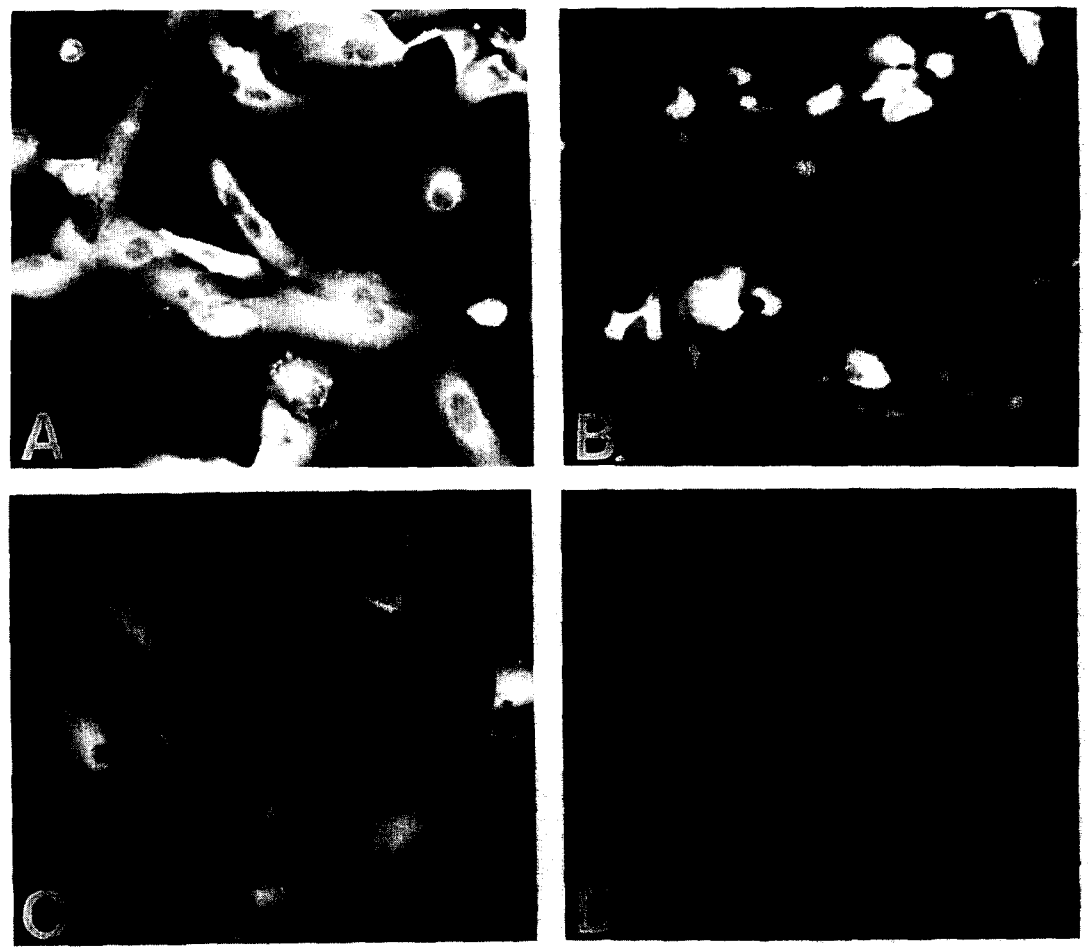

Fig. 2. Immunofluorescence localization of elastin on teleost fibroblasts *in vitro*. Cells were plated on glass coverslips, permeabilized, and exposed to antisera followed by FITC-conjugated anti-mouse IgG (A) or FITC-anti-rabbit IgG (B–D). A: Rainbow trout gonadal fibroblasts, reacted with a monoclonal antibody (Mab G7) against salmon elastin CNBr peptides. B: Trout fibroblasts, reacted with polyclonal rabbit anti-salmon elastin antisera. C: Bluegill fry cells reacted with polyclonal anti-trout elastin antisera. D: As C, in the absence of primary antibody. Bar, 20 μm.

sufficient for detection by immunofluorescence, or alternatively, the crosslinking and assembly of elastin is in some way impaired in these systems *in vitro*.

