

Distributions of Vimentin and Desmin in Developing Chick Myotubes In Vivo. I. Immunofluorescence Study

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ABSTRACT Antibodies against chicken erythrocyte vimentin and gizzard desmin were affinity purified and then cross-absorbed with the heterologous antigen. They were used to study the *in vivo* distributions of these proteins in developing and mature myotubes by immunofluorescence microscopy of 0.5–2- μ m frozen sections of iliopsoas muscle in 7–21-day chick embryos, neonatal and 1-d postnatal chicks, and adult chickens. The distributions of vimentin and desmin were coincidental throughout the development of myotubes, but the concentration of vimentin was gradually reduced as the myotubes matured and became largely undetectable at the time of hatching. The process of confining these proteins to the level of Z line from the initial uniform distribution occurred subsequent to the process of bringing myofibrils into lateral registry: in-register lateral association of several myofibrils was occasionally seen as early as in 7–11-d embryos, whereas the cross-striated immunofluorescence pattern of desmin and vimentin was only vaguely discerned in myotubes of 17-d embryos, just 4 d before hatching. In some myotubes of 21-d embryos, myofibrils were in lateral registry as precisely as in adult myofibers but desmin was still widely distributed around Z line in an irregular manner. Nevertheless, in many other myotubes of prenatal or neonatal chicks, desmin became confined to the level of Z line in a manner similar to that seen in adult myofibers, thus essentially completing its redistribution to the confined state of adult myofibers in coincidence with the time of hatching. In extracts from iliopsoas and posterior latissimus dorsi muscles of adult chickens, we detected a hitherto unidentified protein that was very similar to vimentin in molecular weight but did not react with our antivimentin antibody. We discuss the possibility that this protein was confused with vimentin in the past.

The major subunit proteins of muscle-type (4, 21, 25) and fibroblast-type (4, 6, 7) intermediate filaments are now widely referred to as desmin (21) and vimentin (6), respectively. Bennett et al. (1), studying cultures of myogenic cells and their *in vitro* progression to myotubes, observed by immunofluorescence microscopy that both desmin and vimentin were distributed in the form of longitudinal filaments in immature myotubes, but as the myotubes matured, vimentin gradually disappeared, whereas the distribution of desmin changed to a cross-striated pattern after the emergence of striated myofibrils. In contrast, Granger and Lazarides (14) reported the two proteins to coexist at the periphery of Z disk¹ in isolated myofibrils of adult chicken skeletal muscle by immunofluorescence microscopy. Subsequently, Gard and

Lazarides (8) reported that, in developing myotubes in culture, the distributions of desmin and vimentin were indistinguishable from each other. More recently, however, Osborn et al. (23) reported that adult muscle fibers of several mammalian species were not decorated by vimentin antibody, thus supporting the observations of Bennett et al. (1).

Three questions may be asked in relation to these studies. The first: which of the two diametrically different proposals is correct? The second: what is the temporal relationship *in vivo* between the appearance of cross-striations in the myotubes and the confinement of desmin at the level of Z line? This question was studied *in vitro* by both Bennett et al. (1) and Gard and Lazarides (8). They were, however, unable to observe individual myofibrils in the whole-mount cultured cells and therefore to ask whether or not the distributions of desmin and vimentin were related to the cross-striations of individual myofibrils in the early stages of myogenesis. Su-

¹ *Abbreviations used in this paper:* ALD, anterior latissimus dorsi; IT, iliopsoas; PLD, posterior latissimus dorsi.

perpositions of structures within the thickness of the cultured cells would also have prevented them from clearly observing subtle variations of morphological and immunofluorescence patterns within the myotubes. The third question: how are these events related to the different stages of embryonic development? This physiologically important question could not be studied in past *in vitro* investigations.

To study these questions by immunocytochemical methods, critical evaluations of the antigenic specificity of antibodies to these proteins were essential. A certain degree of homology between desmin and vimentin was demonstrated in a peptide analysis by Gard et al. (7) and more recently by amino acid sequence analysis by Geisler and Weber (11, 12). In fact, a low degree of cross-reaction of antivimentin antibody with desmin was reported by Bennett et al. (1).

In the present series of studies, we used chicken gizzard as the source of desmin (21), as all of the three groups mentioned above did. Bennett et al. (1) and Osborn et al. (23) used fibroblasts or a fibroblast-derived cell line as the source of vimentin. It is generally agreed that fibroblasts do not contain desmin (20, 23) but this is not totally accepted (7, 18). On the other hand, Lazarides' group (8, 14) separated vimentin from desmin by SDS PAGE of extracts of skeletal muscle of 14–16-d chick embryos, excised the vimentin band, and used it as immunogen. Because the molecular weights of desmin and vimentin are quite close, however, the possibility exists that the desmin and vimentin bands might overlap to some degree in the SDS polyacrylamide gel. To exclude any possibility of contamination with desmin, therefore, we used chicken erythrocytes as the source of vimentin (15). We cross-absorbed the antidesmin and antivimentin antibodies with vimentin and desmin, respectively, and used them to study the distributions of these proteins in semithin and ultrathin frozen sections of mildly fixed leg muscles of 7–21-d chick embryos, neonatal and 1-d postnatal chicks, and adult chickens. To preserve the interrelationships among the cells in 7–11-d embryos, fixed muscle pieces were embedded in 5% polyacrylamide gel before freeze sectioning.

In this paper, we report the results of the immunofluorescence observations in semithin frozen sections. We found that throughout the development of myotubes, the distributions of vimentin and desmin were coincidental, but the concentration of vimentin relative to that of desmin was gradually reduced as the myotubes matured and became largely undetectable at the time of hatching. Most myofibrils in the myotubes of 7–11-d embryos were still individually separated, and there was no evidence that the distributions of desmin and vimentin were related to cross-striations of individual myofibrils. In-register lateral association of several myofibrils was occasionally seen even in the myotubes of 7–11-d embryos. Cross-striations of the whole myotube became recognizable in many myotubes of 17-d embryos. In such myotubes, the distribution of desmin also showed a pattern of cross-striation but the desmin bands were still much wider than Z lines. The distribution of vimentin also showed a similar pattern, although its concentration in some myotubes was quite low at this stage. Confinement of desmin to the level of the Z line was seen in many but not all myotubes of prenatal 21-d embryos. In a subsequent paper (K. T. Tokuyasu, P. A. Maher, and S. J. Singer; manuscript in preparation), these immunolabeling experiments have been carried to the electron microscopic level of resolution.

MATERIALS AND METHODS

Immunochemical Reagents

ANTIGEN PREPARATION: Vimentin was prepared from chicken erythrocytes by a modification of the method of Granger et al. (15). Erythrocyte ghosts prepared as described (15) were opened by a combination of sonication and passage through a 23-gauge needle. The nuclei were pelleted at 200 *g* for 30 min and the vesicles of enucleated erythrocyte ghosts were collected from the supernatant at 30,000 *g* for 30 min. Crude vimentin was extracted from these vesicles as described previously (15) and isolated by preparative SDS PAGE. The vimentin band was cut out from the gel, electrophoretically eluted into 0.01 M NH_4HCO_3 , 0.1% SDS, 5% β -mercaptoethanol, lyophilized, redissolved in, and dialyzed against 50 mM NH_4HCO_3 , pH 9.0, 0.05% SDS, 1 mM EDTA. Desmin was purified from chicken gizzard smooth muscle by a modification of the method of Hubbard and Lazarides (16) as described (10).

