Expression of Intermediate Filament–associated Proteins Paranemin and Synemin in Chicken Development

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ABSTRACT   The expression of two intermediate filament–associated proteins, paranemin (280,000 mol wt) and synemin (230,000 mol wt), was investigated with respect to the expression of two core intermediate filament proteins, desmin and vimentin, in various embryonic and adult chicken muscle and nonmuscle cells. All developing muscle cells, regardless of their type, simultaneously express desmin, vimentin, paranemin, and synemin. However, a difference is observed in the expression of paranemin in adult muscle. This protein is removed during differentiation of both fast and slow skeletal muscle, visceral smooth muscle, and the smooth muscle of muscular arteries, but remains in mature myocardial cells, cardiac conducting fibers, and the smooth muscle cells of elastic arteries. Some of these cells express vimentin, others desmin, and still others a mixture of the two. On the other hand, synemin is expressed in all the above types of adult muscle cells except myocardial cells. Adult myocardial cells also lack vimentin, and its presence is gradually reduced after hatching. Since in adult striated muscle all expressed intermediate filament proteins are found predominantly in association with the peripheries of myofibrillar Z discs, these results suggest that a change in the composition of skeletal and cardiac muscle Z discs occurs during chicken development and maturation. Erythrocytes that express synemin and vimentin do not express paranemin, while both embryonic and adult Schwann cells co-express paranemin and vimentin, but not synemin. Endothelial cells of muscular vessels express paranemin, while those of elastic vessels do not, and neither contains synemin. Paranemin and synemin are not expressed in neurons, epithelial, and most glial cells, suggesting that these two polypeptides are expressed only in conjunction with desmin or vimentin. These results suggest that the composition of intermediate filaments changes during chicken development, not only with respect to their core subunit proteins but also with respect to two associated polypeptides, particulary in muscle cells.

Five major classes of intermediate-filament subunits have thus far been identified in higher vertebrates (for recent reviews, see 29, 30). In general, fully differentiated cells express one class of intermediate filament subunit that typifies that cell type, but many exceptions where two subunit classes are co-expressed also exist. One of the main exceptions appears to be the co-expression of desmin and vimentin in avian muscle cells. Mature visceral smooth muscle contains predominantly desmin (22, 31, 37, 44), but vascular smooth muscle (4, 13, 17, 35, 38, 47) and fast and slow skeletal muscle (21, 32) co-express various ratios of desmin and vimentin. In adult cardiac cells the situation is less clear. The impulse-conducting cells of avian and mammalian hearts contain desmin filaments (10, 11), and desmin has been shown to be present in

atrial and ventricular myocardial cells (7, 16, 28, 31). Whether mature myocardial cells or conducting tissue express vimentin is unknown (see below). In development, co-expression of two intermediate filament subunits is common, with vimentin being co-expressed with neurofilament subunits during neuronal differentiation (46) and with desmin during muscle differentiation (2, 3, 6, 18, 32).

In addition to the cell type-specific classes of intermediate filament subunits, a number of intermediate filament–associated proteins have also been identified. In most cases, their function is unknown or the proteins have not been adequately studied. The few that have been studied in detail exhibit an even greater degree of specificity in their expression than do the core proteins of intermediate filaments. One of them,

filaggrin, is expressed specifically in differentiating and fully differentiated epidermal cells and functions to cross-link individual keratin filaments into fiber bundles, known as macrofibrils (45). Two other polypeptides with molecular weights of 145,000 and 200,000 have been shown to be constituents with the 70,000-mol-wt neurofilament subunit in neurons (41–43, 48). Immunoelectron microscopic studies on isolated neurofilaments (49) or neurofilaments in situ (40) have demonstrated that the 200,000-mol-wt polypeptide functions to cross-link these filaments at periodic intervals. During neuronal differentiation, only some neurons express the 200,000-mol-wt polypeptide, and those that do begin expressing it later in development, well after the onset of expression of the 70,000- and 145,000-mol-wt polypeptides (41–43). Furthermore, certain neuronal processes may be devoid of the 200,000-mol-wt polypeptide, suggesting that both the expression of this polypeptide in development and its distribution within a neuron may be closely related to its cross-linking function (41–43).

Two other proteins found in association with intermediate filaments are the 230,000-mol-wt polypeptide, synemin (22), and the 280,000-mol-wt polypeptide, paranemin (5). In chickens, synemin has been shown to be expressed only in cells that express vimentin or desmin, or both, and in particular smooth and skeletal muscle cells and erythrocytes (22–24). Fibroblasts and myoblasts lack synemin (22), even though they express vimentin (2, 18), but upon the onset of fusion the synthesis of synemin, along with that of desmin, is initiated (18, 22). Immunoelectron microscopic localization of synemin along vimentin filaments in erythrocytes has suggested that, like the 200,000-mol-wt neurofilament polypeptide, synemin functions to cross-link intermediate filaments at periodic intervals (23). The specific expression of synemin in certain cell types and in certain stages of differentiation may also be related to its cross-linking function. Paranemin exhibits an expression pattern distinct from that of synemin. In skeletal muscle cells, where its expression has been studied in detail, paranemin is co-expressed with vimentin in myoblasts and with vimentin, desmin, and synemin in myotubes (5). Furthermore, all four polypeptides coordinately change their distribution during myogenesis and associate with the newly assembled Z discs. However, unlike the other three polypeptides, paranemin gradually disappears in the ensuing days of muscle development and maturation and is absent from the adult tissue as shown by immunoautoradiography and immunofluoresence (5).

We have examined the pattern of expression of paranemin with respect to that of synemin, desmin, and vimentin in various chicken embryonic and adult muscle and nonmuscle cells. The results suggest that the expression of paranemin, like that of synemin, is determined by the structural requirements of different cells. They further suggest that there are differences in the functions of intermediate filaments, as evidenced by the heterogeneity in the expression of synemin and paranemin in the adult.

MATERIALS AND METHODS

Antibodies: Polyclonal antibodies to chicken gizzard desmin and synemin and to vimentin from chicken embryonic skeletal muscle have been characterized previously (21–23). Antibodies to paranemin also have been described previously (5). Antibodies to the 170,000-mol-wt neurofilament-associated polypeptide will be described elsewhere (Granger B. L., and E. Lazarides, manuscript in preparation). All four rabbit antisera were purified at 50% ammonium sulfate saturation, dialyzed extensively against phosphate- or Tris-buffered saline (PBS: 137 mM NaCl, 3 mM KCl, 8 mM Na$_2$HPO$_4$, 1 mM

KH$_2$PO$_4$, 5 mM NaN$_3$, pH 7.5) and stored at −70°C after the addition of ε-amino-*n*-caproic acid to 1 mM. The specificity of each antibody preparation has been tested by immunoautoradiography on one- and two-dimensional polyacrylamide gels (5, 21, 22) and shown to cross-react only with the corresponding antigen.

Two-Dimensional Peptide Mapping: Two-dimensional peptide mapping was done according to the procedures of Elder et al. (9), as modified previously (19). Briefly, spots of protein and an area of blank gel, as a control, were excised from two-dimensional gels; then the proteins in the gel slices and the blank gel slice were iodinated with ^{125}I by the Chloramine-T method. Each of the iodinated proteins and the control gel slice were proteolized for 24 h with trypsin-*N*-tosyl-L-phenylalanine chloromethyl ketone (Millipore Corp., Freehold, NJ), chymotrypsin (Worthington Biochemical Corp., Freehold, NJ), or thermolysin (Sigma Chemical Co., St. Louis, MO) using a protease concentration of 0.05 mg/ml. The released peptides were spotted on cellulose sheets (Eastman-Kodak, Rochester, NY) and separated in the first dimension by high-voltage electrophoresis and in the second dimension by thin-layer chromatography. The peptides were visualized by exposing the chromatograms to Kodak X-Omat XR5 film with DuPont Chronex Lightning-Plus screens (E. I. DuPont de Nemours, Wilmington, DE) for 12 h to 6 d at −70°C.

PAGE: One-dimensional PAGE was performed according to the method of Laemmli (27), as modified by Hubbard and Lazarides (25), using a discontinuous buffer system of SDS-Tris-glycine. The separating gels contained 12.5% acrylamide and 0.11% *N,N'*-methylene-bisacrylamide. Two-dimensional PAGE was performed according to the method of O'Farrell (33) as modified by Hubbard and Lazarides (25).

Sample Preparation: To minimize proteolysis in tissue samples to be used for PAGE and subsequent immunoautoradiography, small pieces of tissue were removed from embryonic and adult White Leghorn chickens, rinsed in PBS, and immediately immersed into liquid nitrogen. The tissue pieces were then ground under liquid nitrogen. Most of the liquid nitrogen was then poured off, the tissue particles were scraped into a cooled test tube, and a boiling solution of 0.2% SDS, 0.1 mM phenylmethyl sulfonyl fluoride, 0.2 mM *p*-tosyl-L-arginine methyl ester, and 0.25 mM *o*-phenanthroline was immediately added. Subsequently, an appropriate amount of a 5 × stock solution of SDS-sample buffer (1 × sample buffer: 1% SDS, 40 mM Tris-Cl [pH 6.8], 5% glycerol, 5% β-mercaptoethanol, 0.02% bromphenol blue) was added and the samples were boiled for 5–10 min. Insoluble material was removed by centrifugation at 3,000 *g* for 5 min and portions of the resulting supernatant were used for electrophoresis. Samples were frozen and thawed only once.

The sources of the proteins used for two-dimensional peptide mapping were as follows: desmin, vimentin, and paranemin were obtained from intermediate filaments isolated from skeletal myogenic tissue of 14-d-old chicken embryos by the method described previously (5). Synemin was obtained from the KI-insoluble residue of adult chicken gizzard (22).

Immunoautoradiography: Immunoradiography, using antibodies to desmin, vimentin, and paranemin, was performed as described previously (22). One-dimensional SDS-slab gels were reacted with antibodies diluted 1,000-fold in Buffer I (140 mM NaCl, 10 mM Tris HCl [pH 7.5], 5 mM NaN$_3$, 0.1 mM EGTA). Antiparanemin antisera were preadsorbed for ~4 h with a suspension of acrylamide particles to remove antibodies to acrylamide (5, 22). Gels were exposed on Kodak X-Omat XR5 film with DuPont Cronex Lightning-Plus intensifying screens for 1–6 d at −70°C.