ELISA of both trout and bluegill fry cell culture media produced a positive reaction with Mab G7 as well as with the polyclonal anti-trout and anti-salmon antisera (not shown). Moreover, teleost cells secreted a soluble tropoelastin-like protein into the culture medium that was precipitated by several of the anti-elastin antibodies. In Fig. 3, lanes show total radiolabeled secreted protein from bluegill fry cells (3A) and trout cells (3B). Mab G7 and anti-trout elastin antisera recognized a single radioactive species of M_r 43,000 in the culture media (lanes 2 and 3, respectively). Identical results were obtained with the coho salmon cells (not shown). This apparent molecular weight did not change appreciably in the absence of DTT, and the protein was insensitive to bacterial collagenase (not shown). At the present time we have not identified the bands at the top of the gel (especially in Figure 3B) that co-precipitated with the M_r 43,000 protein. The apparent reactivity might be nonspecific, or the bands might represent aggregates of the secreted elastin monomer.

We attempted to confirm the identity of the M_r 43,000 protein by using the anti-elastin antisera in a Western immunoblot to detect specific peptides in the original CNBr digest of salmon elastin. This digest was fractionated by HPLC and the eluate was analyzed by SDS-PAGE. As shown in Fig. 4(A), a complex elution pattern was obtained; resolution by SDS-PAGE revealed at least 10 distinct peptides. In Fig. 4(B) (lane 1) is shown a pool of the predominant peak fractions indicated by the bar in Fig. 4(A). The predominant polypeptides (M_r 22,000 and 24,000) co-migrated with 2 species from the original CNBr salmon elastin digest. Peptides of similar M_r were also separated from the parent CNBr digest by chromatography on BioGel A-0.5 m (data not shown). However, attempts to fractionate the digest by chromatography on Fractogel, Sephacryl S-500, or Phenyl-Sepharose were unsuccessful.

The 2 polypeptides (22 kD and 24 kD) shown in Fig. 4(B) (lane 1) reacted positively on a Western blot with anti-trout elastin antiserum (lane 2). Although there was apparent immunoreactivity with several bands (which may be aggregates of smaller elastin peptides that occur despite the presence of SDS and

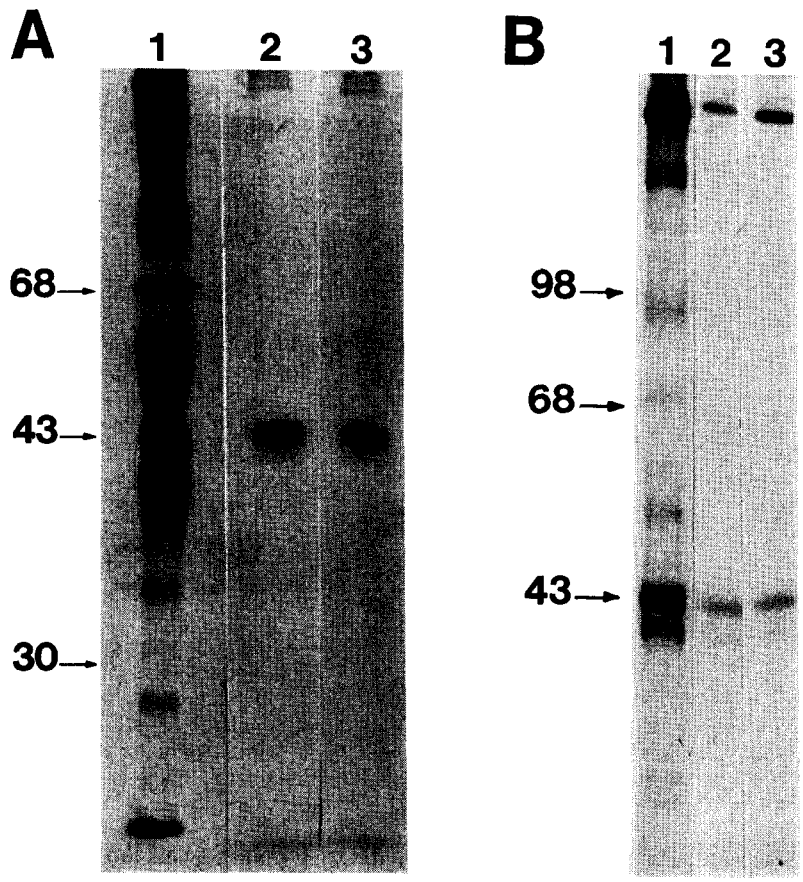

Fig. 3. Synthesis of tropoelastin by teleost fibroblasts. Cells from bluegill fry (A) and trout (B) were metabolically labeled with [^{35}S]-methionine, and the culture medium proteins were precipitated with trichloroacetic acid (lane 1), Mab G7 (lane 2), or anti-trout elastin antisera (lane 3). Precipitates were analyzed by SDS-PAGE (5%/10% gel) in the presence of 50 mM DTT. Molecular weight standards are shown on the left ($M_r \times 10^{-3}$) of two separate gels. Both the Mab and polyclonal antibodies precipitated selectively an M_r 43,000 [^{35}S]-Met labeled protein from the culture media of these cells.