ANTIBODY PREPARATION: Antibodies to desmin were induced in rabbits by lymph node injection of 100 μg of antigen in complete Freund's adjuvant followed by two similar intradermal injections at 2-wk intervals. Antibodies to vimentin were elicited in guinea pigs by intradermal and intramuscular injection of 50 μg of antigen in complete Freund's adjuvant followed by one intradermal injection 2 wk later. Affinity purification of antidesmin and antivimentin antibodies was carried out by using the antigens immobilized on glutaraldehyde-activated Ultrogel AcA 22 (LKB Instruments, Inc., Rockville, MD) (26) and Affi-Gel 10 (Bio-Rad Laboratories, Richmond, CA) (17), respectively. These antibodies were then passed over the heterologous immunoadsorbent one or two times to eliminate any cross-reactivity. The preparation and affinity purification of rhodamine-labeled goat anti-rabbit IgG or anti-guinea pig IgG were as described (9).

TISSUE HOMOGENATE PREPARATION: Fresh skeletal muscle tissue was carefully excised, cleaned of connective tissue, rinsed well in 1 mM NaHCO_3 , pH 8.2, 1 mM phenylmethylsulfonyl fluoride, and homogenized in 10 vol of the same buffer for 30 s at high speed in a Waring blender. The homogenates were filtered through several layers of cheese cloth and centrifuged at 3,000 *g* for 20 min. The pellet was resuspended in 1–5 ml of homogenization buffer and stored in small aliquots at -70°C . The protein concentration of each homogenate was determined by the method of Lowry et al. (22), using bovine serum albumin as the standard.

SDS PAGE: SDS PAGE were run on separating gels containing 7.5, 9, or 10% acrylamide, using a modification of the method of Laemmli (19). Gels were either stained with Coomassie Blue or blotted as described below. Gels for Coomassie Blue staining had 30 μg of total protein per well, whereas gels for immunoblotting had 100 μg of total protein per well. Affinity-purified antibodies were used at 1 $\mu\text{g}/\text{ml}$ to stain the blots. To achieve a higher resolution of the polypeptide bands, we lyophilized 1-mg aliquots of the homogenates, dissolved them in 30 μl of 10% SDS, boiled them for 1 min, diluted them 10-fold with 10 M urea, 2.0% Nonidet NP-40, and 0.5% β -mercaptoethanol, and electrophoresed 10- μl aliquots as described above. For the purpose of comparison, a few gels were prepared as described by Hubbard and Lazarides (16).

IMMUNOBLOTTING: SDS PAGE were blotted onto nitrocellulose and the transfers were stained with amido black as described by Towbin et al. (27). They were destained and then blocked for 2 h at 22°C with 3% bovine serum albumin in Tris-buffered saline, rinsed, and incubated with the antibody in 3% bovine serum albumin/TBS for 4 h at 22°C . Excess antibody was washed out and the transfers were incubated overnight at 22°C with ^{125}I -protein A (2×10^4 cpm/ml) in 3% bovine serum albumin/Tris-buffered saline. Excess reagent was washed out and the transfers were dried and autoradiographed on Kodak X-Omat film (Eastman Kodak Co., Rochester, NY) with an intensifying screen for 24–48 h.

PEPTIDE MAPPING: Two-dimensional mapping of tryptic peptides from proteins separated by SDS PAGE was performed as described (32). The desired Coomassie Blue bands were cut out of the gel, washed with 10% methanol and iodinated in siliconized glass tubes using chloramine T. After tryptic digestion, two-dimensional peptide mapping was carried out on 10×10 cm cellulose-coated glass thin-layer chromatography plates (EM Labs, Elmsford, NY). The electrophoresis buffer for the first dimension was 3:1:16 acetic acid/formic acid/water, and chromatography buffer for the second dimension was 32.5:25:5:20 butanol/pyridine/acetic acid/water with 7% 2,5 diphenylloxazole. Autoradiography was done on Kodak BB-5 x-ray film (Eastman Kodak Co.) with DuPont Cronex Lightening Plus intensifying screens at -70°C for 24 h.

Specimens and Immunofluorescence Microscopy

FIXATION: In 7–11-d embryos, iliopsoas muscle was initially fixed by immersing the entire leg in a 3% paraformaldehyde solution in 0.1 M

phosphate buffer, pH 7.4, for 10–20 min at room temperature. The muscle was then cut off from the leg and fixed further for 1 h in the same fixative. It was subsequently embedded in 5% polyacrylamide gel with the presence of 1% paraformaldehyde as follows (30). Muscle pieces were left in a 5% aqueous solution of acrylamide/bis-acrylamide (37.5:1) containing the initiator, ammonium persulfate (3.7 $\mu\text{g}/\text{ml}$), and 2% formaldehyde, for 1 h in the refrigerator. Subsequently, the catalyst, tetramethylethylenediamine (0.3 $\mu\text{l}/\text{ml}$), was added and the mixture was polymerized overnight in the refrigerator. This measure was taken to preserve the relationship of loosely distributed cells at this stage. The muscle of 17-d embryos contracted when fixed with the same fixative. To prevent contraction, the entire leg was initially fixed while the knee was kept bent. After 10–20 min, the muscle was removed from the leg, dissected to thin strips and fixed further for 1 h. The muscle of prenatal, neonatal, and 1-d postnatal chicks was fixed in the same manner but during the initial fixation, several longitudinal slits were cut into the muscle to accelerate the penetration of the fixative. The muscle of adult chickens was first cut to thin longitudinal strips while the knee was bent. The strips were stretched, tied to wooden sticks, and fixed for 1 h. Sometimes, the muscle of prenatal, neonatal, or 1-d postnatal chicks was also fixed in this manner. Adult chicken gizzard

and intestine were dissected to 0.5–1-mm cubes and fixed in the same fixative for 1 h at room temperature. Chicken erythrocyte ghost cells were attached to glass slides by applying a droplet of a concentrated suspension of the cells, fixed in the same fixative for 10 min, treated with a 0.1% Triton X-100 solution for 3 min, and then used for immunofluorescence studies.

SEMITHIN FROZEN SECTIONS: The method of cutting the fixed tissue pieces of semithin frozen sections was essentially the same as that of cryoultramicrotomy (28, 29). Briefly, short cylindrical or cubic pieces of the fixed tissues, ~0.5 mm in width and length, were infused with 2.3 M sucrose in the phosphate buffer, frozen by immersion in liquid nitrogen, and sectioned at -60°C to the thickness of 0.5–2 μm . After retrieving the sections from the cryochamber and thawing, the sections were transferred onto premarked areas of glass slides by touching the bottom face of the sucrose droplet to the slide surface, on which several circles of ~3 mm in diameter had been scratched. The scratch marks were quite useful for finding the sections as well as serving as a guide for focusing in the microscope.

