Isolation of a New High Molecular Weight Protein Associated with Desmin and Vimentin Filaments from Avian Embryonic Skeletal Muscle

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ABSTRACT Filaments with a diameter of 80–120 Å have been prepared from 14-d-old chick embryonic skeletal muscle, using a physiological salt solution and gel filtration chromatography. The filaments obtained are composed of the two known muscle intermediate-filament proteins, vimentin and desmin, as well as the vimentin- and desmin-associated high molecular weight protein, synemin (230,000 mol wt). In addition, they contain a previously unidentified high molecular weight protein (280,000 mol wt) which differs from synemin by isoelectric point, molecular weight, and immunological reactivity.

Immunofluorescence on cultured myogenic cells, using antisera to the 280,000-dalton polypeptide, has revealed that this protein has the same spatial distribution as desmin, vimentin, and synemin in both early myotubes, where it associates with cytoplasmic filaments, and in late myotubes, where it is associated with myofibril Z lines. Examination by immunofluorescence of frozen sections of developing embryonic skeletal muscle reveals a gradual diminution in the presence of the 280,000-dalton protein. The 280,000-dalton protein is undetectable in adult skeletal and smooth muscle, as shown by immunofluorescence and immunoautoradiography. In chick embryonic fibroblasts grown in tissue culture, only a subpopulation of the cells is reactive with antibodies to the 280,000-dalton protein even though all these cells contain vimentin. In the reactive cells, vimentin and the 280,000-dalton polypeptide exhibit an indistinguishable cytoplasmic filamentous network, which aggregates into filamentous bundles when the cells are exposed to colcemid. These results suggest that this newly identified high molecular weight protein is closely associated with intermediate filaments containing either vimentin alone or vimentin, desmin and synemin. The expression of this protein appears to be developmentally regulated and does not appear to parallel the expression of any of the other three intermediate-filament proteins. The absence of the 280,000-dalton polypeptide in adult muscle cells and its gradual reduction during development implies that is probably not required for the maintenance of Z-disk structure after the assembly of the sarcomere.

The cytoplasm of many higher eucaryotic cells contains a class of filaments whose diameter of 80–120 Å is intermediate to that of actin filaments (60 Å) and microtubules (250 Å) in nonmuscle cells, and intermediate to that of actin filaments and myosin filaments (150 Å) in muscle cells. This class of filaments, known collectively as intermediate filaments, appears morphologically quite homogeneous, although recent biochemical and immunological studies have revealed extensive heterogeneity in their subunit composition in different cell types (for a review, see reference 21). The cytoplasm of smooth, skeletal, and cardiac muscle cells contains numerous intermediate filaments (2, 4, 16, 17, 25). In adult smooth muscle, these filaments are insoluble in buffers containing nonionic detergents and high concentrations of salt that render the majority of myosin and actin soluble (4, 5). The insoluble filaments remain associated with cytoplasmic electron-dense bodies (4, 5) and their subunit proteins can be purified at low pH or using urea (4, 13, 14, 26). Intermediate filaments purified from adult smooth muscle are composed of one major polypeptide, desmin, and two minor copurifying polypeptides, vimentin and synemin (11, 13). In skeletal muscle, pulse-labeling experiments and immunofluorescence experiments have shown that desmin,

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vimentin, and synemin are all present during myogenesis in vitro and exhibit indistinguishable spatial distributions (7, 11). In early myogenesis they are associated with cytoplasmic filaments and in later myogenesis they become associated with myofibril Z disks (7, 11). In adult skeletal myofibrils, these three proteins are localized at the Z disk and in particular at the periphery of this structure as judged by immunofluorescence on isolated intact Z-disk sheets (9–11).

In our attempts to further analyze the biochemical events which culminate in the association of the three intermediate filament proteins with the Z disk, we have sought methods of purifying and analyzing intermediate filaments from embryonic skeletal muscle cells under physiological conditions. We describe here such a technique and we focus our characterization on the association of a new high molecular weight protein, called paranemin, with the isolated filaments in embryonic muscle and during myogenesis in vitro and on its fate in adult muscle.

MATERIALS AND METHODS

Filament Preparation

Thigh and breast muscles (10 g of tissue) were removed from 14- or 16–17-d-old chick embryos and homogenized three times on ice in a Teflon/glass homogenizer in 5–10 ml of a buffer containing 130 mM KCl, 5 mM EGTA, and 20 mM Tris-Cl, pH 7.5. The resulting homogenate was spun for 15 min at 16,000 rpm (30,000 *g*) in a Sorvall centrifuge, Rotor SS-34 (DuPont Instruments–Sorvall Biomedical Div., DuPont Co., Newtown, CT) at 4°C. The supernatant was then filtered through glass wool and spun again for 90 min at 40,000 rpm (145,000 g_{max}; 100,000 g_{av}) in a Beckman Ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) using a Ti-50 rotor at 4°C. The resulting supernatant (30 mg/ml) was loaded directly onto a Biogel A5-m column (49 × 2.5 cm, 240 ml) using a column buffer containing 100 mM NaCl, 0.1 mM EGTA, and 20 mM Tris-Cl, pH 7.5. Fractions from the column were analyzed by gel electrophoresis or applied directly to carbon-coated grids for electron microscope examination.

Myogenic Cultures

Primary cultures were prepared from 10-d-old embryonic chick leg muscle according to the method of Konigsberg (18) as described previously (7). Myogenic cells to be used for immunofluorescence were replated according to Gard and Lazarides (7) on day 2 at a density of 2×10^5 cells/60-mm plate on collagenized cover slips after two pre-platings to remove excess fibroblasts. Collagen was prepared from rat-tail tendons as described by Ehrmann and Gey (6). Cultures enriched in fibroblasts were prepared from the initial pre-plates of the primary cultures and grown for use in immunofluorescence on uncollagenized cover slips at an approximate density of 2×10^5 cells/60-mm plate.

PAGE

One-dimensional SDS polyacrylamide slab gel electrophoresis was based on the discontinuous Tris-glycine system of Laemmli (19), and two-dimensional isoelectric focusing (IEF)/SDS PAGE was performed according to the method of O'Farrell (24) as modified and described by Hubbard and Lazarides (13). For sliver staining, protein samples were analyzed by two-dimensional electrophoresis as described above. Gels were prefixed in 50% ethanol–5% acetic acid for 30 min, washed in distilled water, and then fixed again in 10% unbuffered glutaraldehyde for 30 min. Development of silver stain was carried out according to the method of Merril et al. (22) as modified by Oakley et al. (23), using a fresh ammoniacal silver solution followed by water rinsing and application of reducing agent (0.005% citric acid; 0.019% formaldehyde). The final gel was rinsed extensively in at least six changes of H_2O.

For two-dimensional and one-dimensional preparative electrophoresis of purified filaments, all material was first dialyzed overnight against two changes of 50 mM NH_4HCO_3 to remove excess salt, lyophilized, and stored at −20°C. Dried samples were then resuspended directly in IEF lysis buffer (9 M urea, 2% Nonidet P-40, 0.5% β-mercaptoethanol).

Quanitative Analysis

Lanes from Fig. 1 *a* and similar gels were cut following destaining of the gels and scanned in a Beckman densitometer (Beckman Instruments, Inc.) at 550 nm. Approximate estimates of molar ratios were obtained by dividing peak areas by the approximate molecular weight of the respective protein. Scanning of lanes from different filament preparations gave comparable ratios for the three proteins. Protein concentrations were determined by their absorbance at 280 nm using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as a standard.

Perparation of Antibodies

Antibodies to paranemin were prepared following procedures described elsewhere (10, 11). Intermediate filaments were purified by gel filtration chromatography as described above, and paranemin was further purified from this material by preparative SDS gel electrophoresis (10, 11, 20). The paranemin band was homogenized in a Teflon/glass homogenizer in 2–3 ml of phosphate-buffered saline (PBS), and the packed gel homogenate was further suspended in 2–3 ml of PBS and divided into four aliquots each containing 0.2–0.3 mg total protein; these were administered to a female New Zealand white rabbit by multiple subcutaneous dorsal injections on days 1, 15, 41, and 72. The first injection consisted of an aliquot emulsified with an equal volume of Freund's complete adjuvant, while subsequent injections contained no adjuvant. Sera were collected from the marginal ear vein after the third injection at days 48 and 55, and after the fourth injection at day 78. Sera were precipitated at 50% ammonium sulfate saturation and further purified by passage through a DEAE cellulose column (5 mg protein/ml packed resin) in 0.01 M Tris HCl, pH 7.4. The flow through protein of the column was concentrated at 50% ammonium sulfate saturation, dialyzed against PBS, and stored at −20°C. The paranemin serum showed a single precipitin line against the purified filament preparation in double-immunodiffusion plates containing 1% agarose in 0.5% Triton X-100, 0.1% SDS, 10 mM Tris HCl, pH 7.4, 130 mM NaCl, 10 mM NaN_3, 1 mM EGTA. Antibodies to chicken desmin, vimentin, and synemin were those described previously (10, 11).