urea), the principal band was a CNBr-derived elastin peptide of approximate M_r 24,000. The polyclonal anti-salmon elastin antibody also recognized the 22–24 kD doublet on a parallel Western blot (not shown). The derivation of this fragment from the M_r 43,000 protein immunoprecipitated from fish culture media (Fig. 3) cannot be demonstrated without amino acid sequence information. Nevertheless, we feel that this major M_r 24,000 peptide and the M_r 43,000 secreted form are likely to be closely related.

To corroborate further the nature of the tropoelastin produced by fish cells *in vitro*, we isolated RNA from the bulbi arteriosi of young salmon and per-

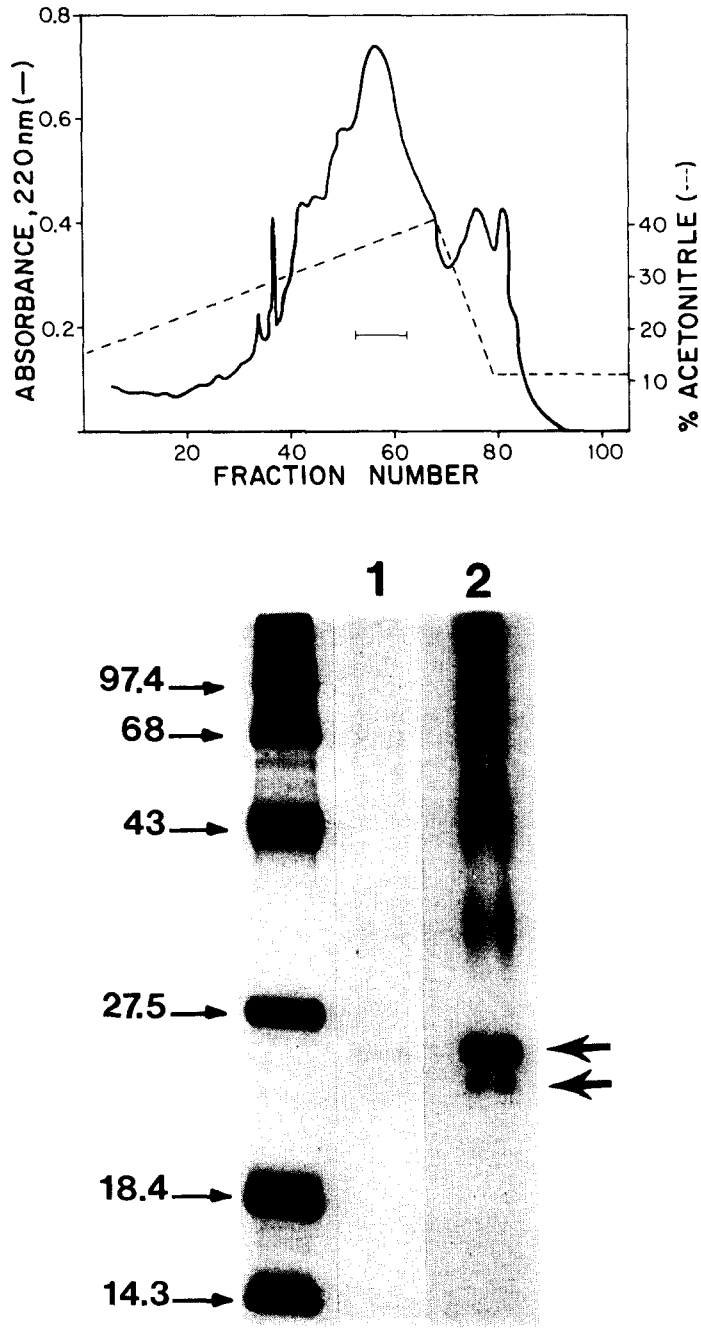

Fig. 4. Fractionation of salmon elastin peptides by HPLC. A: CNBr peptides from salmon elastin were chromatographed on a Vydac C18 column at 60°C with a linear gradient of acetonitrile (20–40%) in 0.1% trifluoroacetic acid. Bar indicates pooled fractions which were analyzed by SDS-PAGE. B: The HPLC fraction shown in Fig. 4A was resolved on a 7.5%/15% SDS-polyacrylamide gel in the presence of DTT and stained with Coomassie Blue (lane 1). In lane 2 is shown an immunoblot of the peptides in lane 1 after incubation with anti-trout elastin antisera, followed by [^{125}I]-Protein A. Protein molecular weight standards are shown on the left. 2 major peptides (M_r 22,000–24,000) (arrows) produced by CNBr cleavage reacted with the antisera.