IMMUNOSTAINING: Primary antibodies and rhodamine-conjugated secondary antibodies were used at the concentration of 10 $\mu\text{g}/\text{ml}$. Immunostaining

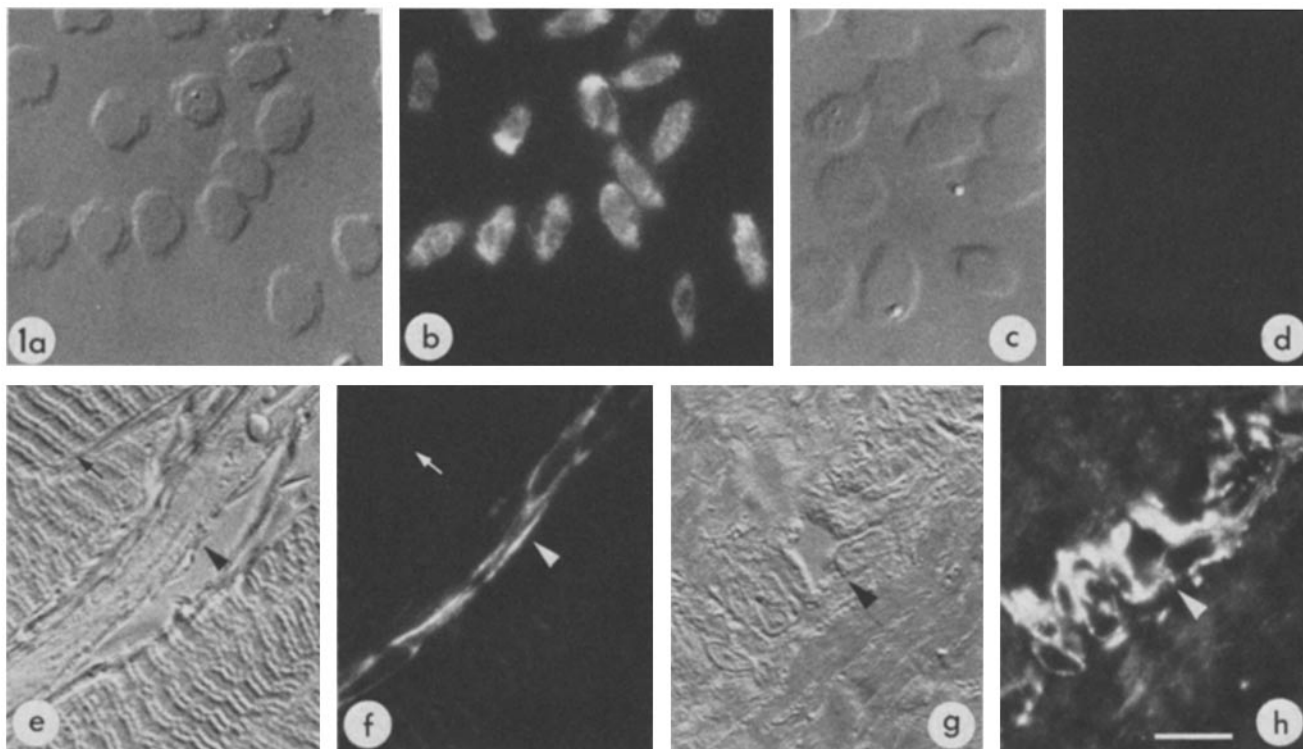


FIGURE 1 Pairs of Nomarski and immunofluorescence (left and right in each pair, respectively) micrographs. Chicken erythrocyte ghosts are strongly stained with guinea pig antivimentin antibody (a and b), but no detectable staining is found in a control that was stained with normal guinea pig IgG (c and d). In frozen sections of chicken iliopsoas muscle (e and f) and gizzard (g and h), the endothelium of blood vessels is strongly stained with this antivimentin antibody before its cross-absorption on a desmin column (arrowheads). A much lesser but positive staining is also seen to occur at the level of the Z line in skeletal muscle (arrows in e and f) and in smooth muscle cells (g and h). Bar, 10 μm . $\times 1,000$.

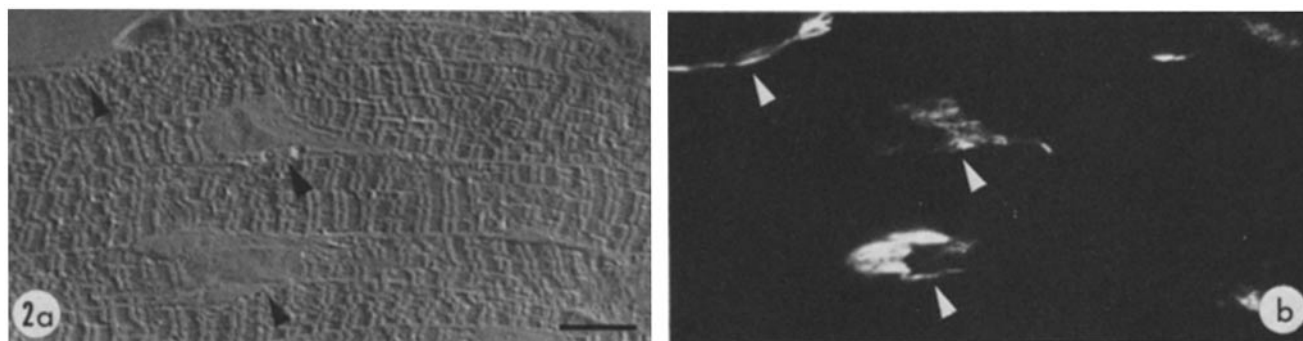


FIGURE 2 Frozen section of skeletal muscle of 1-d postnatal chick, immunostained with antivimentin antibody cross-absorbed with desmin (a, Nomarski, and b, immunofluorescence micrographs). A heavy staining is seen at capillary walls (arrowheads) but no detectable staining is found in muscle fibers. Bar, 10 μm . $\times 1,000$.

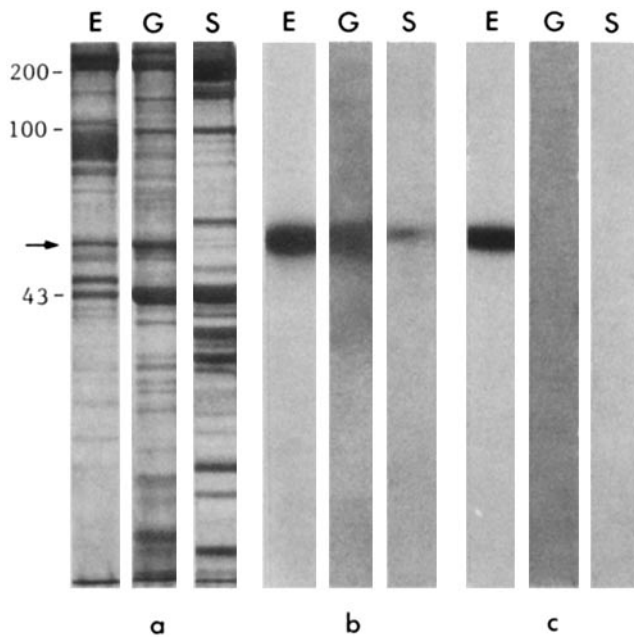


FIGURE 3 SDS PAGE of extracts of erythrocyte ghosts (E), gizzard (G), and leg skeletal (S) muscle (iliotibialis) stained with Coomassie Blue is shown in a. The position of vimentin-desmin is indicated with an arrow, in relation to the positions of myosin (200,000 mol wt), α -actinin (100,000 mol wt) and actin (43,000 mol wt). When blots of the SDS PAGE components on nitrocellulose paper are stained with antivimentin antibodies with or without cross absorption with desmin, followed by 125 I-protein A, autoradiographs show that the preabsorption antibody stains all three preparations at the level of 50,000–60,000 mol wt (b). However, the postabsorption antibody stains only the erythrocyte ghost preparation (c).

taining was carried out essentially in the same manner as for ultrathin frozen sections (29, 31). Briefly, the sections were first conditioned with glycine-containing phosphate-buffered saline to quench remaining aldehyde groups. Immunostaining with the primary antibody was done by covering the sections with the antibody solution for 10–30 min. Between these steps, the surface of the glass slide excluding the section-mounted area was dried by wiping it with tissue paper. The aim of drying was to reduce the volume of the antibody solution needed to cover the sections. After immunostaining, the slide surface was washed several times with phosphate-buffered saline either by immersing in phosphate-buffered saline in a Coplin jar or flooding the surface with phosphate-buffered saline. The surface excluding the section area was dried as described above and the solution of the rhodamine-conjugated secondary antibody was applied onto the section area. Immunostaining was continued for 10–30 min, and the slide face excluding the section area was dried. Throughout these steps, care was taken not to dry the sections at any time. After applying a 90% glycerol droplet onto the section area, it was covered with a coverslip. Immunostaining of erythrocyte ghosts was done in a similar manner. Nomarski and immunofluorescence observations were made in a Zeiss photomicroscope III (Carl Zeiss, Inc., New York).