Immunofluorescence: Pieces of tissues were removed from embryonic or adult chickens, rinsed in PBS, embedded in O.C.T. Compound (Tissue Tek II, Miles Laboratories, Naperville, IL.), and frozen in liquid Freon cooled with liquid nitrogen to −197°C. Tissue was cut into 4–8-μm sections in Tissue Tek II cryostat at −20°C. Sections were fixed and dehydrated in 95% ethanol, and finally immersed in PBS with 2 mM EGTA, 0.5 mM MgCl$_2$, 0.5% Triton X-100. Some sections were fixed for 10 min with 3.7% formaldehyde in PBS subsequent to treatment with 95% ethanol. Some sections were extracted with 0.6 M KI in PBS-Triton.

Indirect immunofluorescence was performed as described previously (5, 18, 21). Double immunofluorescence was performed by the indirect-direct method (26) as described (18). The two directly labeled antibodies were antivimentin conjugated to rhodamine B isothiocyanate and antidesmin conjugated to tetramethyl rhodamine isothiocyanate isomer R, prepared as previously described (21).

RESULTS

Paranemin and Synemin Compared by Two-Dimensional Peptide Mapping

The two-dimensional peptide maps of paranemin purified from embryonic intermediate filaments and of synemin from

adult visceral (gizzard) smooth muscle were compared in order to determine whether the two filament-associated proteins exhibit any structural homology. In addition, peptide maps on desmin and vimentin were prepared, to examine the possibility that the larger proteins were complexes of desmin and/or vimentin. The maps of peptides resulting from digestion by thermolysin, trypsin, and chymotrypsin were compared, but only the two-dimensional maps of radioiodated peptides cleaved by thermolysin are shown here (Fig. 1). The tryptic and chymotryptic maps were more complex and ex-
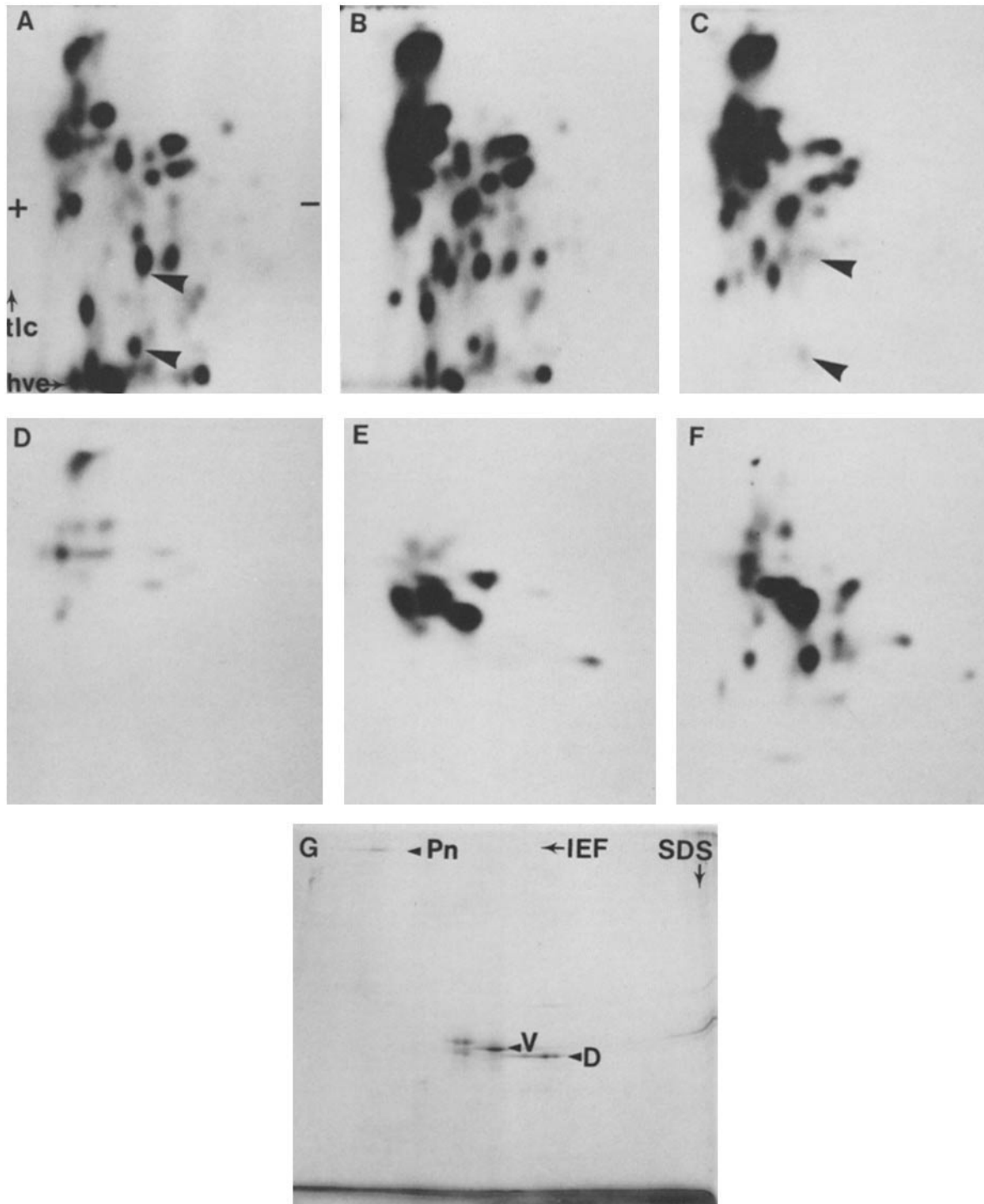

FIGURE 1 Autoradiograms of the two-dimensional maps of radioiodinated peptides from thermolysin digestion of (A) paranemin from intermediate filaments of embryonic skeletal muscle, (B) combination of paranemin and synemin, (C) synemin from adult gizzard muscle, and (D) blank acrylamide, for a control of nonspecific iodination, all exposed for 5 d. The axes of the electrophoretic (hve) and thin-layer chromatography (tlc) gradients are marked. There are two spots on the peptide maps of paranemin and synemin that co-migrate (arrows). The rest are unique, or are spots that co-migrate with those in the control preparation. Peptide maps of desmin (E) and vimentin (F) digested with thermolysin, which were exposed for 8 h, are shown for comparison with the maps of paranemin and synemin. The short exposure of these maps prevents development of the control spots. A two-dimensional polyacrylamide gel (G) of the preparation of intermediate filaments isolated from skeletal myogenic tissue demonstrates paranemin (Pn), desmin (D), and vimentin (V). These proteins, as well as synemin, were used for the peptide maps. (IEF, isoelectric focusing.)

hibited more hydrophobic peptides, in the case of paranemin, which failed to migrate in the second dimension. Nevertheless, all sets of maps yield the same conclusion, namely that paranemin and synemin are distinct molecules with very little peptide homology between themselves (Fig. 1, *A-C*) or with desmin and vimentin (Fig. 1, *E* and *F*).

The thermolysin digest of the iodinated blank gel contained a number of radiolabeled fragments that can be separated by the mapping procedure (Fig. 1*D*). There are eight spots and two complexes of spots on the paranemin and synemin digests that can be overlapped with the spots on the control map, so these spots are discounted from the comparison.

The paranemin thermolysin digest contains 10 major spots, six of which are relatively hydrophobic and thus remain at or near the origin of the chromatographic separation, and six minor ones. The thermolysin digest of synemin gives a different pattern of nine major peptides and nine minor ones, one of which is relatively hydrophobic. From analysis of a map of a mixture of paranemin and synemin digests (Fig. 1, *B*), it

was found that only two spots in the maps of paranemin and synemin digests migrate identically, which means that 2/16–2/18 of total thermolysin peptides of paranemin and synemin are similar.

Immunoautoradiographic Survey of the Presence of Paranemin, Vimentin, and Desmin in Embryonic Muscle

The presence of paranemin in embryonic myocardium, various vessels originating from the heart, and several organs containing large amounts of smooth muscle was ascertained by immunoautoradiography of proteins separated by SDS gel electrophoresis (Fig. 2*A*). The Coomassie Blue-stained gel (Fig. 2*A*) of the embryonic tissues reveals that paranemin is present in too low a quantity to be clearly identifiable. Additionally, paranemin has an electrophoretic mobility closely similar to that of filamin (19) (compare lanes *g*, *i*, and *if* in Fig. 2*A*) and its presence could be masked by the higher
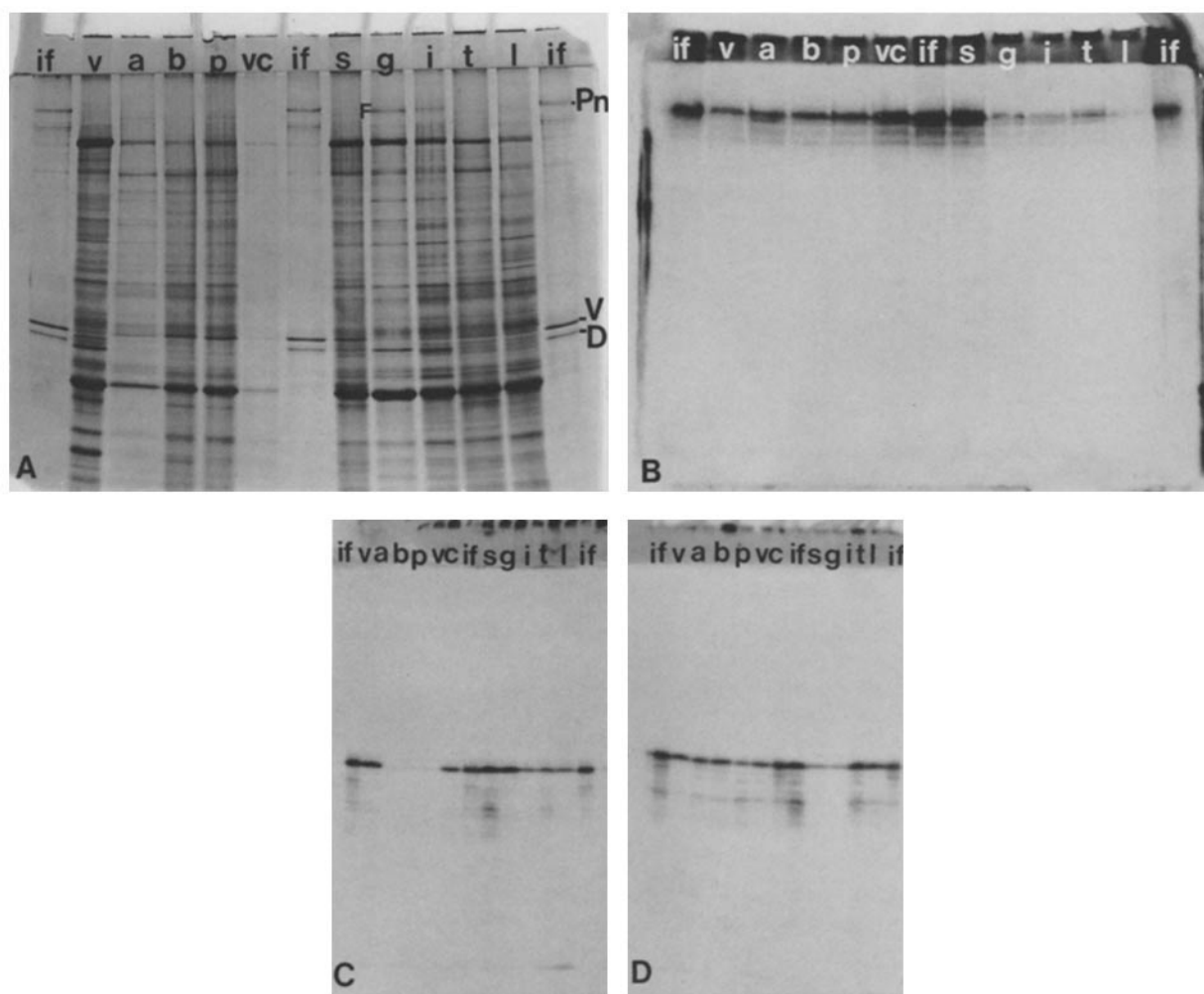