formed cell-free translations in a reticulocyte lysate system. As shown in Fig. 5, a major product of approximate M_r 43,000 was translated by both a total RNA (lane A) and poly A$^+$ RNA preparation (lane B) from this developing vascular structure. This product also incorporated [^{3}H]Pro and [^{3}H]Val with high specific activity and was insensitive to bacterial collagenase (data not shown). We observed this translation product in approximately 10 separate preparations of salmon bulbus arteriosus poly A$^+$ RNA and with several different reticulocyte lysates. By scanning densitometry, the M_r 43,000 band represented from 30–50% of the total translated products that were resolved by SDS-PAGE.

Confirmation of the M_r 43,000 cell-free translation product as newly synthesized tropoelastin was provided by radioimmune precipitation of the translation mixture with anti-teleost elastin antibodies. In Fig. 6, lane A shows the spectrum of polypeptides translated in a cell-free system by salmon bulbus arteriosus poly A$^+$ RNA. After incubation of this mixture with anti-trout elastin IgG or anti-salmon Mab G7 coupled to Sepharose beads, a principal immunoreactive product of M_r 43,000 was observed (lane B). Because of the tendency of proteins to adsorb nonspecifically to Sepharose beads, we precleared the total translation mixture shown in lane A with uncoupled beads and analyzed the absorbed material (lane D) and the proteins remaining in solution (lane C). From these experiments it was clear that the M_r 43,000 band was specifically precipitated by the antibodies and had not been adsorbed by the beads alone.

To determine the size of mRNA directing the

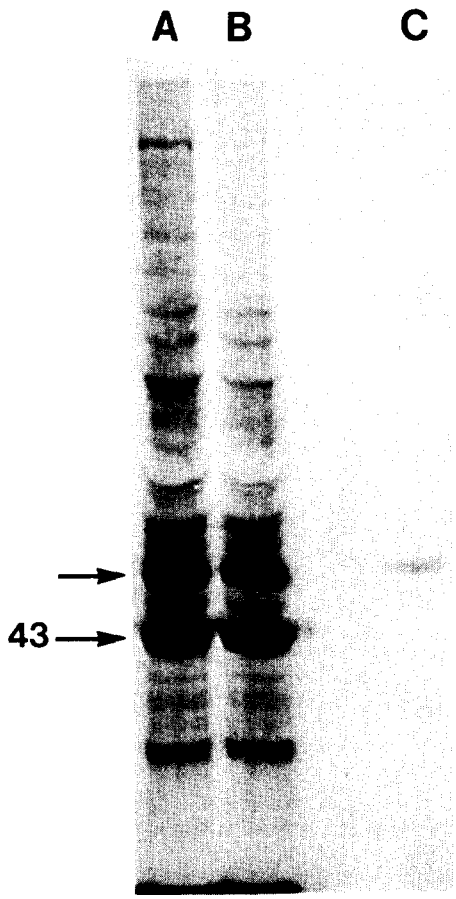

Fig. 5. Cell-free translation of salmon RNA. Total RNA (10 μg) from 1–2 yr old salmon bulbus arteriosus (A) and poly A$^+$ RNA (1.0 μg) (B) were translated *in vitro* in the presence of a rabbit reticulocyte lysate and [^{35}S]-Met. The translation products were analyzed by SDS-PAGE on a 4%/8% gel under reducing conditions. Lane C represents translation in the absence of added RNA. The major translation product of this tissue was an M_r 43,000 polypeptide. Unlabeled arrow indicates a product of the endogenous activity of the lysate.