RESULTS

Specificity of Antibodies

Chicken erythrocyte ghosts were strongly immunostained with the affinity-purified antivimentin antibody (Fig. 1 a and b; compare with a control in c and d that was stained with normal guinea pig IgG in place of the antibody). In adult skeletal muscle, a faint immunostaining with the antivimentin antibody was seen to occur in the muscle fibers at the level of the Z line (example at arrows in Fig. 1 e and f), in addition to a strong staining of the capillary wall (arrowheads). In gizzard, too, a faint staining of smooth muscle cells and a strong staining of the capillary wall was seen with the same antibody (Fig. 1 g and h). In that very little vimentin is present

in gizzard smooth muscle cells (20), these observations suggested that the antivimentin antibody contained a fraction that was reactive with desmin. In fact, affinity-purified antivimentin antibody derived from several individual guinea pigs showed a considerable variation in the staining intensity of the desmin-containing structures, with some of them showing a high staining intensity nearly equal to that observed with the antidesmin antibody. However, when these antibodies were cross-absorbed with desmin by passing them over a desmin-Ultrogel AcA 22 column and then used for immunostaining, no staining was detectable at the level of the Z line, whereas a strong staining was seen at capillary endothelial

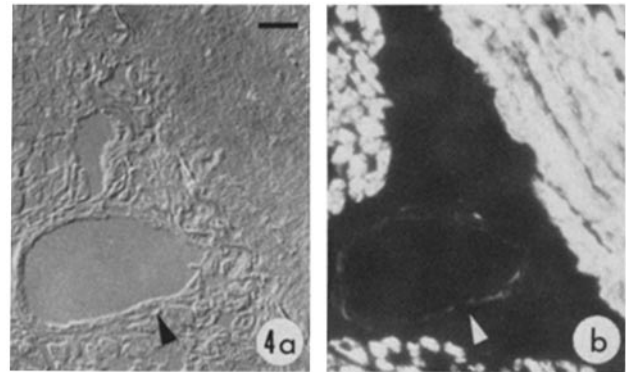


FIGURE 4 Nomarski (a) and immunofluorescence (b) micrographs of a frozen section of chicken intestinal smooth muscle. (b) The affinity-purified antidesmin antibody was used before its cross-absorption on a vimentin column. In addition to a strong staining of muscle cells, the blood vessel endothelium (arrowhead) is positively stained. Bar, 10 μ m. \times 500.

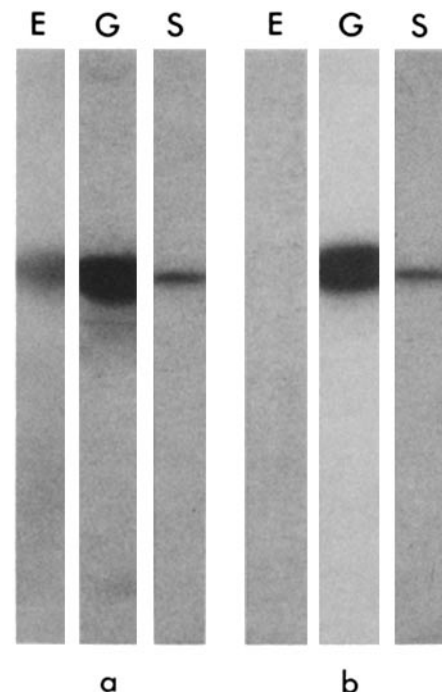


FIGURE 5 Autoradiographs of immunoblots of SDS PAGE components of extracts of erythrocyte ghosts (E), gizzard (G), and skeletal (S) muscle, immunostained with antidesmin antibodies with or without cross-absorption with vimentin, followed by 125 I-protein A. The preabsorption antibody stains the preparations not only of gizzard and skeletal muscle but also that of erythrocyte ghosts at the level of 50,000–60,000 mol wt (a), whereas the postabsorption antibody does not stain the preparation of erythrocyte ghosts (b).