FIGURE 2   Immunoautoradiography of samples of embryonic muscular tissues. (A) Polyacrylamide gel treated with antiparanemin antibodies and radioiodinated protein A before staining with Coomassie Blue. Paranemin (*Pn*), vimentin (*V*), filamin (*F*), and desmin (*D*) are indicated. The control samples are proteins of intermediate filaments (*if*) from embryonic muscle; the other samples are (*v*) ventricle, (*a*) aorta, (*b*) brachiocephalic arteries, (*p*) pulmonary arteries, (*vc*) vena cava, (*s*) skeletal muscle, (*g*) gizzard, (*i*) intestine, (*t*) trachea, and (*l*) lung. *B* is the corresponding antiparanemin autoradiogram. Also shown are autoradiograms of gels stained with antidesmin (C) and antivimentin (D). Electrophoresis of the gel to be stained with antiparanemin was continued for 1 h after the dye-front reached the bottom. Note that paranemin is present in too low a quantity in the samples of Fig. 2*A* to be clearly identifiable by Coomassie Blue staining. The heaviest Coomassie Blue-stained band above myosin in lanes *g* and *i* is most likely filamin.

abundance of filamin. However, immunoautoradiography with antibodies to paranemin (Fig. 2B) clearly demonstrates the presence of paranemin in each muscular tissue associated with the 15-d embryonic heart, including cardiac ventricle (v), aorta (a), the brachiocephalic arteries (b) that branch from the aorta, pulmonary arteries (p), and vena cavae (vc). The paranemin antibodies react with a polypeptide that co-migrates with the immunogen, as can be seen in the lane containing proteins from embryonic skeletal (pectoralis) muscle (s) and the three marker lanes of intermediate filaments isolated from skeletal myogenic tissue. Paranemin is also discerned (Fig. 2B) in the samples of viscera that contain smooth muscle, such as gizzard (g), small intestine (i), and trachea (t). There is a small amount of paranemin in the sample of embryonic lung (l).

In each of the embryonic tissue samples and in the control samples of intermediate filaments, the antibodies bound to a major band of 280,000 mol wt and to a triplet of proteins with slightly lower molecular weights. The triplet proteins are probably products of proteolysis of paranemin, since the number of proteins of lower molecular weight with which antiparanemin reacted was greatly increased in samples that were frozen and thawed several times.

For comparison of the content of the intermediate filament subunits, desmin and vimentin, with the presence of parane-

min, corresponding gels were treated with antidesmin (Fig. 2C) and antivimentin (Fig. 2D) antibodies. Paranemin is present in tissues that have a wide range of desmin/vimentin ratios, as determined qualitatively from the immunoautoradiograms. There is paranemin in the large blood vessels (a, b, and p) that emerge from the embryonic heart, which have a low desmin/vimentin ratio, as well as in the gizzard, which has the highest desmin/vimentin ratio of the embryonic tissues examined.

Comparative Cellular Distribution of Paranemin and Intermediate Filament Proteins in Myogenic Tissues

Since each tissue is comprised of a number of different types of cells and is infiltrated by nerves and blood vessels, only immunohistochemistry can reveal the cellular localization of the antigens in question. Frozen sections of different types of myogenic tissues taken from embryos 12–18 d in ovo were stained indirectly with antiparanemin antibodies and directly with either rhodamine antidesmin or rhodamine antivimentin antibodies so that the cellular distribution of paranemin could be compared with that of each muscle intermediate-filament core protein. Each myogenic tissue was also treated with antisynemin antibodies. Gizzard smooth muscle
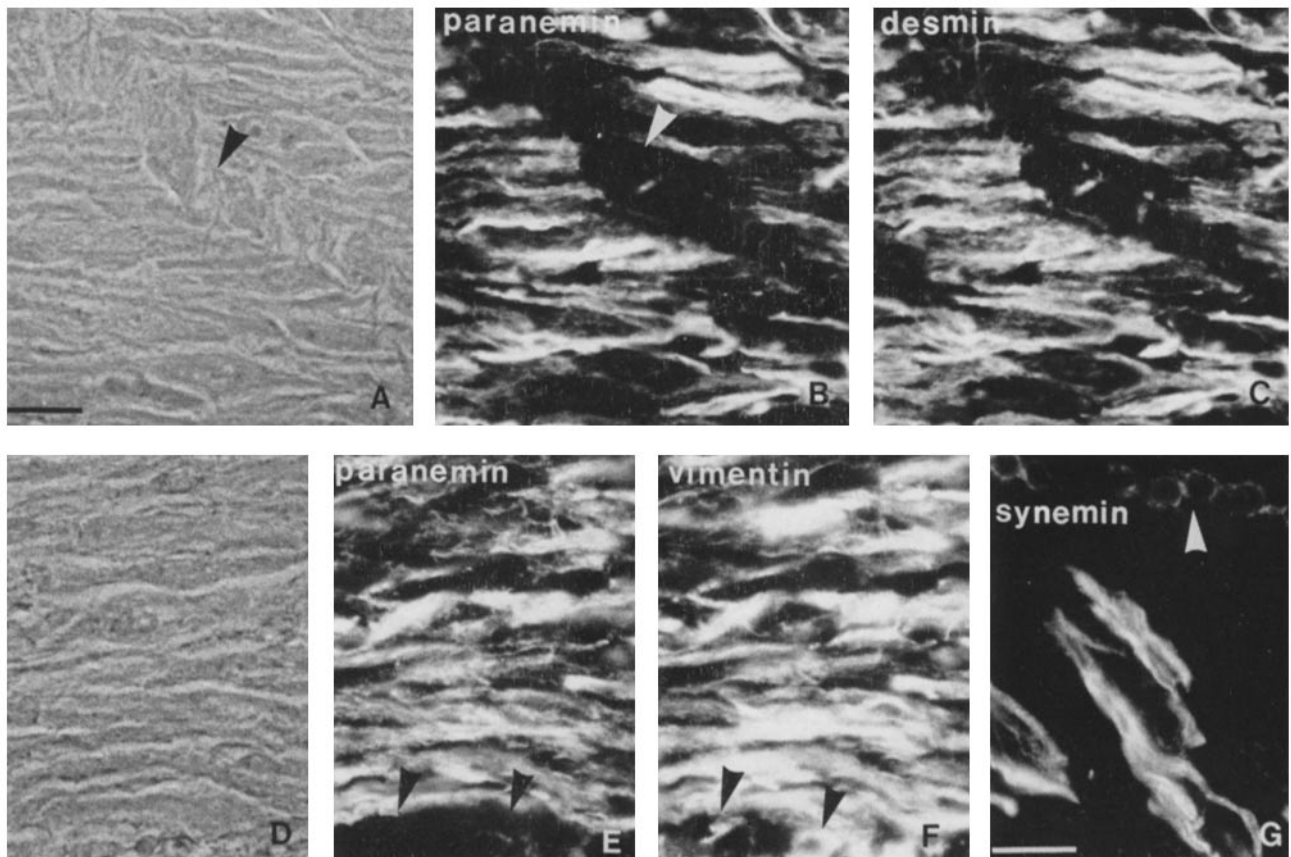

FIGURE 3 Immunofluorescence of embryonic gizzard, demonstrating the presence of paranemin, desmin, vimentin, and synemin in the smooth muscle cells. A phase-countrast micrograph (A) of a frozen section of gizzard, and the corresponding double immunofluorescent micrographs of antiparanemin (B) and antidesmin (C) staining demonstrate the coincidental distribution of paranemin and desmin in smooth myogenic cells. Fibroblasts in the connective tissue layer (arrow, A and B) are not stained by either antibody. A phase-contrast micrograph (D) and the corresponding double immunofluorescent images of antiparanemin (E) and antivimentin (F) staining show that paranemin and vimentin are both present in smooth muscle cells. Other cells (arrows) exhibit bright antivimentin reactivity and little or no antiparanemin staining. Antisynemin (G) binds to smooth myogenic cells in a fibrillar pattern, and to erythrocytes (arrow), but not to connective tissue cells. Bar, 10 μm. × 1,020.

was chosen for representation of the cellular localization of the intermediate-filament proteins in visceral smooth muscle, although similar staining patterns of the developing smooth muscle were obtained with embryonic small intestine.