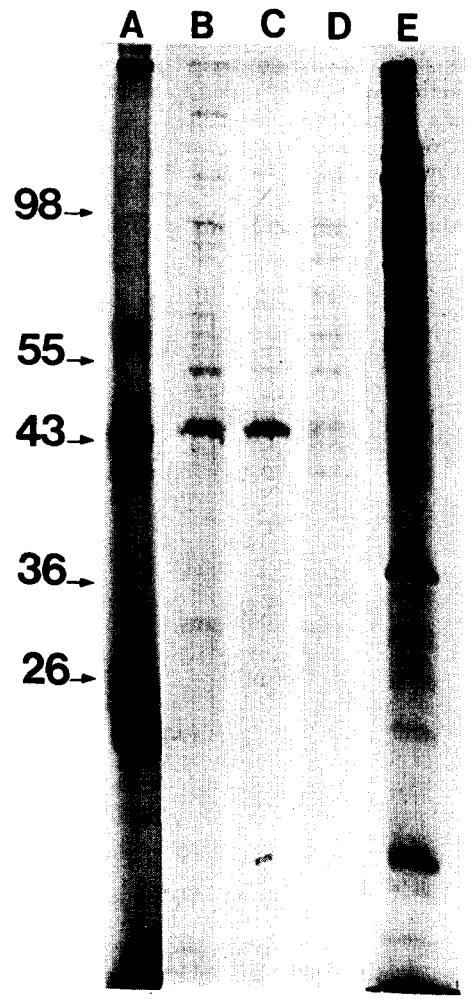

Fig. 6. Cell-free translation of salmon poly A$^+$ RNA and radioimmunoprecipitation of an M_r 43,000 protein with anti-elastin antibodies. Salmon poly A$^+$ RNA was translated in a cell-free system and the products were identified by radioimmune precipitation and SDS-PAGE under reducing conditions. Lane A, total translation products; lane B, lane A material, after incubation with anti-trout elastin antibodies coupled to Sepharose; lane C, lane A material initially precleared with uncoupled Sepharose, representing unadsorbed material; lane D, components which adsorbed to uncoupled Sepharose; lane E, brome mosaic virus RNA-directed translation products. Protein molecular weight standards are shown on the left ($M_r \times 10^{-3}$).

translation of a salmon elastin of M_r 43,000, we fractionated poly A$^+$ RNA from salmon bulbus arteriosus by sucrose density gradient centrifugation and performed cell-free translations with each of the ensuing fractions. A peak of poly A$^+$ RNA with a high translational activity was observed near the position of an 18 S ribosomal RNA marker in the gradient (Fig. 7A). We have estimated that the M_r of RNA in this peak was 1.9–2.1 kb. For comparison, RNA with a sedimentation value of 25 S has a M_r of 1.25×10^6 and contains approximately 3.5 kb.

Examination of translation products directed by fractions 34–38 is shown in the fluorescent autoradiograph in Fig. 7(B). Poly A$^+$ RNA of approximate M_r 2 kb was associated with a cell-free translation product of M_r 40,000–43,000 by SDS-PAGE. The abundance of this 2 kb mRNA in a salmonid tissue known to be enriched in elastin (Sage and Gray, 1979,