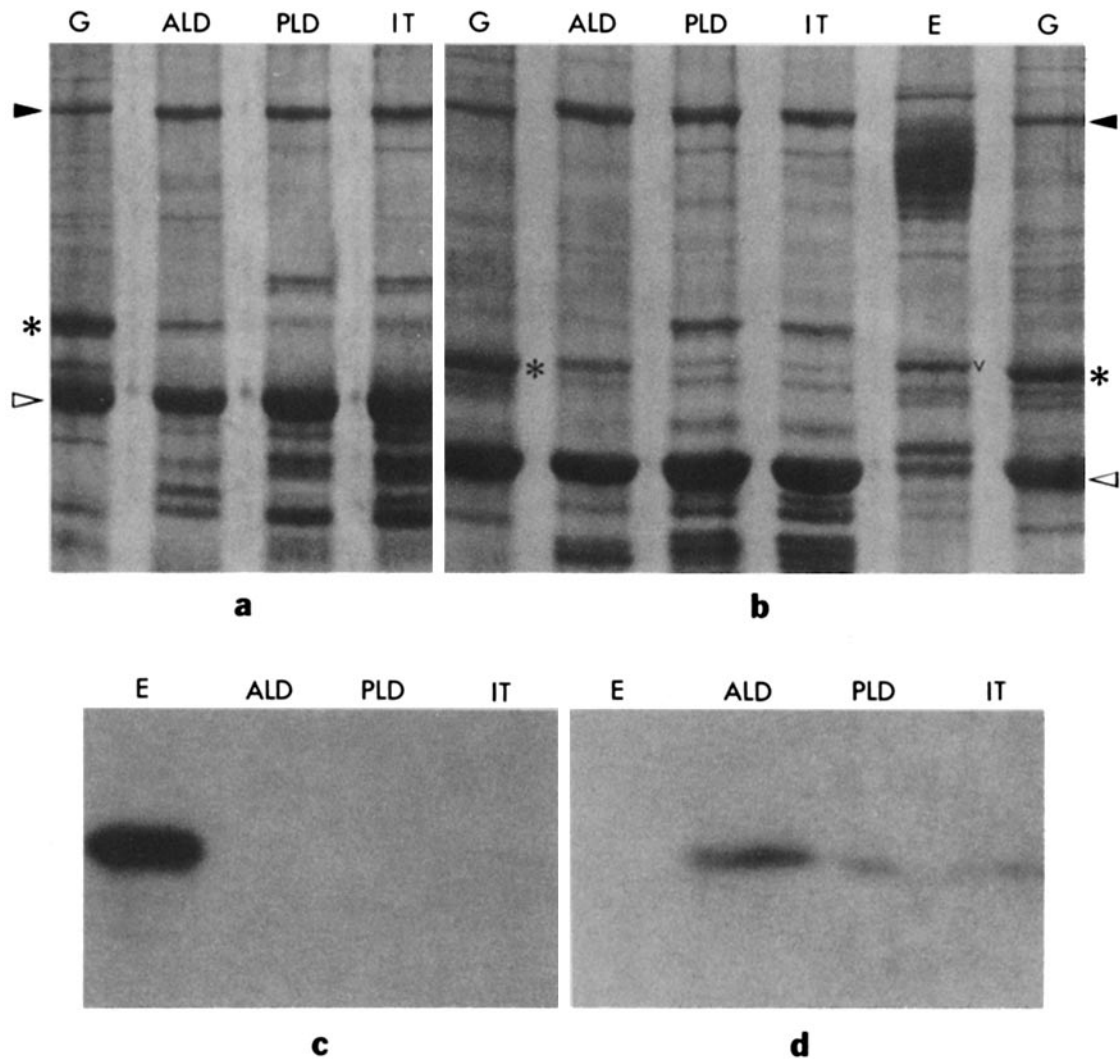


FIGURE 6 In one SDS PAGE system (a) as used by Hubbard and Lazarides (16) extracts of *ALD*, *PLD*, and *IT* muscles show a single band at the position corresponding to desmin (desmin band of gizzard [G] shown with an asterisk). In another system (b, in which the distance between the α -actinin [dark arrowhead] and actin [white arrowhead] bands is expanded by ~20% over that in the first system), the single band of *PLD* and *IT* in the first system is separated to two sub-bands, above and below the desmin band of gizzard (asterisks), while that of *ALD* apparently remains single. The top sub-band is found at a position similar to that of the vimentin band of erythrocyte (*E* lane, indicated with a small v). The density ratio of the top sub-band to the bottom one is about 1:1 in *PLD* and 1:2 in *IT*. When blots of these sub-bands are stained with vimentin-specific antibody, followed by ^{125}I -protein A, autoradiography reveals that neither of these sub-bands is positively stained, whereas the vimentin band of erythrocyte is strongly stained (c). When stained with desmin-specific antibody, the single band of *ALD* and the lower sub-bands of *PLD* and *IT* are seen to be positively stained, whereas the vimentin band of erythrocyte is not (d).

cells, in the skeletal muscle of either adult chicken or 1-d postnatal chick (Fig. 2a and b). These observations were further confirmed by preparing extracts from erythrocyte ghosts, gizzard, and skeletal muscle, separating the polypeptide components by SDS PAGE (Fig. 3a), transferring them to nitrocellulose paper, and staining the transfers with the antibodies before and after cross-absorption. The preabsorption antibody stained all three preparations at the level of 50,000–60,000 mol wt (Fig. 3b), whereas the postabsorption antibody stained only the erythrocyte ghost preparation (Fig. 3c).

The above observations suggested that the antidesmin antibody might in turn contain a fraction that was reactive with vimentin. In fact, staining with this antibody produced a low but positive staining of erythrocyte ghosts (not shown) and the endothelium of blood vessels (Fig. 4). When extracts of erythrocyte ghosts, gizzard, and skeletal muscle were separated

into their polypeptide components by SDS PAGE, transferred to nitrocellulose paper, and stained with the antidesmin antibody, all three preparations were positively stained at the level of 50,000–60,000 mol wt (Fig. 5a). However, when the antibody was cross-absorbed with vimentin and then used for staining, the erythrocyte ghost preparation was not stained, whereas gizzard and skeletal muscle preparations were positively stained as with the preabsorption antibody (Fig. 5b). Furthermore, the fraction of the antibody that was absorbed on the vimentin column and then eluted, strongly stained erythrocyte ghosts and capillary endothelial cells (not shown).

These studies clearly indicated that vimentin and desmin could be unequivocally identified only by using the cross-absorbed antibodies. In all of the immunofluorescence experiments described subsequently (Figs. 8–12) only the affinity-purified and then cross-absorbed antibodies were employed for immunostaining.

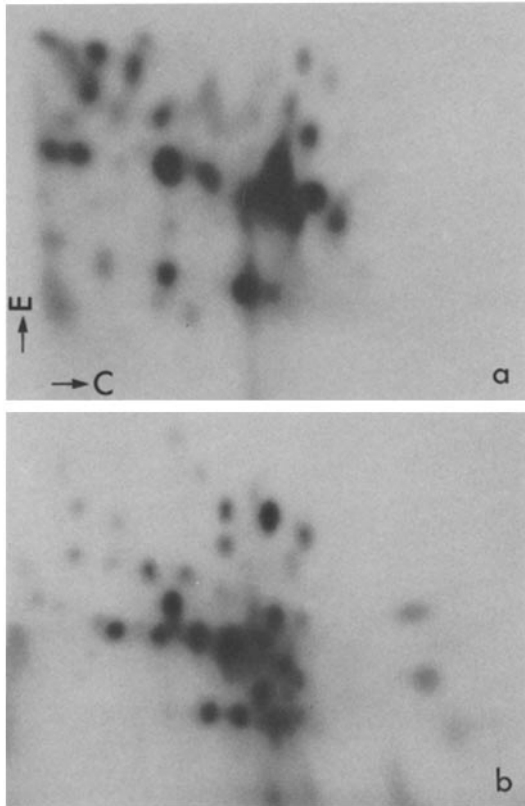


FIGURE 7 Two-dimensional peptide maps of trypsin digests of vimentin (a) and a vimentinlike protein (b) which is the upper one of the two sub-bands at the position of desmin-vimentin in SDS PAGE of PLD extract (see *b* of Fig. 6 for the sub-bands). They are obviously quite dissimilar. Directions of electrophoresis (E) and chromatography (C) are indicated in a. Origin is in the lower left-hand corner.

Granger and Lazarides (14) have demonstrated the presence of a component at the position corresponding to vimentin in two-dimensional gels of an extract from adult chicken pectoralis muscle. Lazarides and co-workers (20) have found the molar ratio of this component (which they considered to be vimentin) to desmin in anterior latissimus dorsi (ALD), posterior latissimus dorsi (PLD), and iliobtibialis (IT) muscles of adult chicken to be 1:7, 1:1, and 1:2, respectively. When we employed the one-dimensional gel that they used (16), we observed a single band at the position of desmin-vimentin in all muscles (Fig. 6*a*). In our gel, on the other hand, the band was separated to two sub-bands in PLD and IT, although it appeared to remain a single band in ALD (Fig. 6*b*). In fact, the molar ratios of the upper (heavier molecular weight) sub-band to the lower one in PLD and IT were approximately equal to the previously reported values of the vimentin-to-desmin ratio in the two muscles (Fig. 6*b*). When these sub-bands were transferred to nitrocellulose paper and stained with the cross-absorbed antibodies, neither one was positively stained with the vimentin-specific antibody (Fig. 6*c*), whereas only the lower sub-band was positively stained with the desmin-specific antibody (Fig. 6*d*). Furthermore, we found that the protein of the upper sub-band was quite different from vimentin in peptide maps (Fig. 7). Thus, this protein, which is very similar to vimentin in molecular weight and which may be the same protein that was identified as vimentin by

Lazarides et al. (20), was immunochemically and structurally distinguishable from vimentin.