Immunoautoradiography

One-dimensional SDS gels (12.5% in acrylamide concentration) and two-dimensional IEF/SDS gels were prepared for immunoautoradiography as described by Granger and Lazarides (10, 11). Antisera or pre-immune sera (partially purified at 50% ammonium sulfate saturation) were applied at 1:1,000 dilution. Both synemin and paranemin antisera were preadsorbed onto a slurry of crushed polyacrylamide gel in Buffer I before immunoautoradiography to remove any anti-acrylamide antibodies (11). Autoradiograms were exposed for 1–3 d at −70°C on Kodak X-Omat RXR5 film with DuPont Cronex Lightning-Plus intensifying screens.

Immunofluorescence

Immunofluorescence on cells grown on cover slips was performed as described previously (7, 11). To determine whether paranemin would remain associated with vimentin-containing filaments in Triton X 100-insoluble cytoskeletons, cover slips were rinsed first in PIPES buffer (0.1 M PIPES, pH 6.8, 0.5 mM $MgCl_2$, 1 mM EGTA, 0.1% albumin, 5 mM NaN_3) and then in PIPES buffer containing 0.5% Triton X-100 (S. I. Danto, personal communication). All subsequent washes and incubations were carried out in the Triton X-100-PIPES buffer. Directly labeled antibodies were prepared according to the methods of Cebra and Goldstein (3) and Brandtzaeg (1) as described previously (7, 11).

Double immunofluorescence was performed according to the indirect/direct method of Hynes and Destree (15). Sequential incubations of fixed or unfixed cells were done with rabbit anti-paranemin, fluorescein-conjugated goat anti-rabbit IgG, normal rabbit serum to block free anti-IgG sites, and finally with rhodamine-conjugated rabbit anti-vimentin or anti-desmin.

Pre-adsorption studies were carried out on paranemin antisera by incubating DEAE cellulose-purified IgG with purified paranemin, vimentin, or desmin obtained by preparative SDS gel electrophoresis (11).

Muscles to be used for frozen sections were stretched, coated in O.C.T. compound (Tissue-Tek II, Miles Laboratories, Inc., Naperville, IL), and quickly frozen in dry ice. Tissues were mounted and cut into 8- to 10-μm sections in a cryostat at −20°C. Sections were placed on glass cover slips and immediately dehydrated in 95% ethanol, rinsed in PBS and then in PBS containing 0.5% Triton X-100, and processed for immunofluorescence.

Cover slips were mounted in 90% glycerol in Tris-buffered saline, examined with a Leitz phase/fluorescence microscope (filter modules K and N2), and photographed on Kodak Tri-X film.

Electron Microscopy

The presence of filaments in 14-d-old embryonic muscle extracts was determined on carbon-coated copper grids (400-mesh) by negative staining. Samples

were applied twice for 1 min each, and the excess liquid was removed with filter paper. Column fractions and the high-speed supernatants were applied directly to the grids. Samples were occasionally prefixed with 2% glutaraldehyde in PBS for 1 min and rinsed with PBS. The grids were then stained for 30 s with a solution of 2% uranyl acetate in 1 mM HCl or 2% phosphotungstic acid (PTA) adjusted to pH 7.0 with NaOH. Grids were viewed in a Philips EM 201 at 60 kV and photographed on 35-mm film.

RESULTS

Preparation of Desmin and Vimentin from Embryonic Muscle

High-speed supernatants (145,000 g_{max}) prepared from 14-d-old chick embryonic breast and thigh muscles in low ionic strength buffer and in the absence of detergents are enriched in actin, α and β tubulin, and the two intermediate-filament proteins, desmin and vimentin, as judged by two-dimensional IEF/SDS PAGE (10). Gel filtration chromatography of these extracts through a column of Biogel A-5m (range 10^4–5×10^6 mol wt) and subsequent analysis of the column fractions by one-dimensional SDS PAGE (Fig. 1*A*) indicates that the void volume fractions of the column are highly enriched in vimentin, desmin, and a new high-molecular-weight protein, which we have named paranemin. Later column fractions contain progressively lesser quantities of these three proteins and increasing amounts of tubulins and actin. As can be seen from Fig. 1*A*, the separation of the desmin- and vimentin-containing material from the rest of the proteins in the extract is substantial. Quantification of the amount of desmin, vimentin, and paranemin present in the void volume fractions of the column indicates that these three proteins account for ~8% of the total protein loaded on the column and 80% of the protein present in these fractions. Little or no desmin and vimentin is detectable in the later fractions of the column as judged by one-dimensional SDS PAGE (Fig. 1*A*) and by two-dimensional IEF/SDS PAGE (not shown). The apparent native molecular weight of the desmin-, vimentin- and paranemin-containing material is well above 5×10^6 (exclusion limit of Biogel A-5m) as shown by the exclusion of these two molecules from Biogel A-15m (exclusion limit 15×10^6 mol wt) (not shown). Comparative one-dimensional SDS PAGE of paranemin present in the void volume fractions with myosin heavy chain (200,000 mol wt) and filamin (250,000 mol wt) indicates ~280,000 mol wt for paranemin (Fig. 1*B*). Quantification of the ratio of vimentin, desmin, and paranemin present in the void volume fractions of the Biogel A-5m column (Fig. 1*A*) from gel scanning yields a molar ratio of vimentin:desmin:paranemin of 15:10:1, assuming normal and linear binding of Coomassie Brilliant Blue R to all three proteins. Coelution of desmin, vimentin, and paranemin is also observed when the high-speed supernatant is adjusted to 0.6 M KCl and chromatographed in 0.6 M KCl (not shown).

Under a typical experimental protocol, high-speed supernatants are prepared for 90 min at 100,000 g_{av} in a Ti 50 rotor. Desmin, vimentin, and paranemin purified by gel filtration from 14-d-old embryonic muscle were spun for two different time periods to determine their sedimentation. When the purified proteins were centrifuged for 60 min at 100,000 g in a Ti 50 rotor, very little protein was recovered in the pellet and all of the protein remained in the supernatant. However, when the proteins were centrifuged for 10 h at 100,000 g, the majority of the protein was recovered in the pellet. Analysis of the supernatant and pellet of the material centrifuged for 10 h shows