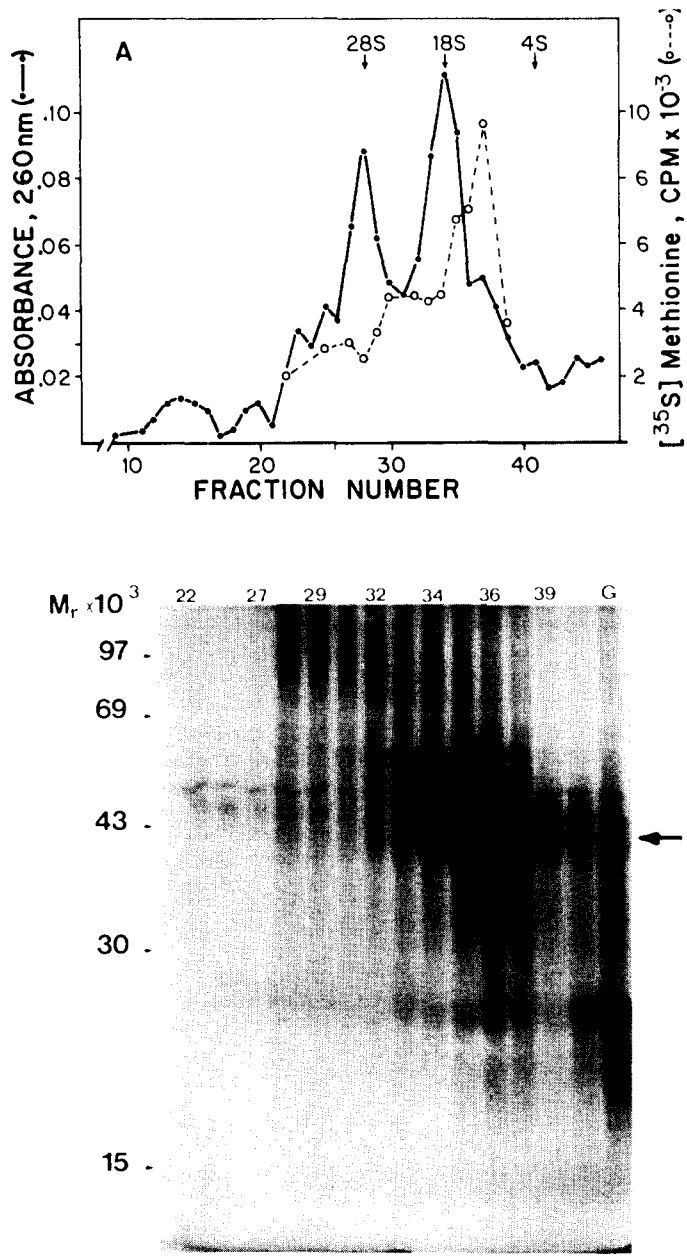

Fig. 7. Fractionation of a salmon poly A$^+$ RNA that directs the translation of an M_r 43,000 polypeptide. Salmon total RNA was separated on a 10–35% sucrose gradient. Fractions were precipitated in ethanol and were subsequently analyzed for poly A$^+$ RNA by cell-free translation and SDS-PAGE (5%/10% gel after reduction of samples with 50 mM DTT). A: The fraction which shows translational activity (○---○) corresponded to an RNA species of approximately 2 kb. ●---●, total RNA, including ribosomal subunits (28 S, 18 S). B: Fluorescent autoradiograph shows major translation products in fractions 34–38 that correspond to species of M_r 40,000–43,000 (arrow). G, translation products of globin mRNA.

1980) and the recognition of a 43 kD translation product by anti-salmon elastin antisera have allowed us to conclude that this mRNA was transcribed from a salmon elastin gene. Consistent with the lack of interspecies crossreactivity among anti-elastin antisera, the human elastin cDNA probe HEL-2 (a gift from Dr J. Rosenbloom, University of Philadelphia, Philadelphia, PA), did not hybridize with any mRNA isolated from salmon bulbus arteriosus under the experimental conditions used in our study. It therefore appears that elastin genes of teleost fish and higher vertebrates have undergone considerable evolutionary divergence.

DISCUSSION

In this study we have shown that salmon elastin mRNA and protein are 63% and 61%, respectively, the size of their mammalian and avian counterparts. Evidence for this conclusion was based largely on the reactivity of several anti-salmon elastin antisera with an M_r 43,000 protein that was secreted by teleost cells *in vitro* and was produced as a cell-free translation product of salmon bulbus arteriosus poly A^+ RNA that had an M_r of approximately 2 kb. The immunogen used to produce the monoclonal and polyclonal antibodies was a CNBr digest of insoluble elastin purified from salmon bulbus arteriosus, an especially resilient tissue invested with a large amount of elastin-containing fibers (Sage and Gray, 1979, 1980). At the level of amino acid composition, insoluble elastins from teleost fish and mammals are similar enough to be included in a family of related genes. However, they are sufficiently unique, especially with respect to the levels of several amino acid residues, to consider separate lines of descent from a common ancestral vertebrate gene.

Evolution of the salmon elastin gene

Since elastin is a periodic protein with unique domains encoded in separate exons, one question we felt could be addressed by studying its structure in the primitive teleost suborder Salmonoidea concerned the mechanisms by which genes with repetitive sequences evolved. The presence of the elastin gene as a single copy in the sheep genome (Olliver *et al.*, 1987) suggests that evolution did not occur by duplication of an entire, functional gene. Rather, a series of point mutations coupled with short-range duplications or deletions in the gene, as well as transcriptional modulation of an existing gene, might be responsible for the inter- and intraspecies diversity of elastin. Karyotyping of Salmonidae has revealed marked variations in diploid chromosome number ($2n$ = 50–90), chromosome arm number, and chromosome polymorphism (Hartley, 1987). Although the evolution of salmonid fishes from a tetraploid ancestor remains an open question, it is clear that a substantial amount of structural rearrangement has occurred to produce the present-day karyotypes (see Hartley, 1987, for a discussion on this point). Possible consequences of such genomic restructuring are the origin of replicate loci which are subject to drift and consequent establishment within a gene family.