Immunofluorescence Microscopy

In 11-d chick embryos, vimentin was found in myotubes as well as in myoblasts and fibroblasts that were present in the intermyotube spaces (Fig. 8*a* and *b*). Desmin, on the other hand, was localized only in myotubes (Fig. 8*c* and *d*). The presence of both vimentin and desmin in the developing leg skeletal muscle of this embryonic stage was further confirmed by immunostaining of polypeptide components in extracts of muscle with the vimentin- and desmin-specific antibodies (not shown).

Many myofibrils in the myotubes of this stage were still individually separated and clearly seen to be cross-striated in 1- μ m-thick frozen sections of the muscle (Fig. 8*a* and *c*). This was expected from the previous morphological observations by electron microscopy of embryonic skeletal muscle (5) but had not been observed in earlier *in vitro* light microscopic studies of whole-mount cultured cells (1, 8). It therefore became necessary to distinguish the striations of individual myofibrils from the wider striations that became recognizable when a large number of myofibrils were laterally associated in register. In the present study, the term "striations" is used to describe the striations of individual myofibrils and "cross-striations" to indicate the striations that were seen across a number of myofibrils. In previous studies of whole mount cultured cells, the term "cross-striations" was similarly used in the latter sense.

In the 11-d embryonic myotubes, both vimentin and desmin were present throughout the cytoplasm, including the area where myofibrils were absent (asterisks in Fig. 8*a-d*). There was no evidence that the distributions of these proteins varied at specific levels of sarcomeres along the individual myofibrils (Fig. 8*a-d*). In some areas in the myotubes, on the other hand, several myofibrils were seen to be already laterally associated with their sarcomeres in register. Such areas were generally lower in immunofluorescence intensity than in myofibril-free areas (Figs. 9*a-d*). In addition, cross-striated patterns of immunofluorescence were often recognized both for vimentin (Fig. 9*a* and *b*) and desmin (Fig. 9*c* and *d*). In fact, such patterns were occasionally recognized even in the myotubes of 7-d embryos (not shown). When distinct landmarks such as the edges of the nuclei were used as the points of reference, however, it was found that it was not the bright striations, but rather the dark ones, that corresponded to Z lines (compare Fig. 9*a* with *b*, and *c* with *d*). This feature, which was opposite to that of adult skeletal muscle fibers, was found to be due not to an altered distribution of desmin or vimentin but to the presence of sarcoplasmic reticulum at the level of the Z line and the consequent exclusion of the structures containing these proteins from the vicinity of the Z line, as will be described in the second paper of this series (K. T. Tokuyasu, P. A. Maher, and S. J. Singer; manuscript in preparation).

In 17-d embryos, the myotubes were packed with myofibrils nearly as fully as in the adult and the pattern of cross-striations began to emerge in many areas (Figs. 10*a-f*). Bright immunofluorescence striations were now found to correspond to the levels of Z lines both in desmin- (compare Fig. 10*e* with *f*) and vimentin-immunostaining (not shown), although the bright striations were still much wider at this stage than those in the adult skeletal muscle (compare Fig. 10*f* with Fig. 12*f*).

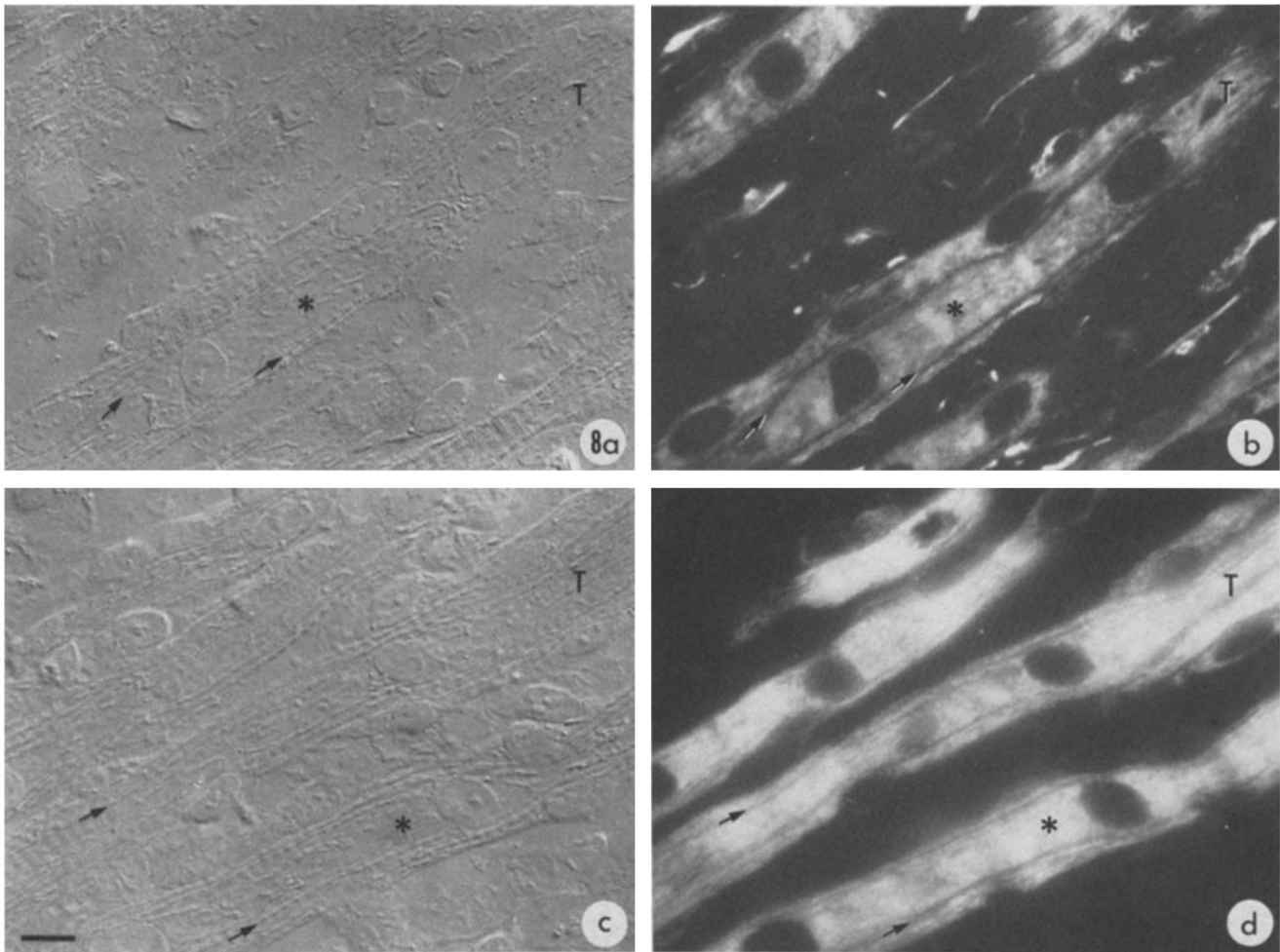


FIGURE 8 Frozen sections of myotubes of 11-d embryos, immunostained for vimentin (a, Nomarski, and b, immunofluorescence micrographs) and for desmin (c, Nomarski, and d, immunofluorescence micrographs). Myotubes of extended morphology (T) show positive immunostaining both for vimentin (b) and desmin (d). Most of myofibrils at this stage are individually separated and the areas of separation show a negative density in the background of immunofluorescence (arrows in a and b, and in c and d). Mononucleated cells in the spaces between myotubes are seen to be positively stained for vimentin (a and b) but not for desmin (c and d). Striations of individual myofibrils are clearly visible in Nomarski micrographs (a and c). No modification of vimentin-desmin-distribution is recognized in relation to the striations (b and d). Immunofluorescence is observed even in the areas where myofibrils are absent (asterisks). Bar, 10 μ m. \times 700.