The immunofluorescent images of antiparanemin and antidesmin staining of any myogenic tissue are almost identical, as demonstrated in double immunofluorescent pairs of developing gizzard (Fig. 3, A-C), skeletal myogenic tissue (Fig. 4, A-C), and developing heart muscle (Fig. 5, A-B), each taken from a 14-d embryo. Similarly, double immunofluorescence with antibodies to paranemin and vimentin reveals the co-distribution of these proteins in each of the myogenic cell types (Figs. 3, 4, and 5, D-F). Antibodies to synemin bind to networks of filaments within the smooth muscle cells of embryonic gizzard (Fig. 3 G). The distribution of synemin in developing skeletal muscle has been demonstrated (22); synemin is localized in cytoplasmic filaments in immature myotubes and at the assembling Z discs in more mature myotubes. The same pattern of intercellular distribution is evident in cardiomyogenic cells (Fig. 5 H), although there appears to be much less synemin in the developing heart cells, compared with skeletal or smooth myogenic cells. The developing muscle cells of visceral, skeletal, and cardiac tissue therefore co-express the two intermediate-filament proteins, desmin and vimentin, and both associated proteins, paranemin and synemin. These proteins are localized in a diffuse fibrillar pattern in the cytoplasm of the myogenic cells, as well as at the levels of the Z discs in skeletal and cardiac myogenic

cells. The Z-disc staining is difficult to demonstrate in sections of embryonic skeletal muscle tissue, although it is clear in the micrographs of cardiomyogenic tissue (Fig. 5).

Each of the myogenic tissues includes fibroblastic cells which express vimentin (2, 6, 14) but lack paranemin and synemin. Embryonic chondrocytes likewise contain vimentin without either filament-associated protein (data not shown). Erythrocytes contain vimentin filaments with associated synemin (Fig. 3 G) as shown previously (24), but no paranemin (see below, Fig. 10 D). In addition to these cells, there is a small population of unidentified cells that express both vimentin and paranemin (5). Two cell types that are easily identified by their location in peripheral nerves and blood vessels, respectively, are Schwann cells and endothelial cells. Vimentin and paranemin are present in these cell types throughout embryogenesis and in the adult (see also reference 1, 12, and 15 for vimentin), as is demonstrated in sections of adult tissue (see below).

Immunoautoradiographic Survey of Paranemin, Desmin, and Vimentin in Adult Muscular Tissues

The major difference between the steady-state amounts of paranemin in embryonic and adult muscular tissues is the reduced reactivity of antiparanemin antibodies with proteins from adult skeletal (s) muscle and adult viscera, including gizzard (g), small intestine (i), trachea (t), and lung (l) (Fig.
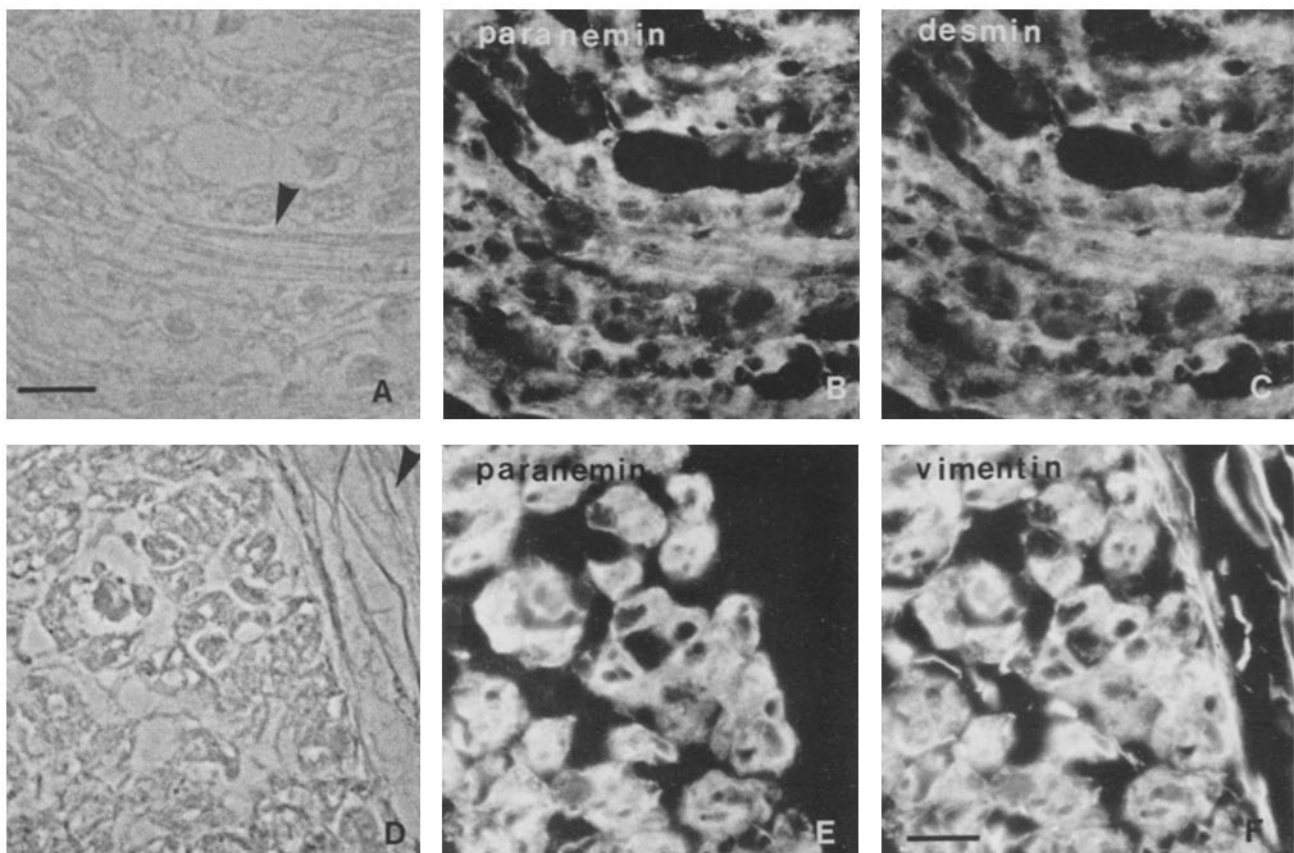

FIGURE 4 Double immunofluorescence of frozen sections of embryonic skeletal muscle tissue. Phase-contrast micrograph (A) of longitudinal section, with striated myofibrils (arrow), and corresponding antiparanemin (B) and antidesmin (C) micrographs of a cross-section including a connective tissue layer of perimysium. A phase-contrast micrograph (D) and the matching antiparanemin (E) and antivimentin (F) micrographs are shown. The distribution of antiparanemin is identical to that of antidesmin and antivimentin except that fibroblasts in the connective tissue (arrow) react only with antivimentin antibodies. Bar, 10 μm. × 1,020.
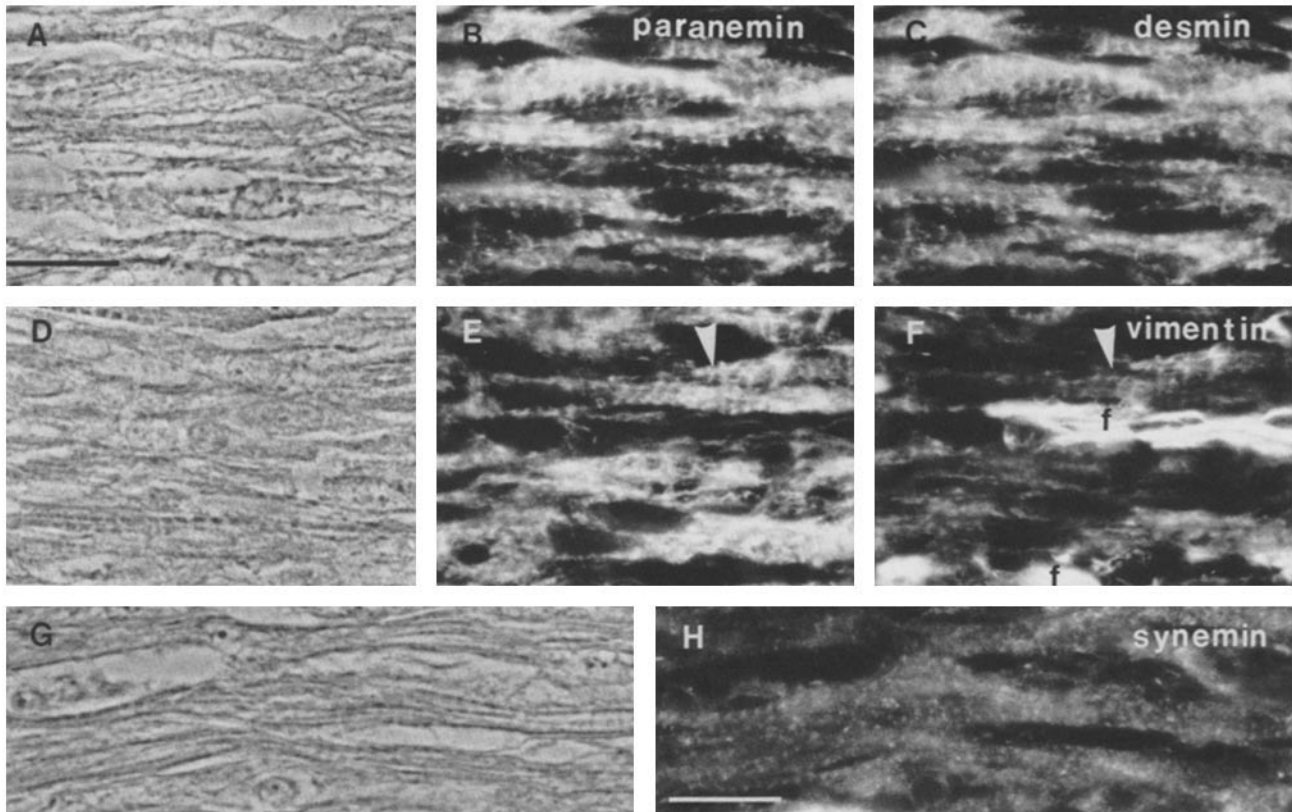
FIGURE 5 Immunofluorescence of embryonic cardiac tissue. Phase-contrast micrograph (A) of a frozen section through the longitudinal axis of 14-d ventricle and the corresponding pair of antiparanemin (B) and antidesmin (C) fluorescence images, which are nearly identical. Phase-contrast micrograph (D) and corresponding antiparanemin (E) and antivimentin (F) fluorescence micrographs, demonstrating less vimentin than paranemin in the cardiomyogenic cells (arrows). Fibroblasts (f) are stained only with antivimentin. All four antisera stain at the area of Z discs within the developing heart cells. Bar, 10 μm. × 1,020.