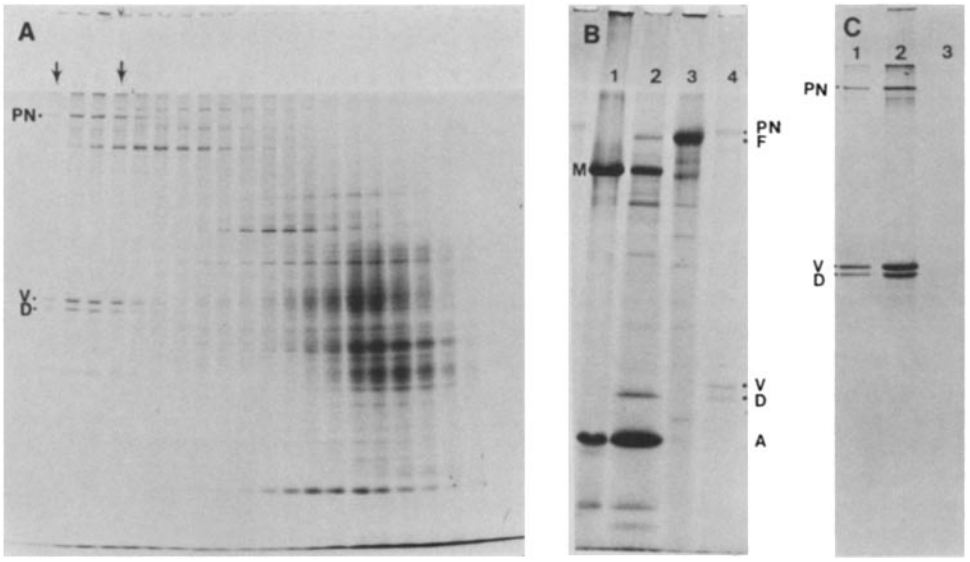

FIGURE 1 Analysis of isolated filaments from 14-d-old embryonic muscle by one-dimensional SDS PAGE. (*A*) Elution profile of a high-speed supernate of 14-d-old embryonic skeletal muscle following gel chromatography on a Biogel A-5m column. Every other fraction from the column was analyzed by one-dimensional SDS PAGE. Arrows indicate the void volume fractions. (*B*) Comparative electrophoresis of high molecular weight proteins with the paranemin polypeptide present in the filaments isolated from 14-d-old embryonic muscle. Lane *1*: chicken pectoralis myofibrils. Lane *2*: low ionic strength Tris HCl-EGTA extract of chicken gizzard smooth muscle prepared as described previously (11). Lane *3*: Purified filamin preparation from chicken gizzard smooth muscle. The polypeptides below filamin are formed during protein storage and are most likely degradation products of filamin (8). Lane *4*: a filament preparation from 14-d-old chick embryonic skeletal muscle. (*C*) Sedimentation analysis of purified filaments from 14-d-old embryonic muscle. Lane *1*: Filament preparation after Biogel A5-m chromatography. This material was centrifuged for 10 h in a Ti 50 rotor at 100,000 g_{av}. The resulting pellet (lane *2*) contains >90% of the total protein and all three major bands. The supernatant (lane *3*) contains little detectable amount of protein. Protein bands referred to are paranemin (*PN*), filamin (*F*), myosin heavy chain (*M*), vimentin (*V*), desmin (*D*), and actin (*A*).

that the composition of the pellet is the same as that of the starting material (Fig. 1 *C*). It contains predominantly desmin, vimentin, and paranemin, and the ratio of these three proteins in the pellet is the same as that in the original supernatant before centrifugation. Examination of the pelleted material by electron microscopy showed the presence of numerous filaments with a diameter of 80–120 Å (see below).

Analysis by two-dimensional IEF/SDS PAGE and Coomassie Blue staining of the material in the void volume of the Biogel A-5m column obtained from 14-d-old embryonic chick skeletal muscle shows the predominant polypeptides to be vimentin, desmin, and paranemin (Fig. 2*A*). On IEF, paranemin focuses as two closely spaced variants with an average isoelectric point of 4.5. In the second (SDS) dimension, paranemin migrates as two closely spaced polypeptides of very similar apparent molecular weights. These two closely spaced polypeptides are also frequently detected on one-dimensional SDS analysis of paranemin. To detect other polypeptides which coelute with desmin, vimentin, and paranemin and which exist in too low a concentration to be detected by Coomassie Blue, a duplicate gel of that depicted in Fig. 2*A* was stained with silver nitrate, which enhances the sensitivity of protein detection ~100-fold (22). This technique clearly reveals paranemin, desmin, and vimentin as the major proteins in the sample (Fig. 2*B*). It also reveals a number of polypeptides of lower molecular weight and more acidic isoelectric point than desmin and vimentin which have been previously identified by immunoautoradiography as degradation products of the parent molecules (10). In the silver nitrate-stained gel, paranemin is resolved more clearly as two closely spaced polypeptides which do not focus with a discrete isoelectric point but focus as a streak with the main variants primarily in the pH range of 4.0–4.5. This technique also reveals the presence of a small number of other polypeptides, most notably actin, two polypeptides with a molecular weight of 68,000 previously identified as the avian 68,000 heat-shock proteins thermin A and thermin B (27), and a small number of unidentified polypeptides. Of interest is a major protein (designated by an asterisk) which focuses diffusely and more acidic than paranemin. Apparently, this protein binds Coomassie Blue poorly but its presence is clearly revealed by silver nitrate staining. The origin and function of this protein is presently unknown. Some of the polypeptides which focus as streaks above this protein and below paranemin are most likely degradation products of paranemin or oxidation aggregates of desmin and vimentin, all of which can be shown to exist in this material by immunoautoradiography (see below).

Electron Microscope Examination of Polymeric Desmin and Vimentin

To further investigate whether the extracted desmin and vimentin material was in a filamentous form we examined the various extracts by electron microscopy. Samples from the two centrifugation steps (30,000 and 100,000 *g*) were directly applied to grids. Fig. 3*D* shows that the high-speed supernatant contains numerous filaments with a diameter of 80–120 Å and a morphology (Fig. 3*E*) closely similar to that observed with the purified filaments. Similar results were obtained with the lower speed supernatant (not shown).

Examination of the desmin- and vimentin-enriched void volume fractions by negative staining in electron microscopy reveals the presence of filaments with variable lengths and diameters ranging from ~80 to 120 Å. As can be seen in Fig. 3*A* the surface contour of the purified filaments is irregular rather than smooth, and an individual filament exhibits slight variations in diameter along its length. Two characteristic features of the isolated filaments are their bifurcation (see Fig. 3*B*) and the presence of increased stain densities at irregular intervals along the filaments (Fig. 3*C*). At higher magnification the two forked branches of a single filament often appear to contribute half the diameter of the parent filament, suggesting a "splitting" of the parent filament. Further substructure cannot be resolved by this technique, although the filaments occasionally seem to be associated with vesicular structures at their tips or at points along their length.

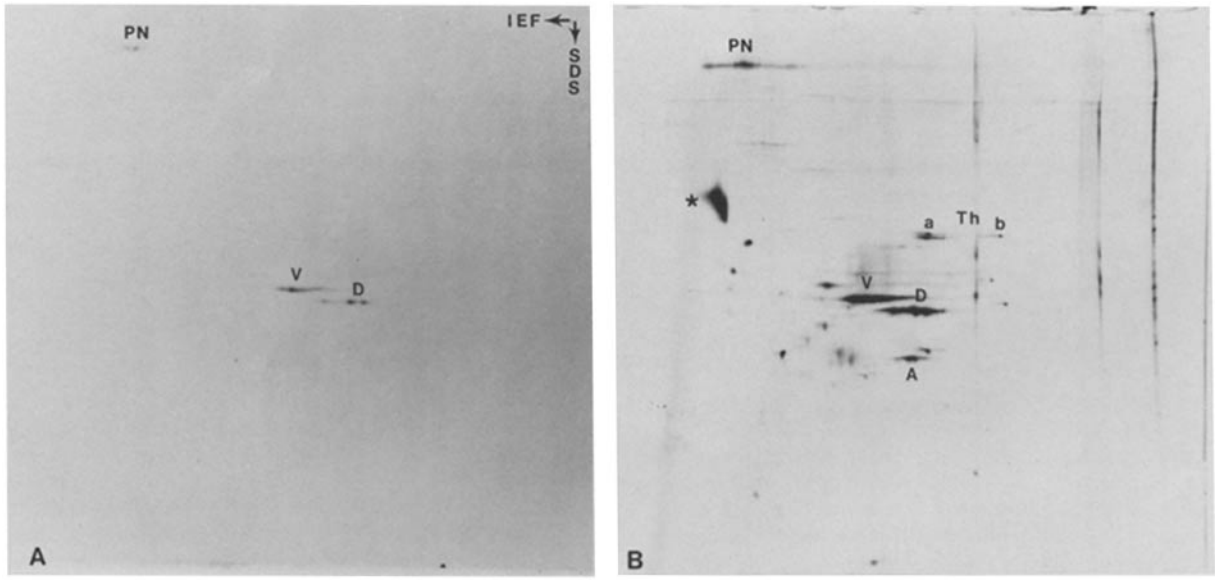

FIGURE 2 Coomassie Blue and silver-nitrate staining of filament protein purified from 14-d-old embryonic muscle and analyzed by 2D IEF/SDS PAGE. (*A*) Coomassie Blue-stained gel; (*B*) silver nitrate-stained gel. Proteins referred to are as in Fig. 1. thermin a (*Th, a*) and b (*Th, b*). Actin (*A*). In these and all subsequent two-dimensional gels the acidic end of the IEF dimension is on the left of each figure.