In the Salmonidae, members of the protamine gene superfamily are highly homologous to one another, and at least one of them exhibits intergenus conservation of 2 structurally different repetitive elements (Moir and Dixon, 1988). Fish that live in freezing seawater have another multigene family comprised of repetitive antifreeze proteins. Genes for both the wolffish and the winter flounder antifreeze proteins, which are structurally different, are arranged in a tandem series with each repeat containing 2 of these genes in an inverted orientation (Scott *et al.*, 1988). A tandem pattern could originate through unequal crossing-over in meiosis, facilitated by mismatching of chromatids prior to recombination. In an analysis of tandem-repetitive mini-satellite regions in human DNA, Jeffreys *et al.* (1988) demonstrated significantly high mutation rates to new length alleles. These changes in gene length were similar to those expected as a result of unequal exchange during meiosis. We infer from these studies that repetitive elements within a gene predispose that DNA to allelic variations that, by mechanisms largely unknown, result in gene products of different molecular size. Our data showing differences in molecular weight between teleost and mammalian elastin mRNA and protein could be explained by the insertion or loss of certain, possibly repetitive, elements during the evolution of the salmonid elastin gene.

Comparison of elastin protein and mRNA in teleosts and homeotherms

It is now clear that alternative splicing of a single elastin gene transcript accounts for some of the multiple molecular weight forms of tropoelastin secreted by bovine and human cells *in vivo* and *in vitro* (Indik *et al.*, 1987; Raju and Anwar, 1987; Wrenn *et al.*, 1987; Yeh *et al.*, 1987). Three separate mRNAs have been described in fetal calf nuchal ligament that encode 3 forms of elastin containing 747, 733, and 713 amino acids, respectively (Raju and Anwar, 1987). Similarly, Wrenn *et al.* (1987) demonstrated three distinct tropoelastins (M_r 67,500, 65,000, and 62,000) in fetal calf ligament fibroblasts and chondrocytes. Two tropoelastin isoforms (M_r 63,000 and 65,000) were found as primary translation products of fetal sheep nuchal ligament (Olliver *et al.*, 1987). Recent studies in the chicken have confirmed the presence of 3 distinct tropoelastins in lung tissue (Rich and Foster, 1987). Synthesis of these isoforms of tropoelastin appears to be mediated by mRNA of 3.5 kb, which in mammals is comprised of approximately 2.2 kb of coding sequence and 1.3 kb of 3′ untranslated sequence (Indik *et al.*, 1987; Deak *et al.*, 1988; Olliver *et al.*, 1987).

The use of antibodies specific for discrete domains of tropoelastin has enabled investigators to address several functional aspects of this protein (Grosso and Mecham, 1988). The specificity of these reagents appears to be paralleled by the species specificity of most polyclonal anti-elastin antibodies described in the literature. In an early study, Kucich *et al.* (1981) reported that antibodies to lung and aortic elastin crossreacted within a single species but did not exhibit interspecies reactivity. Moreover, antibodies to elastin peptides solubilized by digestion with oxalic acid did not recognize peptides solubilized by elastase, although antibodies specific for these digestion products would react with the insoluble elastin from

which the peptides were derived (Kucich *et al.*, 1981). We had also observed that anti-salmon elastin antibodies did not crossreact with bovine elastin, and that teleost elastin was in turn not recognized by antibodies raised against elastins from higher vertebrates. In addition, antisera against CNBr-solubilized salmon elastin peptides also recognized epitopes on undigested, insoluble salmon elastin. The ability of the anti-trout elastin antisera to crossreact with CNBr-solubilized salmon elastin was most probably due to a subpopulation of antibodies specific for epitopes on insoluble trout elastin, rather than for those created by oxalic acid cleavage. The failure of either the anti-salmon or anti-trout elastin antisera to recognize human and bovine elastins attests to the uniqueness of the teleost elastin sequences that are antigenic. It has been shown that antibodies to the Ala-rich, potentially cross-linking domains of tropoelastin exhibited a higher degree of interspecies crossreactivity than was seen with antibodies to the less-conserved, hydrophobic domains, which tended to be species-specific (Mecham and Lange, 1982). If CNBr cleavage created antigenic sites on teleost elastin, it may therefore follow that Met is present largely in the hydrophobic sequences, as the antisera elicited to these elastin peptides did not crossreact with elastins from other vertebrates.