6, *A* and *B*). Since the vast majority of the mass of adult gizzard tissue is composed of smooth muscle cells, it would appear that the mature smoooth muscle cells are devoid of paranemin. If the autoradiogram in Fig. 6*B* is over-exposed, then a small amount of reactivity can be seen in the lanes containing proteins of adult skeletal muscle and organs. This limited reactivity of antiparanemin antibodies can be accounted for by selective staining of certain nonmuscle cells in the tissues, as will be demonstrated later.

The similarity between the antiparanemin immunoautoradiograms of embryonic and adult muscular tissues (Fig. 6*B*) is the strong reactivity of the antibodies with the samples of the ventricles and with those of the vessels that emanate from the heart, such as the aorta (*a*), brachiocephalic arteries (*b*), pulmonary arteries (*p*), and vena cavae (*vc*). In both antiparanemin immunoautoradiograms the antibodies bind predominantly to a 280,000-mol-wt protein and to three proteins of slightly lower molecular weight (Figs. 2*B* and 6*B*) that are presumed to be products of proteolysis of paranemin (see above). However, as noted above, paranemin is present in too low a quantity to be detectable by Coomassie Blue staining and the band that co-migrates with paranemin in Fig. 6*A*, lanes *a*, *b*, and *p*, is most likely filamin. Taken together, the two antiparanemin immunoautoradiograms indicate that paranemin is present in all the embryonic muscular tissues, and that it remains in the adult cardiac and great vascular tissues but is eliminated from adult skeletal muscle and visceral smooth muscle.

Since smooth muscle is the main cellular component of the adult aorta and the brachiocephalic and pulmonary arteries, the large amount of paranemin detected in these tissues is most likely a component of vascular smooth muscle, as will be shown below by immunofluorescence. This indicates that there is heterogeneity in the expression of paranemin, not only between the two types of striated muscle, skeletal and cardiac, but also between the two types of smooth muscle, visceral and vascular.

However, within different types of adult skeletal muscle, there is homogeneity in the lack of expression of paranemin. In a previous study (5), it was shown by immunoautoradiography and immunofluorescence that paranemin is eliminated from fast skeletal muscle (thigh) during the posthatching stages of development. In the present study, it was found that paranemin is also absent from slow skeletal muscle (anterior latissimus dorsi) of the adult (data not shown).

The presence of desmin and vimentin in each of the adult muscular tissues is confirmed by the antidesmin (Fig. 6*C*) and antivimentin (Fig. 6*D*) immunoautoradiograms. The presence of paranemin cannot be predicted on the basis of the desmin/vimentin ratio. For example, both cardiac ventricular tissue and gizzard have high desmin/vimentin ratios, but paranemin is detected in large amounts only in the ventricle. Desmin is also clearly present in skeletal muscle (lane *s*) as expected (20, 21) when these autoradiograms are exposed for longer periods or a higher quantity of protein is electrophoresed.
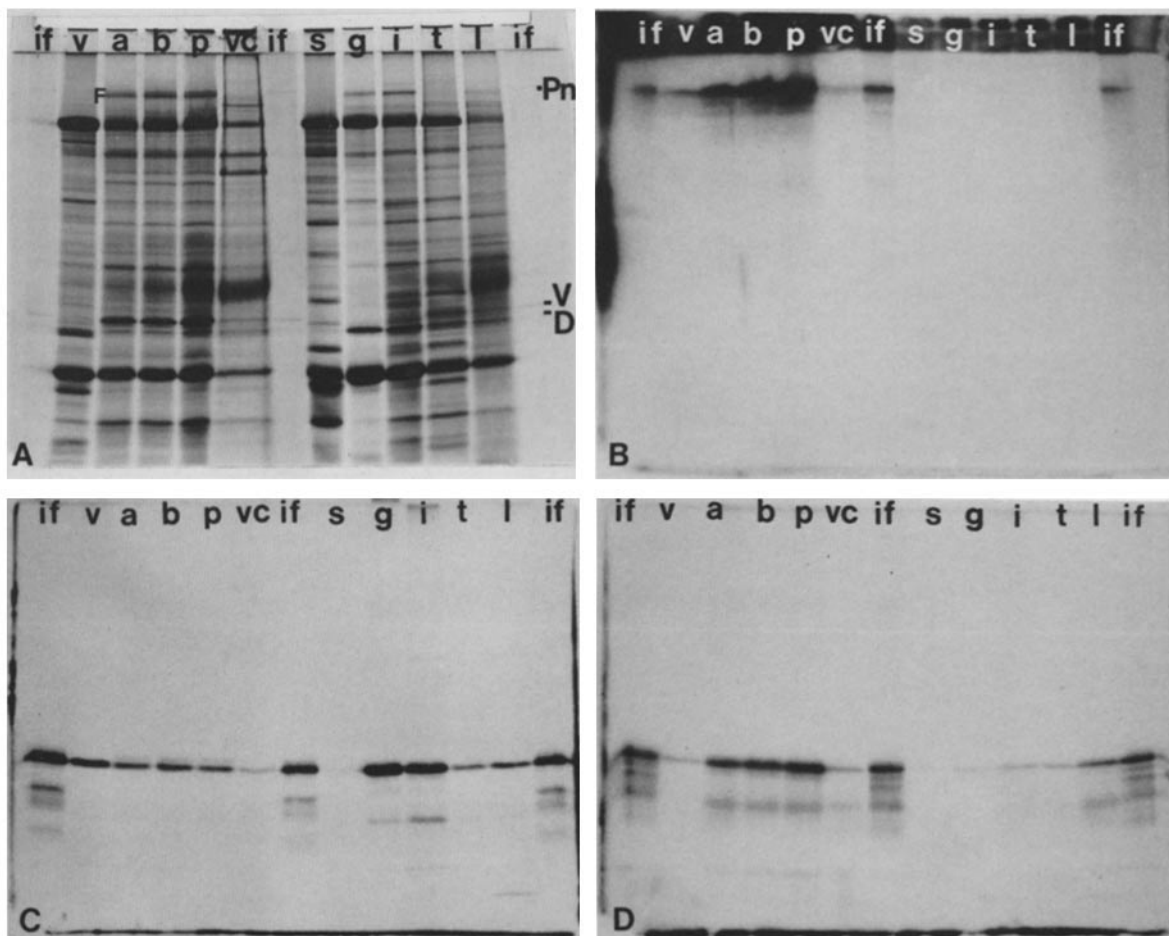
FIGURE 6   Immunoautoradiography of adult muscular tissues. (A) Polyacrylamide gel of samples of intermediate filaments (if) from embryonic muscle, used for a control, and adult ventricle (v), aorta (a), brachiocephalic arteries (b), pulmonary arteries (p), vena cava (vc), skeletal muscle (s), gizzard (g), intestine (i), trachea (t), and lung (l). The gel was treated with antiparanemin antibodies and radio-iodinated protein A before being stained with Coomassie Blue. (B) Corresponding autoradiogram showing the distribution of antibodies to paranemin. Paranemin is detected in the samples of heart, the large vessels associated with it, and in the control filament samples. Antiparanemin binds to a major band and to three minor bands of lower molecular weight. Antidesmin (C) and antivimentin (D) autoradiograms are displayed for comparison of the presence of these two filament proteins and paranemin in adult tissues. As noted in Fig. 2, paranemin exhibits an electrophoretic mobility closely similar to that of filamin but is present in too low a quantity to be detectable by Coomassie Blue staining. The band above myosin in A, lanes a, b, and p, is most likely filamin (F).

Immunofluorescence of Adult Organs Containing Smooth Muscle

Immunofluoresence, using antibodies to desmin, vimentin, and paranemin, established the cellular distribution of these antigens in adult viscera. Micrographs of the gizzard sections presented here (Fig. 7) are representative of the staining pattern of any visceral smooth muscle. Neither antiparanemin (Fig. 7, B and D) nor antivimentin (Fig. 7 F) antibodies stain the smooth muscle, which is the main cellular component of the organ, while antidesmin (Fig. 7 H) and antisynemin (not shown) antibodies react with a fine filamentous network within these cells.

In the nonsmooth muscle cells of the gizzard, the staining patterns of antiparanemin and antivimentin antibodies are generally similar. Both antibody preparations intensely stain the endothelial cells of the capillaries within the gizzard (Fig. 7, A-F; 15). Vimentin (24), but not paranemin, is also detected in erythrocytes and in the fibroblasts (Fig. 7 F).The epithelial cells of the small intestine, which contain keratin intermediate filaments (14, 39), lack both paranemin (data not shown) and vimentin (14, 39).

Distribution of Paranemin in Adult Vasculature

Paranemin is differentially distributed in the elastic and muscular vessels of adult vasculature. The distribution of paranemin in the endothelium and smooth muscle is reversed for the two classes. In the relatively noncontractile elastic vessels that emanate from the heart and constitute the primary through the tertiary branches from the aorta, there is paranemin in the smooth muscle cells. This is shown by immunoautoradiography of the aorta, brachiocephalic arteries (primary branches), and pulmonary arteries (Fig. 6 B), and by immunofluorescence of the aorta (Fig. 8, B and D). The intermediate filaments of the smooth muscle cells in elastic vessels contain desmin (Fig. 8 C) and relatively less vimentin (Fig. 8 E). In addition to paranemin, synemin (Fig. 8 F) is also present in aortic smooth muscle. Paranemin is absent from the smooth muscle of adult muscular vessels (Fig. 10 J, for example), including arteries and veins in all peripheral regions and in viscera both above and below the heart.