Analysis of Purified Filaments by Immunoautoradiography

To further assess the composition of the isolated desmin- and vimentin-containing embryonic material and compare it to adult muscle cells, we assayed for the presence of different intermediate-filament proteins using one-dimensional SDS immunoautoradiography. A comparison of a Coomassie Blue–stained gel (Fig. 4*A*) with a corresponding autoradiogram shows that antibodies to chicken desmin and vimentin react, respectively, with a 50,000- (Fig. 4*B*) and a 52,000-dalton (Fig. 4*C*) protein in filaments isolated from 14- and 16-d-old embryonic skeletal muscle as well as in whole myofibril extracts. In addition, the antibodies reveal the presence of a family of reacting polypeptides with a molecular weight <50,000 in the case of desmin and <52,000 in the case of vimentin. Previous studies have indicated that this family of reacting polypeptides are degradation products of the parent desmin or vimentin proteins (10). The antibodies also reveal the presence of a very low quantity of higher molecular weight polypeptides with an approximate molecular weight of 310,000 daltons. These cross-reacting polypeptides have an electrophoretic mobility higher than that of paranemin, filamin, or synemin, and are most likely higher molecular weight aggregates of desmin and vimentin which are not dissociated by SDS and β-mercaptoethanol (see also reference 11).

Previous studies have indicated that the desmin- and vimentin-associated protein, synemin (230,000 mol wt), exists in very low concentrations in adult smooth (gizzard) and skeletal muscle (synemin:desmin ratio 1:50–100) and is not easily detectable by Coomassie Blue staining. The presence of this protein can be clearly demonstrated by immunoautoradiography on whole SDS extracts of adult smooth muscle and cultured chicken embryonic skeletal myotubes (11). The presence of small quantities of synemin in the material isolated from 14-d- and 16-d-old embryonic muscle can be detected with anti-

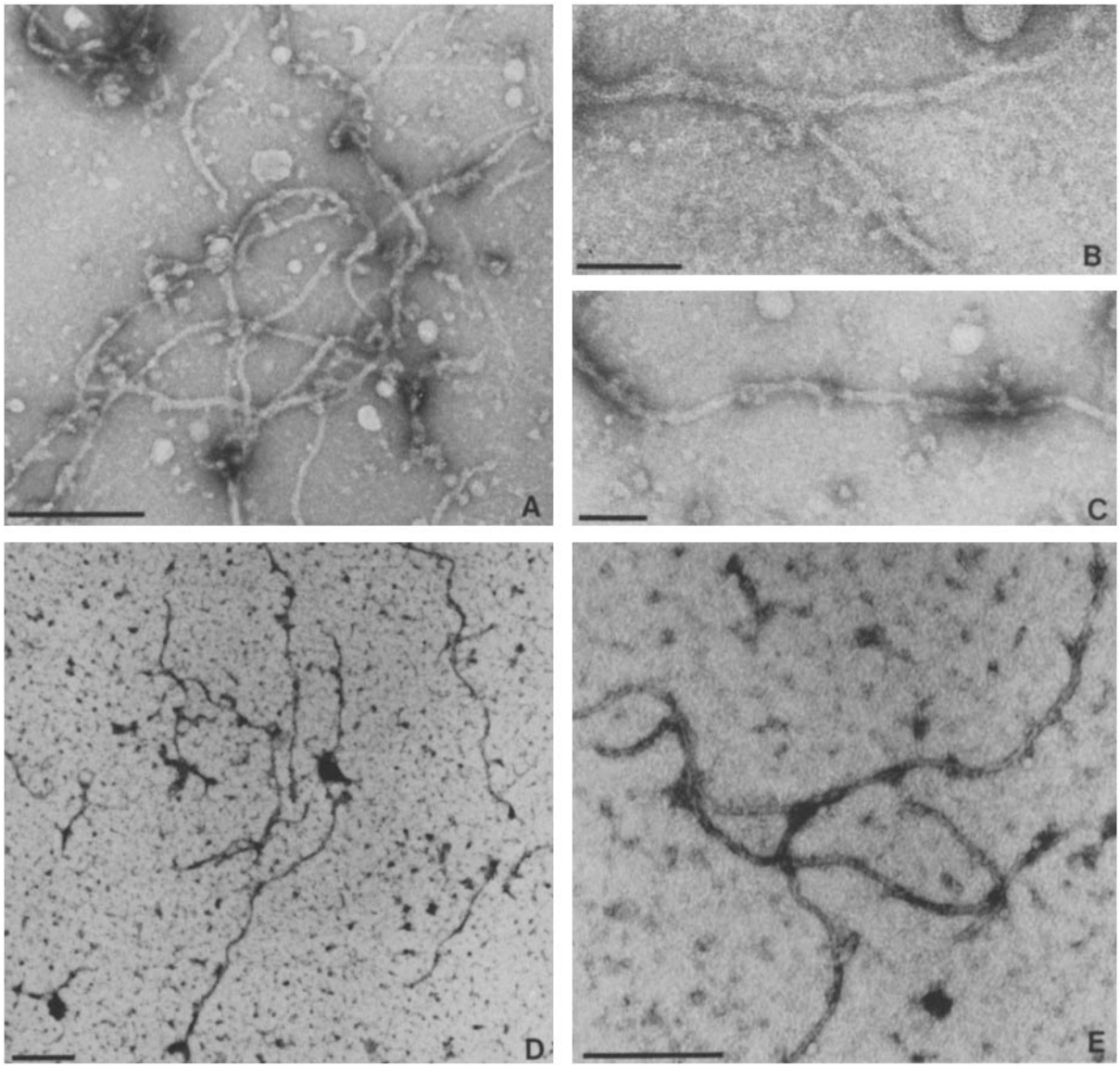

FIGURE 3 Electron micrographs of filaments isolated from 14-d-old embryonic skeletal muscle. Negatively stained filament preparations following gel chromatography using uranyl acetate (*A*, *D*, and *E*) and PTA (*B* and *C*) as stains. (*A*) Low magnification. (*B* and *C*) Higher magnification. Note the splitting of the filament and the periodic staining densities along the filaments. Before gel chromatography the high speed supernate contains numerous filaments. (*D*) Low power micrograph. (*E*) Higher power micrograph. (*A*, *D*, and *E*) Bar, 300 nm; for *B* and *C* Bar, 100 nm.

synemin in Fig. 5 *C* 1–2. Synemin is also detected in whole SDS extracts of gizzard (Fig. 5 *C*, lane *4*). The absence of synemin from whole extracts of skeletal myofibrils (Fig. 5 *C*, lane *3*) is probably due to the low concentration of this protein and to its extreme susceptibility to proteolysis during myofibril storage (see reference 11).

Anti-paranemin reacts with a 280,000-dalton protein in 14-d- and 16-day-old filamentous material isolated from chick embryonic muscle (Fig. 4 *D*, lanes *1–2*, and Fig. 5 *B*, *1–2*). No other protein, including synemin, desmin, and vimentin, reacts with this antibody in either sample. However, paranemin is undetectable in whole extracts of skeletal myofibrils and smooth muscle (gizzard) at the protein concentrations used for electrophoresis and subsequent immunoautoradiography, even after prolonged exposure of the autoradiograms (Fig. 4 *D*, lane *3*, and Fig. 5 *B*, lanes *3–4*). The specificity of the paranemin antibodies can also be demonstrated by two-dimensional IEF/SDS immunoautoradiography of filamentous material isolated from 14-d-old embryonic muscle. When labeled with anti-paranemin the gels show a 280,000-dalton streak which extends throughout the isofocusing gel with a higher amount of reactivity in the acidic end of the gel (Fig. 6). This is consistent with the isofocusing behavior of paranemin as shown by Coomassie Blue and silver staining (see below). The antibodies also reveal the presence of a small number of doublet streaks of lower molecular weight which are presumably degradation products of paranemin. The paranemin preimmune sera exhibit no detectable reactivity either with the filamentous material isolated from 14-d- and 16-day-old embryonic muscle or with extracts from skeletal myofibrils (Fig. 4 *E*).