An anti-trout elastin antisera has been recently used to identify elastin-like material in certain respiratory and circulatory cartilages in lampreys (Wright *et al.*, 1988). Since we have shown that a similar antiserum contains a population of antibodies that precipitate specifically a teleost tropoelastin, it is possible that the fibers observed by Wright *et al.* (1988) in these primitive agnathan fish contained elastin. Previous studies had shown, by biochemical criteria, that elastin was absent from the aortae of cyclostomes (lampreys and hagfish) (Sage *et al.*, 1979, 1980). The major protein component of lamprey annular cartilage is a novel protein termed lamprin (Wright *et al.*, 1983). We have recently found Verhoeff- and resorcein-positive structures in sections of lamprey notocord sheath (J. Mallatt, M. Chow, and H. Sage, unpublished experiments). However, extraction of hagfish notocord and its surrounding sheath did not produce protein with Des/Ide or an amino acid composition resembling elastin (M. Chow and H. Sage, unpublished experiments).

Structure–function relationships in elastin

Transcriptional and independent regulation of 3 tropoelastin isoforms has recently been demonstrated in fetal, neonatal and adult bovine elastic tissues (Parks *et al.*, 1988). In the developing chick embryo, levels of elastin mRNA within a given organ were found to be temporally and tissue-specific (Tokimitsu *et al.*, 1987). In addition to developmental regulation, the elastin gene also demonstrated increased transcriptional activity in adult pulmonary artery tissue in response to pulmonary hypertension (Mecham *et al.*, 1987). These experiments demonstrate sensitivity of the elastin gene to local environmental situations of physiological relevance to the organism.

In the circulatory systems of vertebrates, elastin varies with respect to abundance, distribution, amino acid composition, and thermodynamic properties (Spina *et al.*, 1979; Sage, 1982, 1983). Selection for an increasingly hydrophobic elastin was correlated with the development of warm-blooded animals with higher blood pressures. The hydrophobic elastins of homeotherms polymerized into aggregated structures (coacervated) at 37°C, while the more hydrophilic elastins of teleosts did not (Sage, 1982). When stretched in a microcalorimeter, elastins with the highest hydrophobic indices demonstrated interaction with the aqueous solvent, a thermodynamic behavior different from that of conventional elastomers (Sage, 1982). The bulbus arteriosus of teleost fish is a specialized structure that effectively damps the high pressure pulses from the cardiac ventricle before the deoxygenated blood reaches its initial destination, the delicate vessels of the gills. This elastin in salmonids has been characterized by higher levels of polar amino acids, fewer crosslinks, and a lower Young's modulus than the corresponding bovine elastin (Spina *et al.*, 1979). Since the $(Ala)_n$ Lys sequences are required for crosslink formation and are highly conserved in birds and mammals, we predict that hydrophobic, Gly-rich domains in salmon elastin will exhibit the most interspecies variability and will reflect the greatest degree of divergence from elastins of higher vertebrates. Raju and Anwar (1987) have shown that the hydrophobic pentapeptide repeat PGVGV, which forms a putative β-spiral, occurs infrequently in chick tropoelastin relative to its levels in the pig and bovine proteins. These authors therefore proposed that this peptide repeat was not essential for the function of elastin, a conclusion that would be consistent with our data on the salmon protein. This hypothesis could perhaps be tested in a disease model of advanced lipoprotein-related atherosclerosis which occurs relatively rapidly in spawning, anadromous salmon (Eaton *et al.*, 1984). In this case the response of the elastin network to increases in local blood pressure from luminal occlusion, as well as the effect of lipid accumulation on the resilience of elastin, could be addressed with respect to both the crosslinking and the hydrophobic domains.

SUMMARY AND CONCLUSIONS

We have shown that the cells of salmonid and other teleost fish secret soluble elastin of M_r 43,000.

mRNA from the bulbi arteriosi of developing salmon directed the cell-free translation of an M_r 40,000–43,000 protein that was recognized by antibodies raised against salmon elastin.

The M_r of this mRNA was approximately 2.0 kb and was consistent with the size of the translation product.

Both the salmon elastin protein and mRNA were significantly smaller than those previously described for avian and bovine elastins. These differences, coupled with the variations in amino acid composition observed between teleost and mammalian elastins, are consistent with a divergence of at least two elastin genes at an early stage of vertebrate evolution from a common ancestral prototype.

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