The concentration of desmin was relatively uniform in different myotubes (Fig. 10d) but that of vimentin became variable among different myotubes (Fig. 10b) and appeared to be reduced as the formation of the cross-striations progressed (compare myotube A with B-D in Fig. 10a and b).

In prenatal 21-d embryos or neonatal chicks, cross-striations became as precisely defined as in the adult myofibers (compare Figs. 11a and c and 12a and c with Fig. 12e). A faint staining of vimentin was still recognized in some myotubes at the level of the Z line (arrowheads in myotube 1 in Fig. 11a and b), whereas in adjacent myotubes, staining was totally undetectable (compare myotube 2 with the background [asterisk] in Fig. 11a and b). In most parts of the muscle, however, no staining was seen over wide areas (Fig. 11c and d).

In some myotubes at this stage, desmin immunofluorescence was still widely distributed around the Z line (Fig. 12a and b), whereas in many others, it became confined at the level of the Z line (Fig. 12c and d) in a manner similar to that seen in the myofibers of adult muscle (Fig. 12e and f).

The wide immunofluorescence bands were seen to consist of filaments of various lengths spanning across or beyond the bands (Fig. 12b), whereas the narrow bands were found to be composed of dots (Fig. 12d and f). The filamentous form of immunofluorescence was not caused by overstretching of myotubes, as evidenced by the fact that the sarcomere length in the myotubes showing the filament-shaped fluorescence (double-headed arrow in Fig. 12a) was not longer than the length in those showing the dot-shape fluorescence (double-headed arrows in Fig. 12c and e).

DISCUSSION

Pruss et al. (24) and Gown and Vogel (13) found, by using monoclonal antibodies, that different classes of intermediate filaments share common antigenic determinants. Geisler and Weber (11, 12), in turn, analyzed the amino acid sequences of the different classes of filaments and found considerable homologies among them. In particular, they found an 80–90% homology between desmin and vimentin in the central