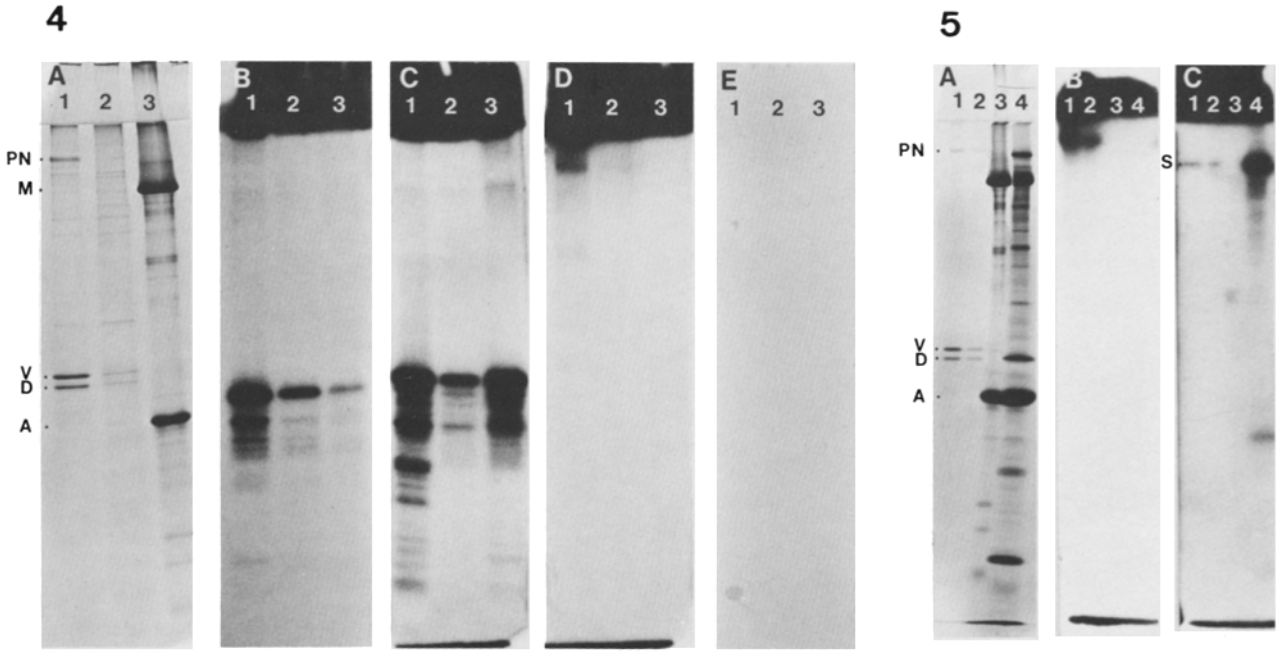

FIGURE 4 Immunoautoradiography of 14- and 16-d-old filament preparations as compared to adult chicken skeletal myofibrils. Lane *1*: filaments from 14-d-old embryonic muscle. Lane *2*: filaments from 16- to 17-d-old embryonic muscle. Lane *3*: chicken skeletal myofibrils. Column *A*, Coomassie Blue-stained gel; *B*, anti-desmin; *C*, anti-vimentin; *D*, anti-paranemin; and *E*, anti-paranemin preimmune serum. Gels identical to those shown in *A* were reacted with the antibodies. Only a representative Coomassie Blue–stained gel is shown.

FIGURE 5 *A* is a representative Coomassie Blue–stained gel of *B* (antiparanemin) and *C* (anti-synemin). *S*, synemin. Lane *1*: filaments from 14-d-old embryonic muscle. Lane *2*: filaments from 16-d-old embryonic muscle. Lane *3*: chicken skeletal myofibrils. Lane *4*: whole-extract of adult chicken gizzard.

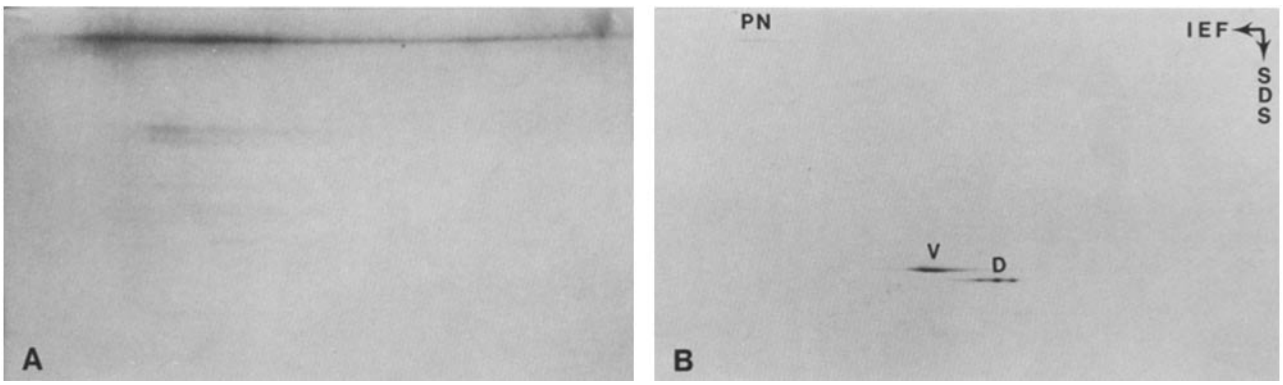

FIGURE 6 Two-dimensional immunoautoradiography of filaments isolated from 14-d-old embryonic muscle using paranemin antibodies. (*A*) Autoradiogram and (*B*) its corresponding Coomassie Blue-stained gel.

Paranemin in Nonmuscle Cells

To investigate the distribution of paranemin in nonmyogenic cells and its putative association with vimentin-containing intermediate filaments, we examined its distribution in the fibroblastic cells which remain adherent to petri plates during the preparation of myogenic cultures. Fig. 7 *A* and *B* are lower power micrographs of primary myogenic cells (fibroblasts and myotubes) reacted with anti-vimentin and anti-paranemin using double immunofluorescence. Vimentin can be seen in all the cells in the culture; paranemin, however, is present in only a subpopulation of the cells. The number of reactive cells with anti-paranemin varies in different fibroblastic preparations and thus far the origin of the positive cells has not been determined. At higher magnification, double immunofluorescence shows that the distribution of vimentin and that of paranemin in the anti-paranemin positive cells is indistinguishable (Fig. 7 *C* and *D*). This coincidental distribution of the two antigens can be shown even more dramatically in cells exposed to Colcemid (5 μM, 18 h). Double immunofluorescence with anti-vimentin and anti-paranemin on cells exposed to Colcemid shows that the two antigens exhibit an apparent coincidental distribution within the aggregated filament bundles (Fig. 8 *A* and *B*).

Paranemin during Myogenesis In Vitro

Double-immunofluorescence microscopy was performed at various times after plating of chick myogenic cells to follow the distribution of paranemin during myogenesis and to compare it with that of vimentin or desmin. In these cells, desmin, vimentin, and synemin exist initially as seemingly random cytoplasmic filaments but begin to associate with the Z line within a week after myoblast fusion (7, 11). Fig. 9 *A* and *B* shows a myotube 4–5 d after the onset of fusion, stained with anti-paranemin and anti-vimentin. The two antigens exhibit coincident distributions in the myotubes in the form of cytoplasmic filaments; this pattern is also characteristic of desmin and synemin at this stage of development (7, 11). Fig. 9 *C* and *D* shows a segment of a fully differentiated myotube (10 d after plating) stained in double immunofluorescence with anti-desmin and anti-paranemin. Both paranemin and desmin are distributed predominantly along the Z lines at this stage of differentiation. Examination of earlier time-points has shown that the association of paranemin with the Z line coincides both spatially and temporally with that of desmin and vimentin (not shown).

To determine whether paranemin shares the solubility properties of desmin, vimentin, and synemin in cultured cells, the cells in Fig. 9 *C* and *D* were lysed with Triton X-100 and processed for double immunofluorescence without fixation with formaldehyde. Both cytoplasmic filaments (not shown) and Z line–associated forms of paranemin and desmin resist extraction with detergent. Fig. 9 *E* and *F* shows a well-differentiated myotube stained in double immunofluorescence with anti-desmin and the preimmune serum from the animal im-