The endothelial cells that line the elastic vessels contain vimentin (Fig. 8 E) but lack the two high molecular weight proteins. In contrast, the vimentin filaments in the capillary
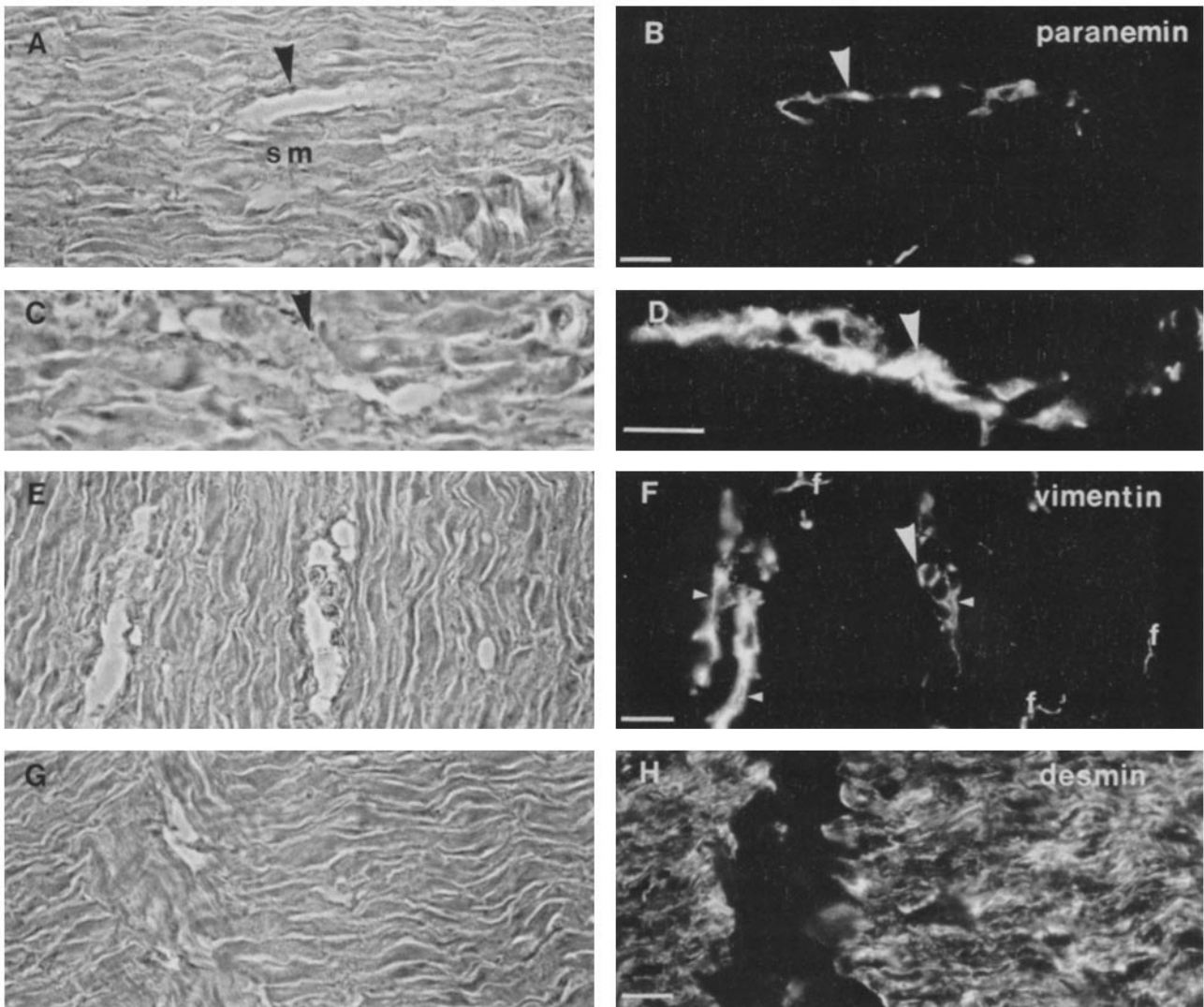
FIGURE 7   Indirect immunofluorescence of adult gizzard. As can be seen in phase-contrast micrographs (*A, C, E,* and *G*) of frozen sections of adult gizzard, the main cell type is smooth muscle (*sm*). Antiparanemin (*B* and *D*) reacts with a filamentous meshwork in capillary endothelial cells (arrows). Antibodies to vimentin (*F*) also bind to endothelial cells (small arrows) as well as to erythrocytes (large arrow) and to fibroblasts (*f*). Only antidesmin (*H*) antibodies react with visceral smooth muscle cells. Bar, 10 μm. *A, B,* and *E–H,* × 715; *C–D,* × 1,130.

endothelium within the adult gizzard (Fig. 7, *B* and *D*) appear to have paranemin associated with them, as do the vimentin filaments of the endothelium in the relatively contractile muscular vessels.

Distribution of Paranemin in Adult Skeletal Muscle

Paranemin is eliminated from both slow and fast skeletal muscle several weeks after hatching of the chicken, as indicated by the loss of reactivity of antiparanemin antibodies detected by immunoautoradiography as well as by immunofluorescence (Figs. 9, *A* and *B*). The lack of antibody staining of adult skeletal muscle appears to be due to absence of paranemin rather than masking by other proteins, since paranemin is not detected in sections of adult muscle from which the majority of proteins have been extracted with high salt (0.6 M KI) (5). Throughout the late embryonic stage and into adulthood, desmin and vimentin are detected at the myofibrillar Z discs in frozen sections of muscle stained with the

corresponding antibodies (Figs. 9, *C–F*). However, paranemin is localized in several nonmuscle cells within the muscular tissue, including the endothelial cells of capillaries within skeletal as well as smooth muscle (see above). Another nonmuscle cell that co-expresses vimentin (1, 8, 48) and paranemin is the Schwann cell, as can be seen in large peripheral nerves cut in longitudinal section (Figs. 9, *G–I*). Cross-sections of peripheral nerves treated with antiparanemin and antivimentin antibodies demonstrate a pattern of staining that correlates with the cytoplasm of the Schwann cells (Figs. 9, *K* and *L*) and that surrounds the circular profiles of neurons revealed with antibodies to the 170,000-mol-wt neurofilament protein (Fig. 9*M*). Paranemin and vimentin appear to be absent from neuronal axons (for the absence of vimentin from neurons, see also 8, 36, 39, 46). The fibroblasts within the perineurium contain vimentin filaments (Figs. 9, *H* and *N*) without associated paranemin. In nervous tissue, desmin is localized only in the smooth muscle of blood vessels (Fig. 9*J*); the same is true for synemin (data not shown). The peripheral nerves that innervate the embryonic skeletal muscle exhibit identical immunofluorescence staining patterns.
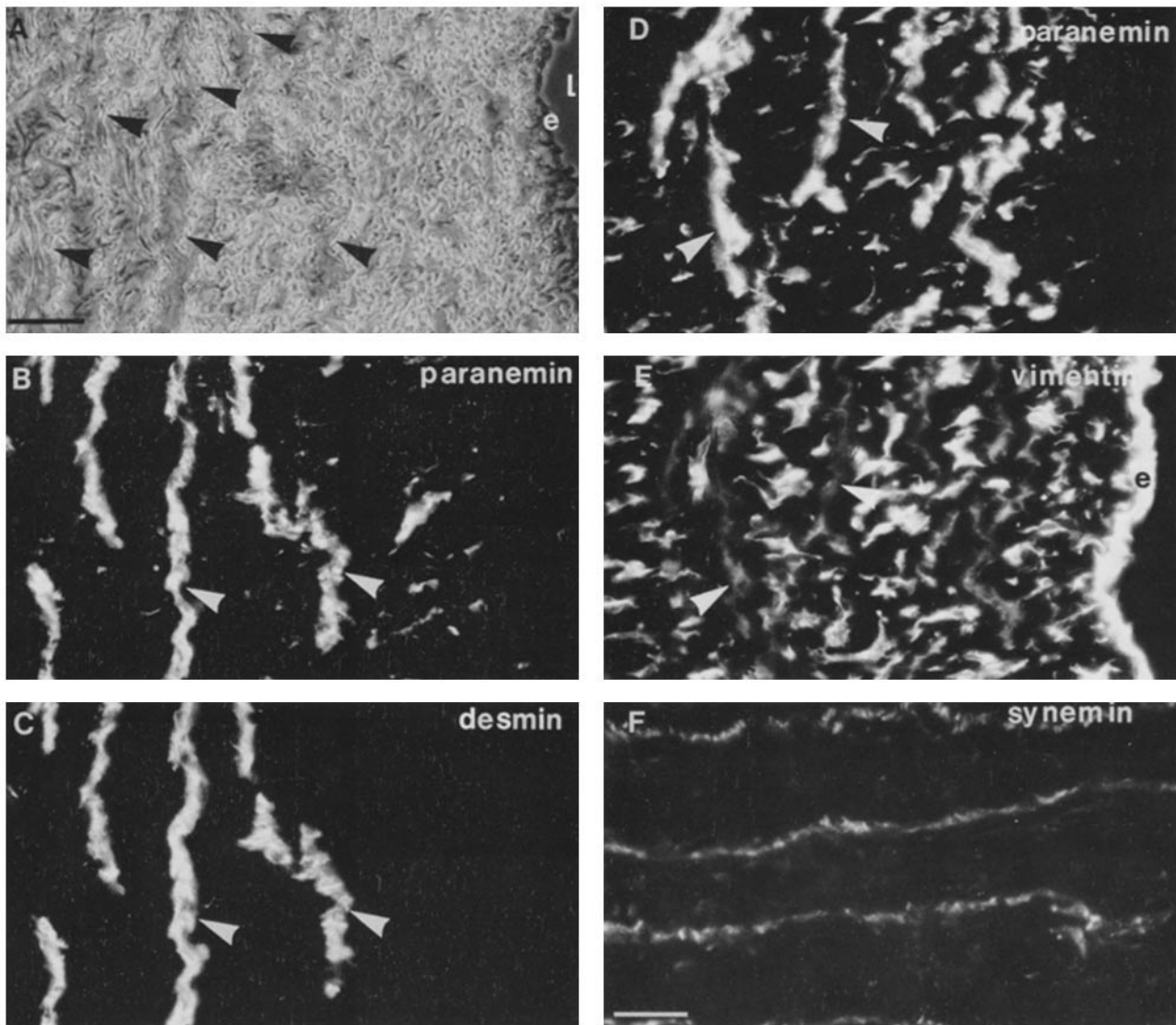
FIGURE 8   Immunofluorescence of adult chicken aorta (A). Phase-contrast micrograph of frozen cross-section of aorta, taken proximal to the point where the brachiocephalic arteries branch. Ribbons of smooth muscle (arrows) are separated by layers of fibroblasts and highly refractile bundles of elastin. The endothelium (e) and aortic lumen (l) are indicated. Double immunofluorescence with antiparanemin (B) and antidesmin (C) showing the coincidental staining of smooth muscle cells (arrows) (the antidesmin staining of the right-most cell is barely discernible). Antibodies to paranemin (B and D) also label some fibroblasts. Double immunofluorescence with antiparanemin (D) and antivimentin (E) demonstrates that smooth muscle cells (arrows) also contain vimentin. Endothelial cells (e) react with antivimentin but not antiparanemin. Antisynemin (F) weakly reacts with the ribbons of smooth muscle cells. Bar, 20 μm. × 524.