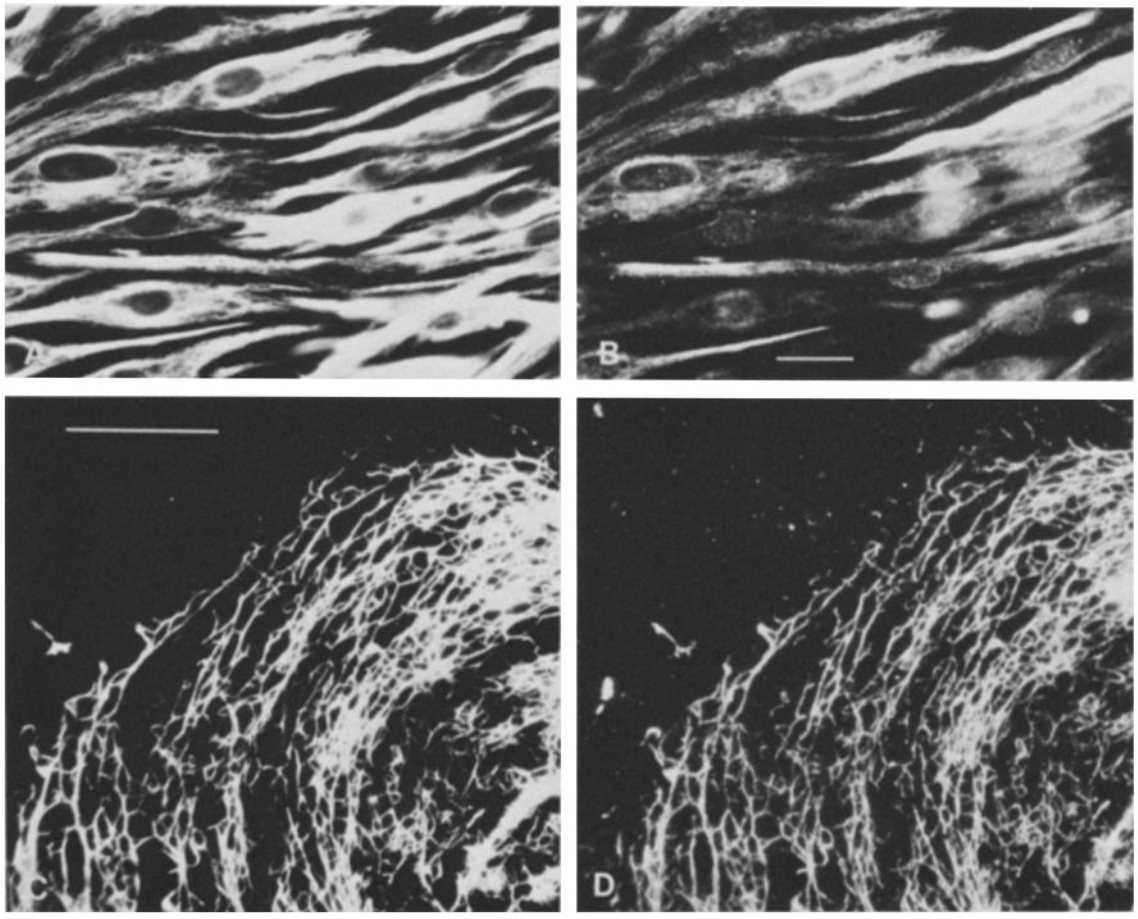

FIGURE 7 Double immunofluorescence on primary myogenic cultures, (*A* and *B*) Lower power micrograph (× 40 lens); (*C* and *D*) higher power micrograph (× 63 lens) of a section of a large cell exhibiting well-splayed-out filaments. (*A* and *C*) Anti-vimentin. (*B* and *D*) Anti-paranemin. In *A* and *B*, note that some fibroblastic cells do not react with antiparanemin. In *D* the staining of anti-paranemin appears punctate on some of the filaments. For A and B: bar, 20 μm. *C* and *D*: bar, 20 μm.

munized with paranemin. The anti-desmin reveals the presence of desmin in Z lines, while the paranemin preimmune serum shows no fluorescent reaction.

To ensure that the anti-paranemin used in our study was reacting with its respective antigen and not cross-reacting with either desmin or vimentin, the immunofluorescence and immunoautoradiographic results were supplemented with appropriate adsorption controls. Fig. 10 *A–B* show a well differentiated cell stained with anti-paranemin. In Fig. 10 *C* and *D*, adsorption of anti-paranemin with purified paranemin blocks fluorescence. However, adsorption with purified vimentin (Fig. 10 *E* and *F*) or desmin (Fig. 10 *G* and *H*) has no effect on the immunofluorescence observed.

Paranemin in Mature Skeletal Muscle

In adult skeletal muscle, desmin, vimentin, and synemin are components of the myofibril Z disk as shown by immunofluorescence, and their presence in myofibril extracts can be demonstrated by immunoautoradiography (see above and also references 10 and 11). However, immunoautoradiography indicated that paranemin is undetectable in extracts of skeletal myofibrils and gizzard smooth muscle (see above Figs. 4 and 5). To investigate this point further we examined the distribution of paranemin and desmin in frozen sections of skeletal muscle (thigh) in various stages of embryonic development and in adult tissue. In Fig. 11 *A* and *B*, cross-sections of 15-d-old embryonic muscle reveal the presence of both paranemin and desmin; at this stage of differentiation, neither of the two molecules exhibits a clear association with Z disks. In Fig. 11 *C* and *E*, cross-sections of 19-d-old muscle reveal the presence of both antigens in a honeycomblike arrangement indicative of their association with the periphery of Z disks. In Fig. 11 *H*, a longitudinal section of 1-wk-old chick thigh muscle (7 d after hatching) clearly shows the association of desmin with Z lines. However, similar sections reacted with anti-paranemin (Fig. 11 *G*) show a marked reduction in the fluorescence of this antigen both in the cytoplasm and at the Z line. Longitudinal frozen sections of adult chicken muscle (Fig. 11 *I* and *J*) show that paranemin is barely detectable (very little fluorescence) but desmin is clearly associated with Z disks. To examine whether paranemin and desmin share the same solubility properties, frozen sections of 19-d-old embryonic muscle were incubated for 60 min in a buffer containing 0.6 MKI, 0.5% Triton X-100 and then processed for immunofluorescence. Fig. 11 *D* and *F* show cross-sectional profiles of fibers extracted in this manner. Both paranemin and desmin still exhibit their distribution at the peripheries of Z disks; both antigens are also present at the periphery of the fiber close to the plasma membrane.

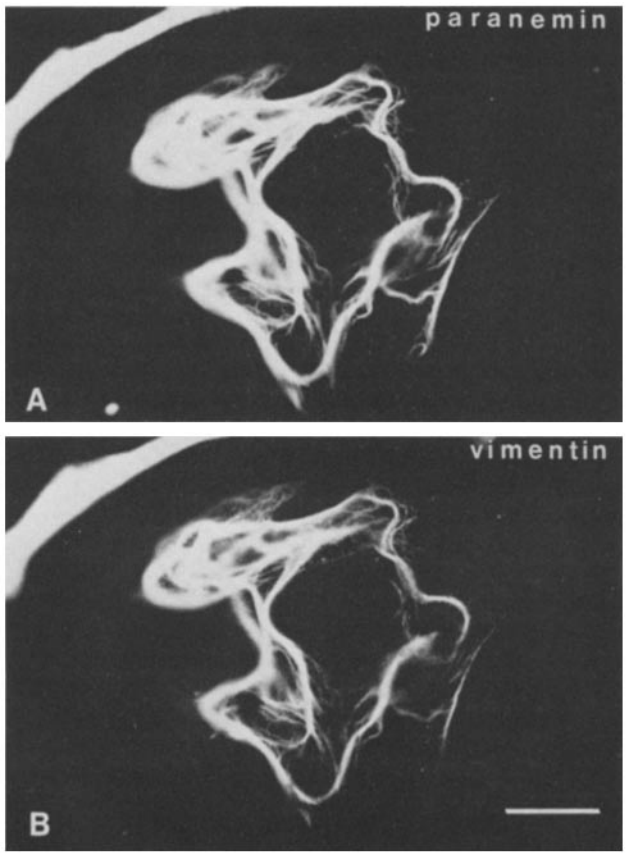

FIGURE 8 Double immunofluorescence on Colcemid-treated fibroblastic cells. Anti-paranemin (*A*) and anti-vimentin (*B*). Bar, 20 μm.

DISCUSSION

Isolation of Filaments

To begin investigating the biochemical events that culminate with the association of desmin, vimentin, and synemin with the periphery of the Z disk, we sought a method of obtaining intermediate filaments from embryonic muscle without the use of high-salt buffers and nonionic detergents. We observed that high speed supernatants (100,000 g_{av}) of 14-d-old embryonic muscle contain considerable amounts of vimentin and desmin. Gel filtration chromatography has shown that these two proteins can be recovered in a very high molecular weight form. The recovered material is highly enriched in vimentin, desmin and a previously undescribed high molecular weight protein (called here paranemin from the Greek para: with, beside, and nema: filament) and when examined in the electron microscope it contains numerous filaments which measure 80–120 Å in diameter. The majority of the vimentin, desmin, and paranemin recovered from the column were in this high molecular weight form, and examination of the included fractions of the column revealed very little desmin, vimentin, and paranemin in a small oligomeric or monomeric form. Examination of the high speed supernatant in the electron microscope revealed the presence of numerous filaments whose morphology was indistinguishable from that of the filaments recovered in the void volume of the Biogel A-5m column. This observation suggests that at least some of the filaments recovered in the void volume of the column are already present in the high speed supernatant. As judged by Coomassie Blue staining and immunoautoradiography, the three proteins, desmin, vimentin, and paranemin, co-elute from the Biogel column and co-sediment when the filaments are pelleted by centrifugation. Taking into consideration that the isolated filaments contain synemin (as shown by immunoautoradiography) and that all four proteins colocalize in immunofluorescence (see above and reference 7, 10–12), these results indicate that all four proteins are associated with the isolated filaments.

The origin of the filaments is difficult to establish unambiguously, as 14-d-old embryonic muscle contains, in addition to muscle cells, a substantial number of nonmuscle cells. However, the presence of high quantities of desmin in the purified filaments indicates that a substantial fraction of the filaments are of muscle origin since nonmuscle cells contain very low

quantities of this protein (7). This conclusion is strengthened further by the observation that intermediate filaments in skeletal myotubes differentiating in tissue culture contain both vimentin and desmin in approximately equal amounts (7).

Characterization of Paranemin

By electrophoretic and immunological criteria, paranemin copurifies with filaments isolated from 14-d-old embryonic muscle but is undetectable in adult skeletal and smooth (gizzard) muscle. This polypeptide is also present in a subpopulation of the fibroblastic cells in tissue culture of chick embryonic cells. In both myogenic and nonmuscle cells this protein has a cytoplasmic distribution indistinguishable from that of the intermediate-filament subunits desmin and vimentin as well as from that of the muscle intermediate filament–associated protein, synemin. Furthermore, all four proteins resist solubilization by nonionic detergents or dissociation by high salt concentrations. From these observations it appears that paranemin is associated with intermediate filaments in cells. Its absence from some fibroblastic cells where vimentin is present suggests that paranemin is not required invariably for the formation of intermediate filaments, but rather it may perform a specific role in the structure of intermediate filaments during differentiation. Furthermore, since it can be found in cells which

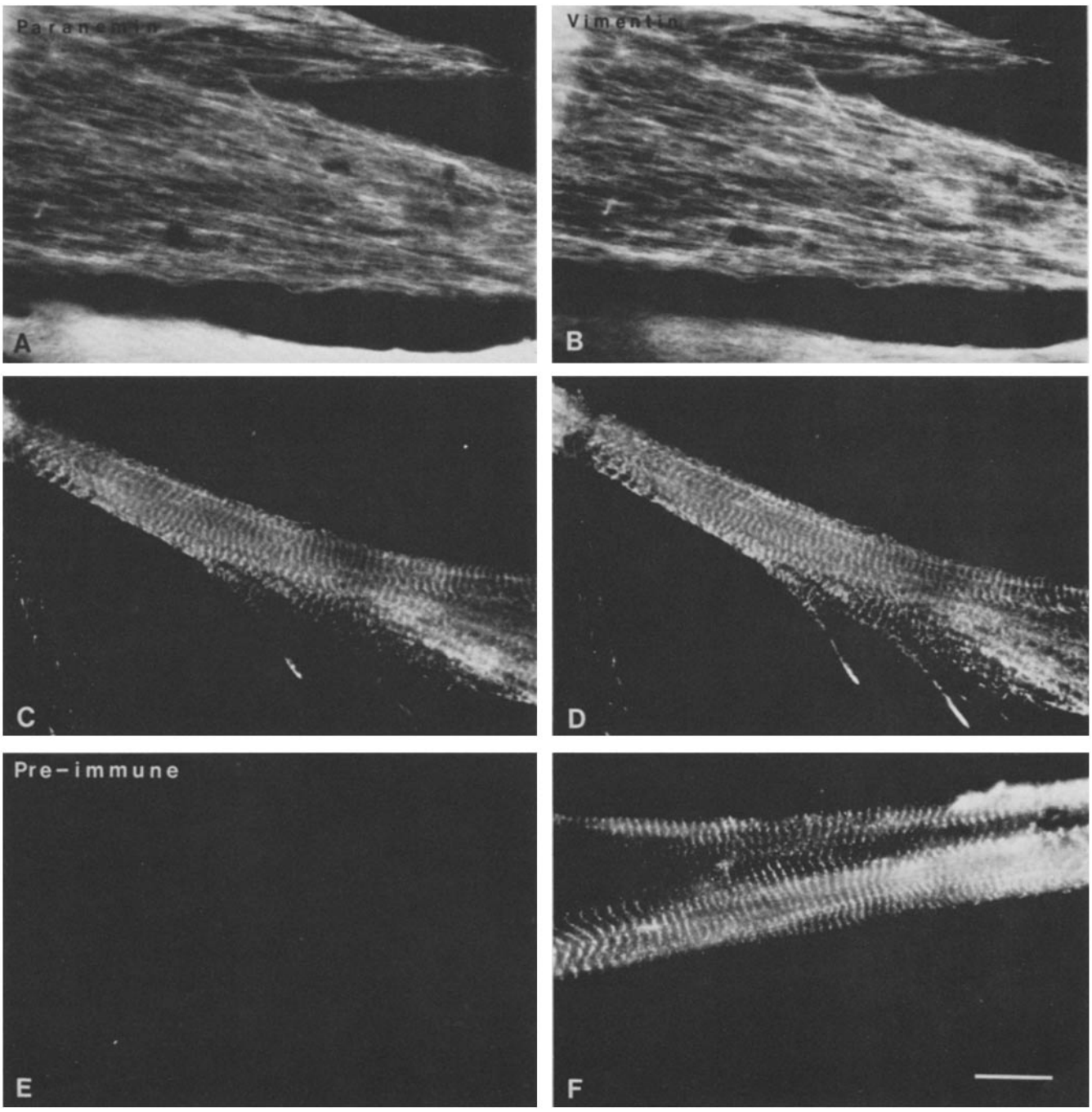

FIGURE 9 Double immunofluorescence of myotubes at different stages of differentiation. 4-d (*A* and *B*) and 10-d (*C–F*) myotubes were doubly labeled for paranemin (*A* and *C*) and vimentin (*B* and *D*) or the anti-paranemin preimmune serum (*E*) and vimentin (*F*). The patterns for the two antigens (*A–D*) are indistinguishable from each other. Bar, 20 μm.