Localization of Paranemin in Adult Myocardium

The results of staining adult myocardium with antibodies to paranemin are in striking contrast to those obtained with adult skeletal muscle. While paranemin is not detected in adult skeletal muscle, it remains at the myofibrillar Z discs of mature myocardial cells (Fig. 10, A, B and D), in both the atria and the ventricles. The myocardial cells also contain desmin (Fig. 10 C), which is localized at the myocardial Z discs, but not vimentin (Fig. 10 E). The staining pattern revealed with antibodies to synemin (not shown) is almost identical to the antivimentin staining pattern except that fibroblasts contain only vimentin; it appears that both vimentin and synemin are largely eliminated from the myocardial cells during their maturation in the adult. Desmin and paranemin resist extraction by high salt (0.6 M KI), which removes

much of the contractile proteins, indicating that paranemin is tightly bound to the Z discs. Paranemin and desmin appear to be localized at the periphery of the myocardial Z discs, as has been clearly demonstrated to be the case for the intermediate filament proteins of skeletal muscle (5, 20–22).

The impulse-conducting fibers of the heart exhibit very bright, diffuse immunofluorescence with antiparanemin (Fig. 10, G–K). The presence of intermediate filaments in these cells has been demonstrated by electron microscopy (10, 34) and antidesmin immunofluorescence (11). In this study, both antidesmin (Fig. 10 H) and antivimentin (Fig. 10 K) antibodies, as well as antisynemin antibodies (not shown), bound to a dense meshwork of cytoplasmic filaments within the conducting fibers. The paranemin and vimentin of these cells contribute to the amounts of those proteins detected by immunoautoradiography of ventricle samples (Fig. 6, B and D).
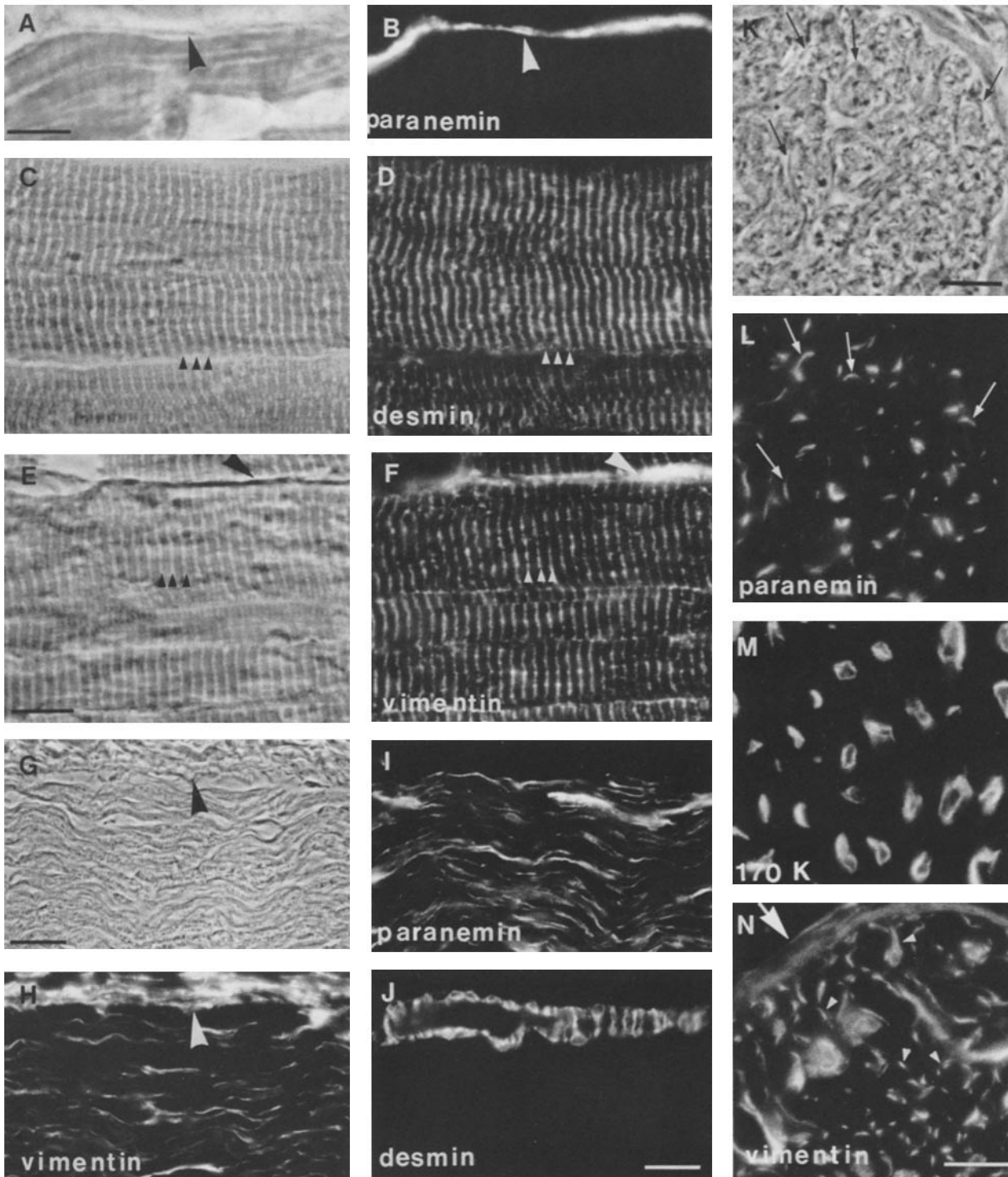
FIGURE 9 Immunofluorescence of frozen sections of adult skeletal muscle and peripheral nerves. (A) Phase-contrast and corresponding fluorescence (B) images of tissue stained with antiparanemin antibodies, demonstrating paranemin within the peripheral nerve (arrow) but not within skeletal muscle tissue. Phase-contrast (C and E) and corresponding fluorescence micrographs of skeletal muscle with antidesmin (D) and antivimentin (F) staining at the myofibrillar Z discs (arrowheads). The Z discs overlap the I bands, which appear light in the phase-contrast images (arrowheads) of the partially contracted muscle. Note the antivimentin staining of fibroblasts (large arrow). Bar, 10 μm. × 1020. (G) Phase-contrast and antivimentin fluorescence (H) micrograph of a peripheral nerve, showing vimentin in the fibroblasts of the perineurium (arrows) and in the Schwann cells surrounding neruons. Antibodies to paranemin (I) give similar staining of Schwann cells alone. Antibodies to desmin (J) stain only the smooth muscle cells of vessels within the nerve. Bar, 20 μm. × 445. Phase-contrast (K) and corresponding fluorescence (L) micrograph of a cross-section of a peripheral nerve, with the perineurium, stained with antiparanemin antibodies to demonstrate the interrupted circular staining of the Schwann cell cytoplasm (arrows). For control, (M) a fluorescence micrograph of a nerve in which the neurofilaments were stained with antibodies to the 170,000-mol-wt neurofilament protein (170 K). Note that the profiles of the neurons would fit within the profiles of Schwann cells stained with antiparanemin (K–L), Bar, 10 μm. × 1015. Antibodies to vimentin (N) stain the Schwann cell cytoplasm (arrowheads) as well as perineuronal fibroblasts (arrow). Bar, 10 μm. × 635.
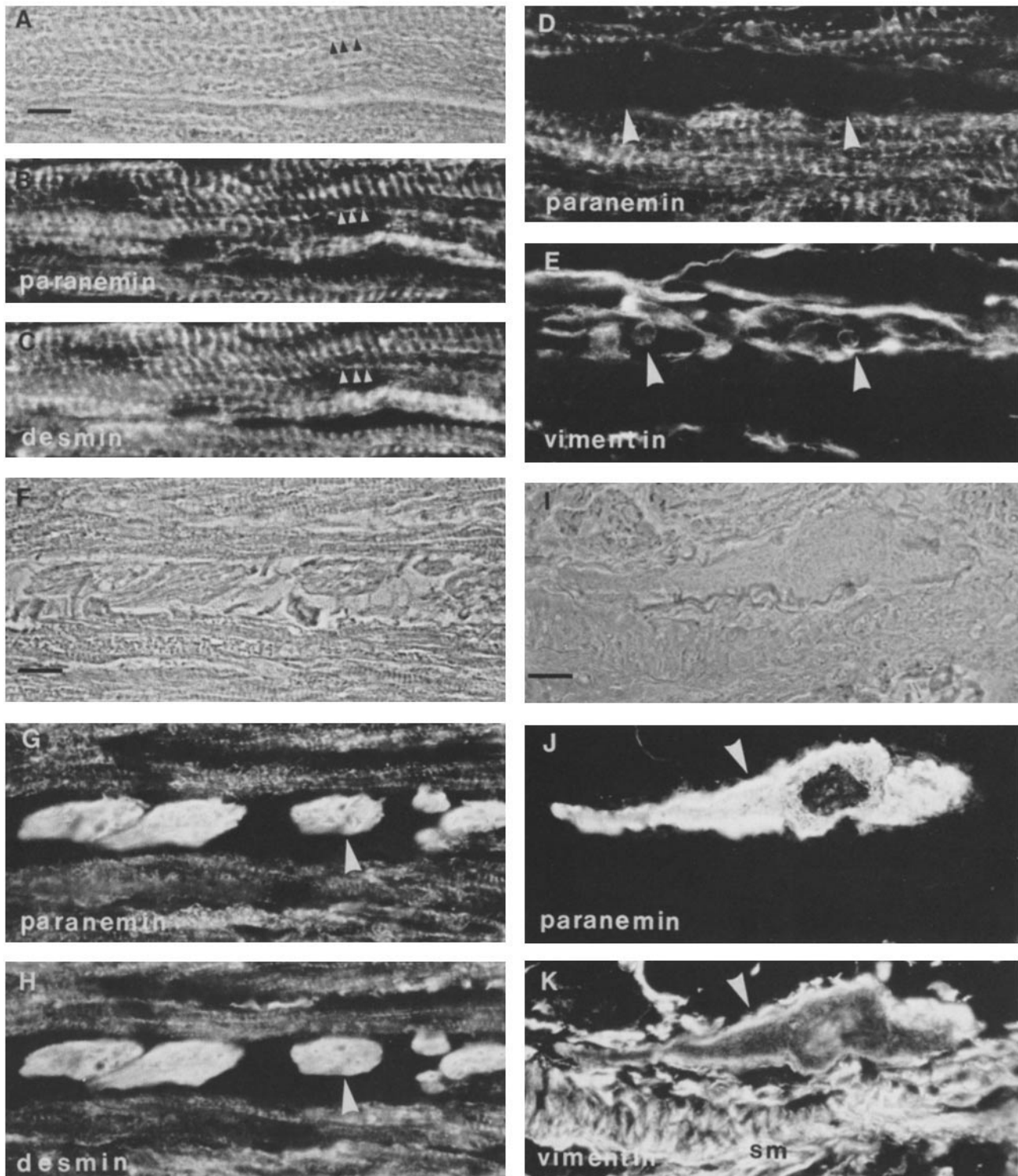
FIGURE 10 Immunofluorescence of adult myocardium. (A) Phase-contrast and corresponding double immunofluorescence micrographs, showing the co-distribution of antiparanemin (B) and antidesmin (C) antibodies along the myocardial Z discs (arrowheads). Double immunofluorescence micrographs of myocardium stained with antiparanemin (D) and antivimentin (E) antibodies, demonstrating the absence of vimentin in mature myocardial cells. Fibroblasts and erythrocytes, unstained by antiparanemin, are stained by antivimentin (arrows, D) antibodies. Bar, 1 μm. × 1020. Phase-contrast (F) and corresponding double immunfluorescence micrographs of myocardium in a longitudinal section, showing the co-localization of paranemin (G) and desmin (H) along intermediate filaments of conducting fibers (arrows). Phase-contrast (I) and corresponding double immunofluorescence micrographs of a large conducting fiber (arrow, J and K) near a blood vessel in this cross-section of ventricular myocardium, demonstrating the fibrillar staining pattern of antiparanemin (J) and antivimentin (K) antibodies in the conducting cells. Paranemin is absent in the vascular smooth muscle cells (sm) that contain vimentin. Bar, 10 μm. × 715.