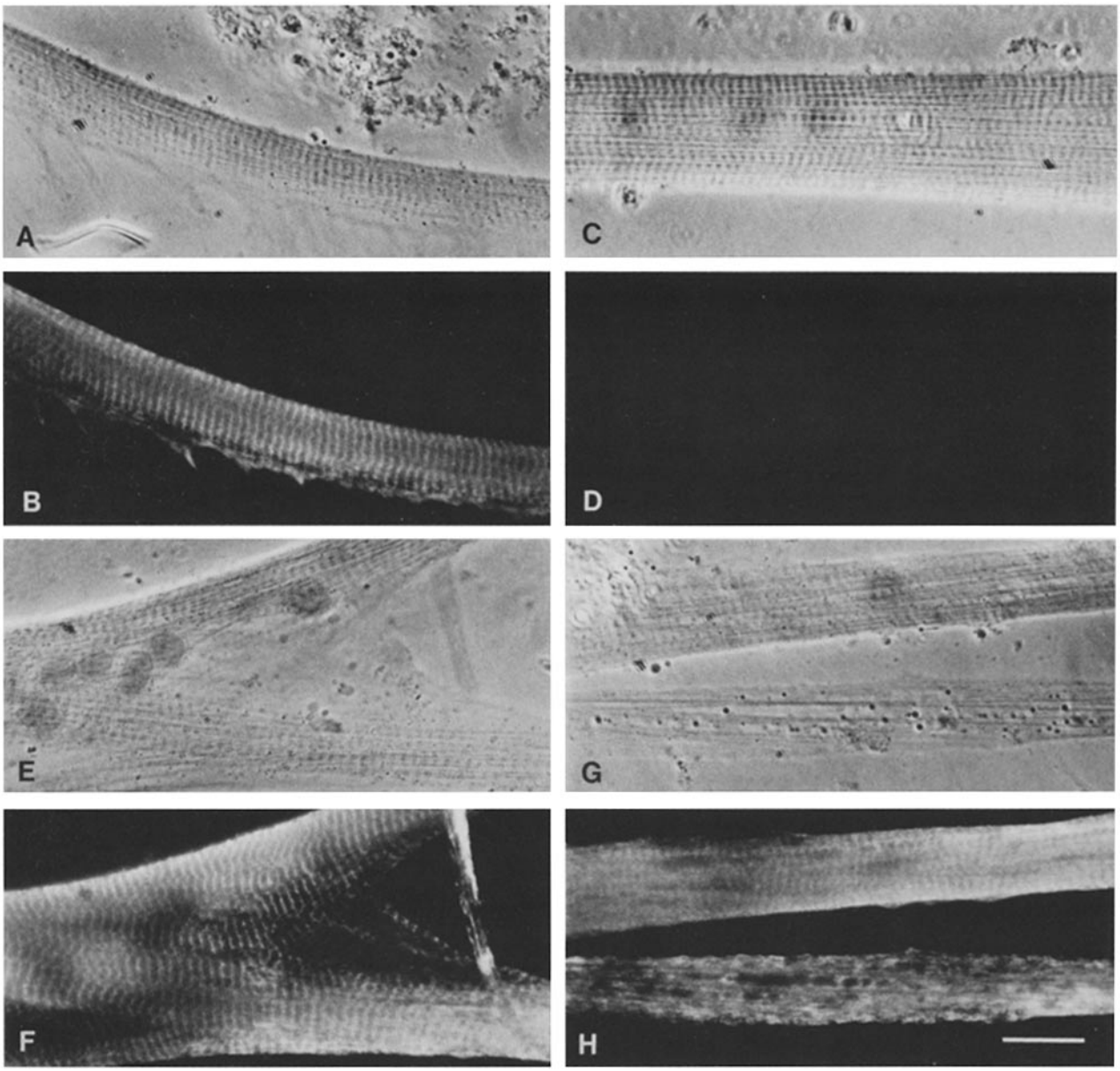

FIGURE 10 Immunofluorescence using antisera preadsorbed with purified antigens. 10-d myotubes were labeled with anti-paranemin that had been preadsorbed with no protein (control) (*B*), or with purified paranemin (*D*), or with purified desmin (*F*), or with purified vimentin (*H*). Phase-contrast micrographs (*A*, *C*, *E*, and *G*) and fluorescence micrographs (*B*, *D*, *F*, and *H*). Bar, 20 μm.

contain either vimentin (nonmuscle cells) or vimentin, desmin and synemin (myogenic cells), paranemin is not necessarily expressed in conjunction with or in response to certain other intermediate-filament proteins. Its absence from adult skeletal muscle and its gradual reduction during development suggest further that its function may be regulatory and that it is required for the subsequent ordered association of the other three intermediate-filament proteins with the Z disk but is reduced in quantity (or removed) in the ensuing days of muscle maturation. It should be emphasized, however, that evidence on the absence of paranemin from some nonmuscle cells and from adult muscle cells based on immunological criteria should be considered tentative since the protein may be present but may not contain the antigenic determinant(s) detected by the antibodies used.

The results presented here and previously (7, 10–11) suggest that the composition of muscle intermediate filaments is developmentally regulated and changes in response to the different cytoplasmic associations of their two major subunit proteins, desmin and vimentin, and that associated proteins such as paranemin and synemin may regulate changes in their structure in response to their different cytoplasmic associations.

We thank B. L. Granger, R. H. Gomer, J. Gelles, and Dr. E. Repasky for their help in various aspects of this work and Drs. E. Repasky, B. Granger, D. Gard, I. Sandoval, and C. Colaco for their many valuable comments on the manuscript. We also thank Ilga Lielausis for expert technical assistance.

This work was supported by grants from the National Institutes of Health (NIH) (PHS-GM 06965), the Muscular Dystrophy Association of America, the National Science Foundation, and a Biomedical Research Support Grant from U. S. Public Health Service. J. Breckler was supported by a postdoctoral fellowship from the American Heart Association Greater Los Angeles Affiliate. E. Lazarides is a recipient of a NIH Research Career Development Award.

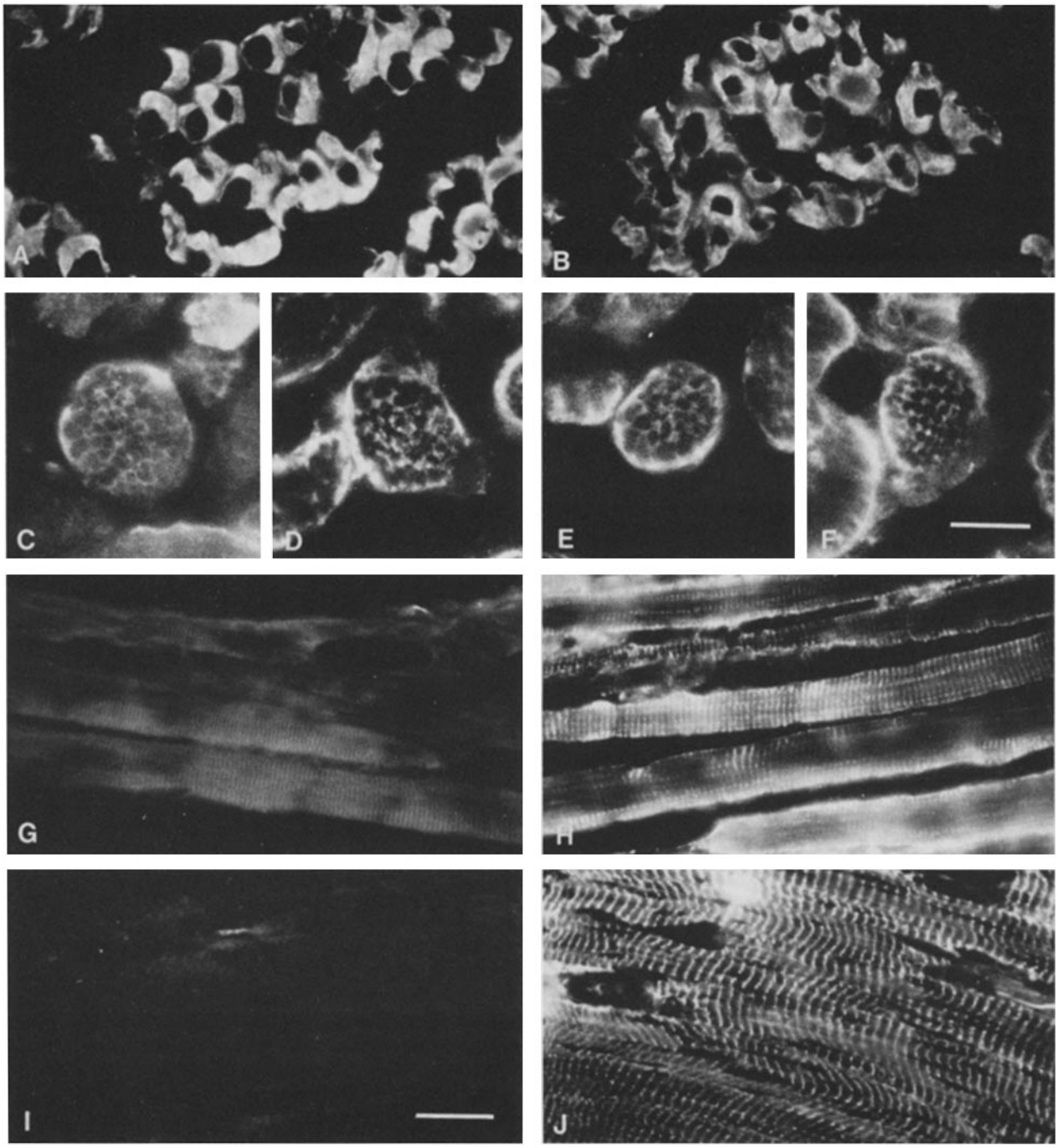

FIGURE 11 Immunofluorescence on frozen sections of skeletal muscle at various stages of differentiation. (*A* and *B*) Cross-sections of 15-d-old chick embryo thigh muscle. (*C–F*) Cross-sections of 19-d-old chick embryo thigh muscle. (*G* and *H*) Longitudinal sections of thigh muscle from a chick 7 d after hatching. (*I* and *J*) Longitudinal sections of thigh muscle from a fully grown chicken. The sections were reacted with anti-paranemin in *A*, *C*, *D*, *G*, and *I* with anti-desmin in *B*, *E*, *F*, *H*, and *J*. The sections in *D* and *F* were incubated for 45 min at room temperature in a buffer containing 0.6 M KI, 0.5% Triton X-100, 0.02 M sodium thiosulfate, 0.02 M Tris HCl pH 7.8, before immunofluorescnece. (*A*, *B*, and *G–J*) Bar, 20 μm. (*C–F*) Bar, 10 μm.

Received for publication 15 June 1981, and in revised form 21 September 1981.

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