DISCUSSION

Paranemin and Synemin Are Distinct Polypeptides

Previous studies from this laboratory have identified two high molecular weight polypeptides in association with vimentin and desmin filaments in chicken muscle cells: paranemin (280,000 mol wt) (5) and synemin (230,000 mol wt) (22). These two polypeptides are antigenically distinct, and in addition to their difference in molecular weight they have distinct isoelectric points as judged by their migration in the presence of urea, paranemin having an apparent pI of 4.3 (5) and synemin a pI of 5.43 (24). As shown here, paranemin and synemin also have distinct two-dimensional peptide maps, thus firmly establishing that they are distinct gene products. Furthermore, the peptide maps demonstrate that the two high molecular weight proteins are distinct from both desmin and vimentin.

Heterogeneous Developmental Regulation of the Expression of Paranemin

In the initial study on the identification and expression of paranemin in embryonic and adult skeletal muscle, it was found that expression of paranemin in fast skeletal muscle is developmentally regulated (5). In myogenic cells differentiating in tissue culture, paranemin exhibits a pattern of expression similar to that of vimentin (3, 18); it is expressed in myoblasts and continues to be expressed after the onset of fusion. This pattern of expression is different from those of synemin (22) and desmin (2, 18) whose syntheses are initiated after myoblast commitment and upon the onset of fusion. In skeletal myotubes, all four proteins exhibit indistinguishable cytoplasmic distributions, first in association with cytoplasmic filaments and later in association with Z discs. However, while the expression of desmin, vimentin, and synemin persists into adulthood, that of paranemin is apparently turned off so that adult fast skeletal muscle lacks paranemin as judged both by immunoautoradiography and immunofluorescence (5).

The results of the present study indicate that the expression of paranemin is developmentally regulated in a heterogeneous manner in different muscle cells. We have demonstrated that paranemin and synemin are co-expressed with desmin and vimentin in all types of embryonic muscle cells, whether smooth, skeletal, or cardiac. The subsequent fate of paranemin in a particular muscle cell depends on the morphological type of the cell. The results are summarized in Table I. They imply that in some mature muscle types the composition of intermediate filaments is similar to that in myogenic cells (e.g., impulse-conducting muscle cells), while in others it changes as evidenced by the cessation of the expression of paranemin (e.g., adult fast and slow skeletal muscle, visceral smooth muscle, and the vascular smooth muscle of muscular vessels) or synemin and vimentin (e.g., adult myocardium). Thus, the intermediate-filament proteins of adult striated cardiac muscle have paranemin associated with them, but lack synemin, while the reverse is true of adult striated skeletal muscle. Visceral smooth muscle and smooth muscle in muscular vessels lack paranemin, yet vascular smooth muscle in elastic vessels contains it; both types of vascular smooth muscle contain synemin. It is also evident from these observations that the expression of paranemin and synemin in

TABLE I
Composition of Intermediate Filaments in Various Cell Types

	Paranemin	Desmin	Vimentin	Synemin
All myogenic cells	+	+	+	+
Adult conducting fibers	+	+	+	+
Adult skeletal muscle	−	+	+	+
Adult myocardium	+	+	−	−
Adult visceral smooth muscle	−	+	−	+
Adult vascular smooth muscle				
Elastic vessels	+	+	+	+
Muscular vessels	−	+	+	+
Endothelium				
Elastic vessels	−	−	+	−
Muscular vessels and capillaries	+	−	+	−
Schwann cells	+	−	+	−
Fibroblasts (95–99%)	−	−	+	−
Erythrocytes	−	−	+	+
Neurons (mature)	−	−	−	−

adult muscle cells is not mutually exclusive in all classes of muscle since synemin and paranemin are co-expressed in the vascular smooth muscle of elastic vessels and in the cardiac impulse-conducting cells.

Pattern of Expression of Paranemin in Nonmuscle Cells

The common feature of the cell types that express paranemin or synemin is that they contain either of the core intermediate-filament proteins, vimentin or desmin, or both, as judged by immunoautoradiography and immunofluorescence. We have found that paranemin and synemin are absent from embryonic and adult neurons and epithelial cells (Table 1), which have been shown to express unique cell type-specific intermediate-filament proteins other than desmin or vimentin (8, 41–43, 48). Thus, expression of desmin or vimentin is obligatory for expression of paranemin, but not sufficient, as is clear from the examples of adult muscle (visceral smooth muscle and skeletal muscle) in which paranemin is eliminated. This conclusion is reinforced by the observation that chicken erythrocytes, certain endothelial cells, and most connective tissue fibroblasts contain vimentin filaments but lack paranemin. As is the case with adult muscle cells, expression of paranemin in some classes of nonmuscle cells is also heterogeneous since the endothelial cells of muscular vessels contain paranemin but those of elastic vessels do not.

Correlation of the Expression of Paranemin and Synemin with That of Desmin and Vimentin

It has been previously demonstrated (1, 32) that in adult chickens the subunit composition of intermediate filaments varies in different types of muscle. Visceral smooth muscle (e.g., gizzard, intestine) contain predominantly, if not exclusively, desmin, while skeletal muscle contains both desmin and vimentin in various ratios depending on the muscle. As we have shown here, the impulse-conducting fibers of the chicken heart co-express desmin and vimentin, while myocardial cells (atrial and ventricular) express predominantly, if not exclusively, desmin. A number of vascular smooth muscle

cells, both in mammals and in chickens, also co-express desmin and vimentin in various ratios depending on the proximity to the heart (4, 13, 17, 35, 38, 47). However, there is no clear correlation of the expression of paranemin, or of synemin, with the expression of either core filament proteins, as can be seen in Table 1. Thus it is evident that expression of both paranemin and synemin is regulated independent from that of desmin or vimentin in muscle cells. Furthermore, paranemin can associate with muscle intermediate filaments containing either desmin, or vimentin, or both. The same conclusion was reached for synemin from its expression in chicken erythrocytes, gizzard, and skeletal muscel (22, 23).

Functional Implications of the Heterogeneous Expresssion of Paranemin and Synemin in Muscle and Nonmuscle Cells

Studies from this laboratory on the immunoelectron microscope localization of synemin have suggested that one of the functions of synemin is to cross-link vimentin filaments at periodic intervals (23). This function of synemin appears to be analogous to that of the 200,000-mol-wt neurofilament-associated polypeptide (49). By analogy to its function in erythrocytes, synemin may also function to cross-link desmin- or vimentin-containing intermediate filaments in adult muscle cells. As previously shown in adult skeletal muscle, synemin exhibits a localization indistinguishable from that of desmin and vimentin at the peripheries of Z discs (22). Of interest is the obervation that synemin is not expressed in adult myocardial cells. Myocardial Z discs appear to be remodeled during development, with both vimentin and synemin being eliminated. Thus the mature cardiac Z discs are structurally analogous but biochemically dissimilar to skeletal muscle Z discs. If indeed synemin functions at the peripheries of Z disc to cross-link desmin and vimentin filaments, then its absence from cardiac muscle suggests either that a different polypeptide performs this function, or that, for some physiological reason, cardiac intermediate filaments need to remain uncross-linked and hence the synthesis of synemin is turned off during cardiac muscle development. This latter argument also applies to all nonmuscle types where synemin is not expressed, such as Schwann cells, endothelial cells, and fibroblasts.

The function of paranemin is presently unknown. Its loss from the skeletal muscle Z discs during muscle maturation implies that the skeletal Z discs are also remodeled subsequent to their assembly in embryos (5). Perhaps one of the functions of paranemin is to modulate the extent of intermediate-filament cross-linking mediated by synemin, and its removal reflects the need for the filaments to be maximally cross-linked in adult skeletal muscle. Future studies on the immunoelectron microscopic localization of paranemin may help elucidate the function of paranemin and explain the heterogeneous expression of this protein in adult chicken cells.

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