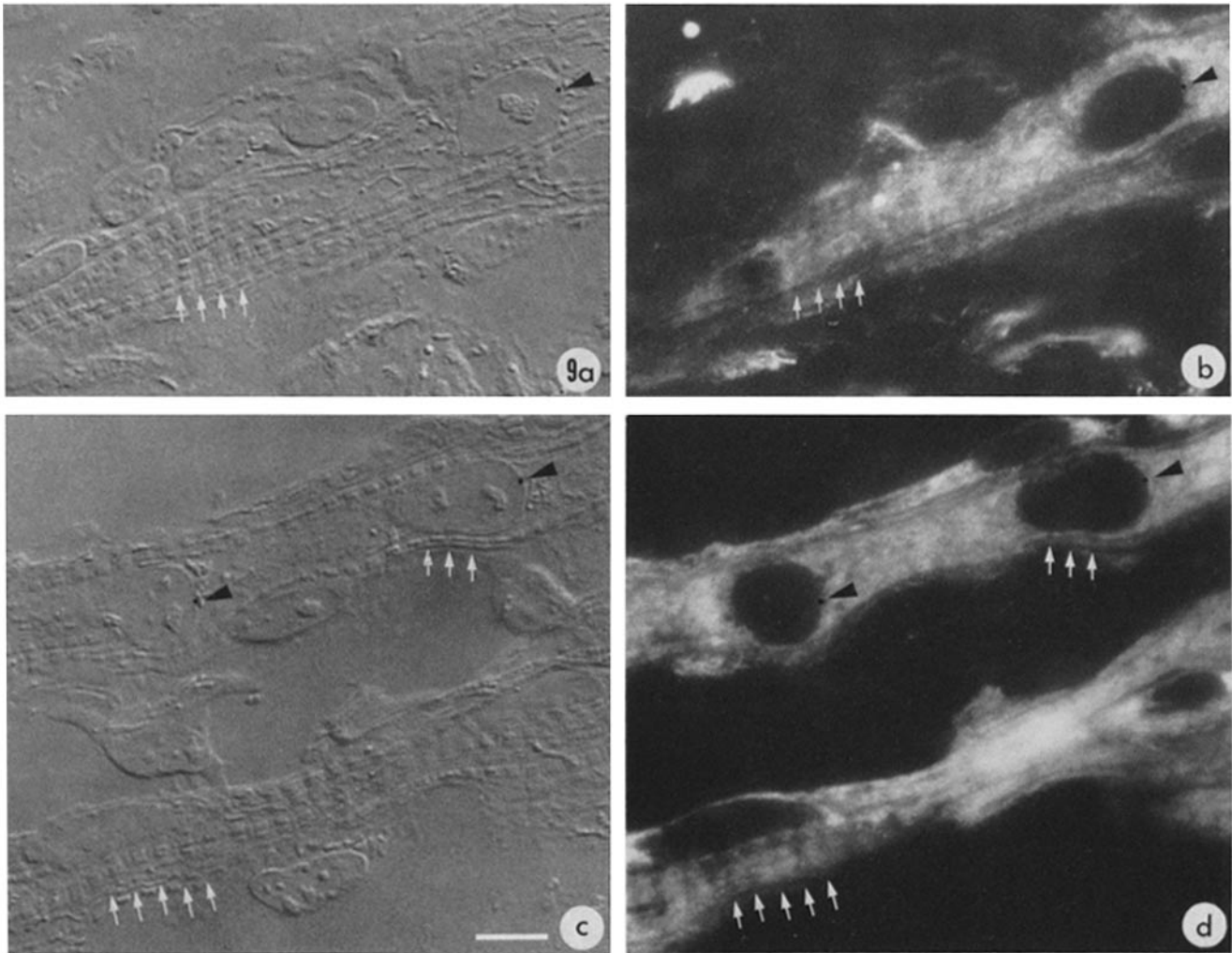


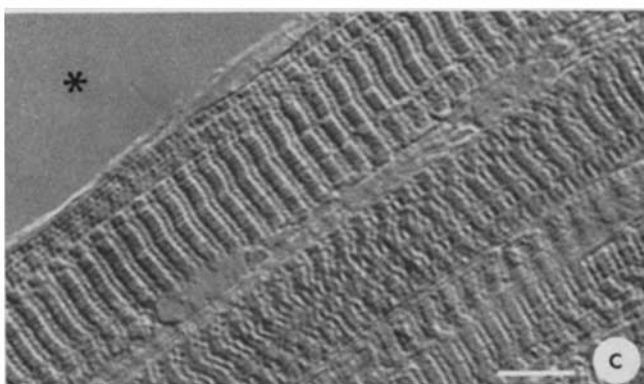
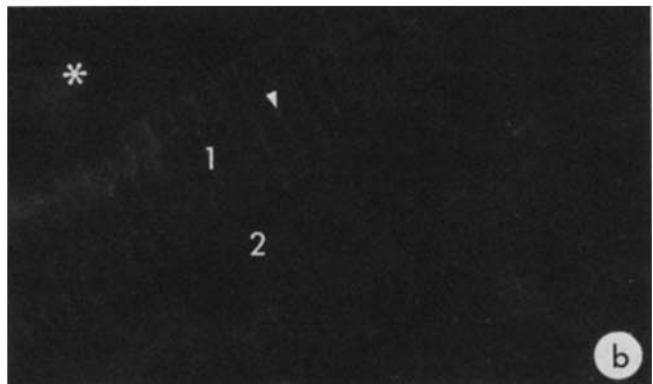
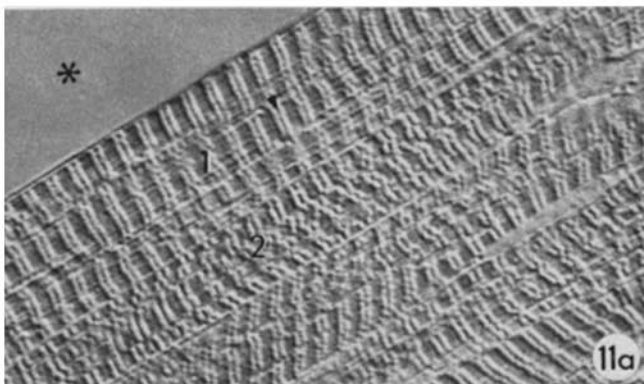
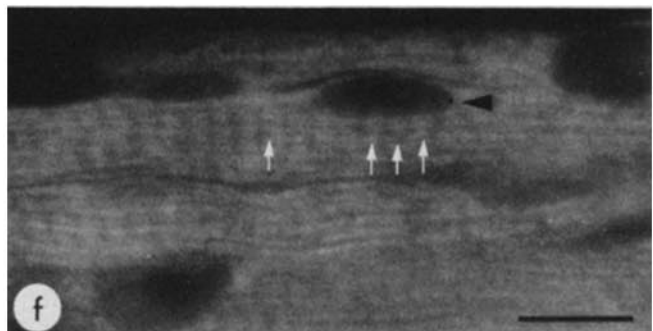
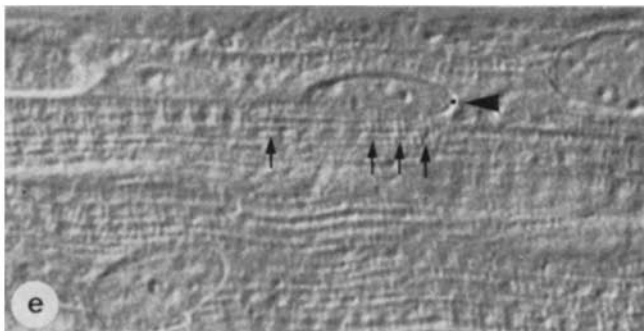
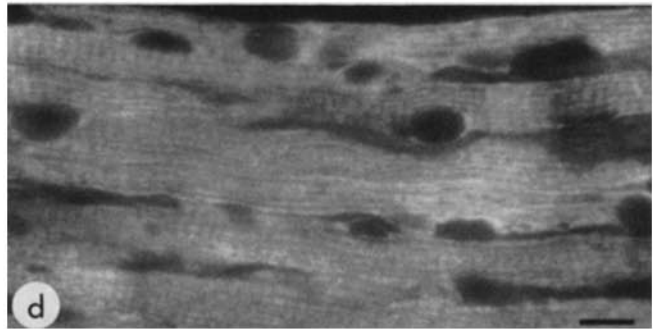
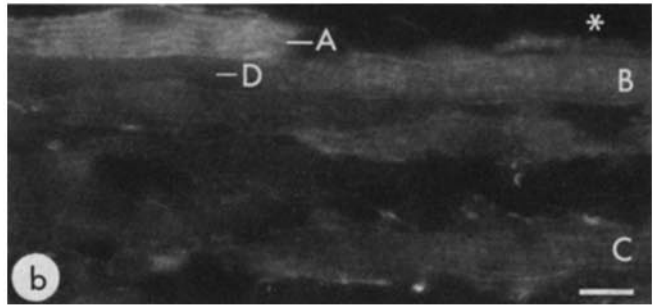
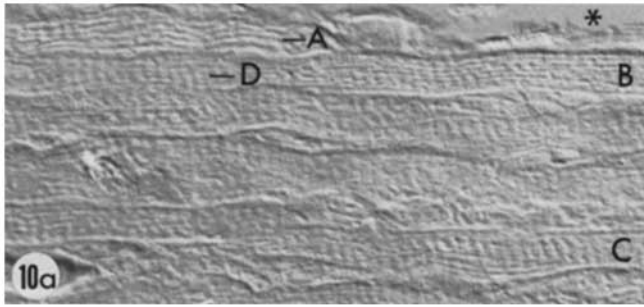
FIGURE 9 Frozen sections of myotubes of 11-d embryos, immunostained for vimentin (a, Nomarski, and b, immunofluorescence micrographs) and for desmin (c, Nomarski, and d, immunofluorescence micrographs). The areas where several myofibrils are in lateral registry (in-register Z lines shown with arrows in a and c) are lower in the overall intensity of immunofluorescence than in the rest of the myotube. In addition, dark cross bands are seen in the background of immunofluorescence (arrows in b and d). When the edges of the nuclei (dark dots indicated with arrowheads in a-d) are used as the points of reference, the dark striations are found to correspond to the in-register Z lines (compare a and b, and c and d). Bar, 10 μm . $\times 1,000$.

helical region of the two molecules. It is therefore to be expected that polyclonal antibodies to the two proteins may cross-react to varying extents. It is also well known that different animals within a species vary in immune response to the same antigen. We, in fact, found that the antibodies

against vimentin derived from different guinea pigs showed an extreme variation in their cross-reactivity with desmin, from a virtually null level to a high level nearly equal to the reactivity of the anti-desmin antibody itself, both in immunofluorescence and immunoelectrophoretic analyses. Our

FIGURE 10 Myotubes of 17 d embryos are full of myofibrils (compare myotubes in a and c with those of 11-d embryos in Fig. 8a and c). (a) Nomarski micrograph, the degree of cross-striation formation in a myotube as a whole is seen to be less advanced in myotube A than in myotubes B-D. (b) immunofluorescence micrograph of the same field, the degree of vimentin immunostaining is found to be greater in A than in B-D (compare with the background indicated with an asterisk). (c and d) Nomarski and immunofluorescence micrographs of a section immunostained for desmin, the intensity of immunofluorescence is approximately uniform in all myotubes. (e and f) Enlarged portions of corresponding areas of c and d, respectively. The measurement of distance from the reference point, edge of a nucleus (dark dot indicated with an arrowhead in each micrograph), indicates that the positions of in-register Z lines (arrows in e) correspond to the mid-levels of the bright areas of the fluorescent striations (white arrows in f). Bars, 10 μm . (a-d) $\times 700$. (e and f) $\times 1,500$.

FIGURE 11 Myotubes of prenatal embryos immunostained for vimentin. In one field of a frozen section (a and b), staining is seen to be slight but positive at the level of the Z line in a myotube (arrowhead in myotube 1) but totally negative in an adjacent myotube (myotube 2; compare it with the background indicated with an asterisk). In another field of the same section (c and d), staining is completely undetectable (compare the outside (asterisk) and the inside of the muscle). Bar, 10 μm . $\times 1,000$.



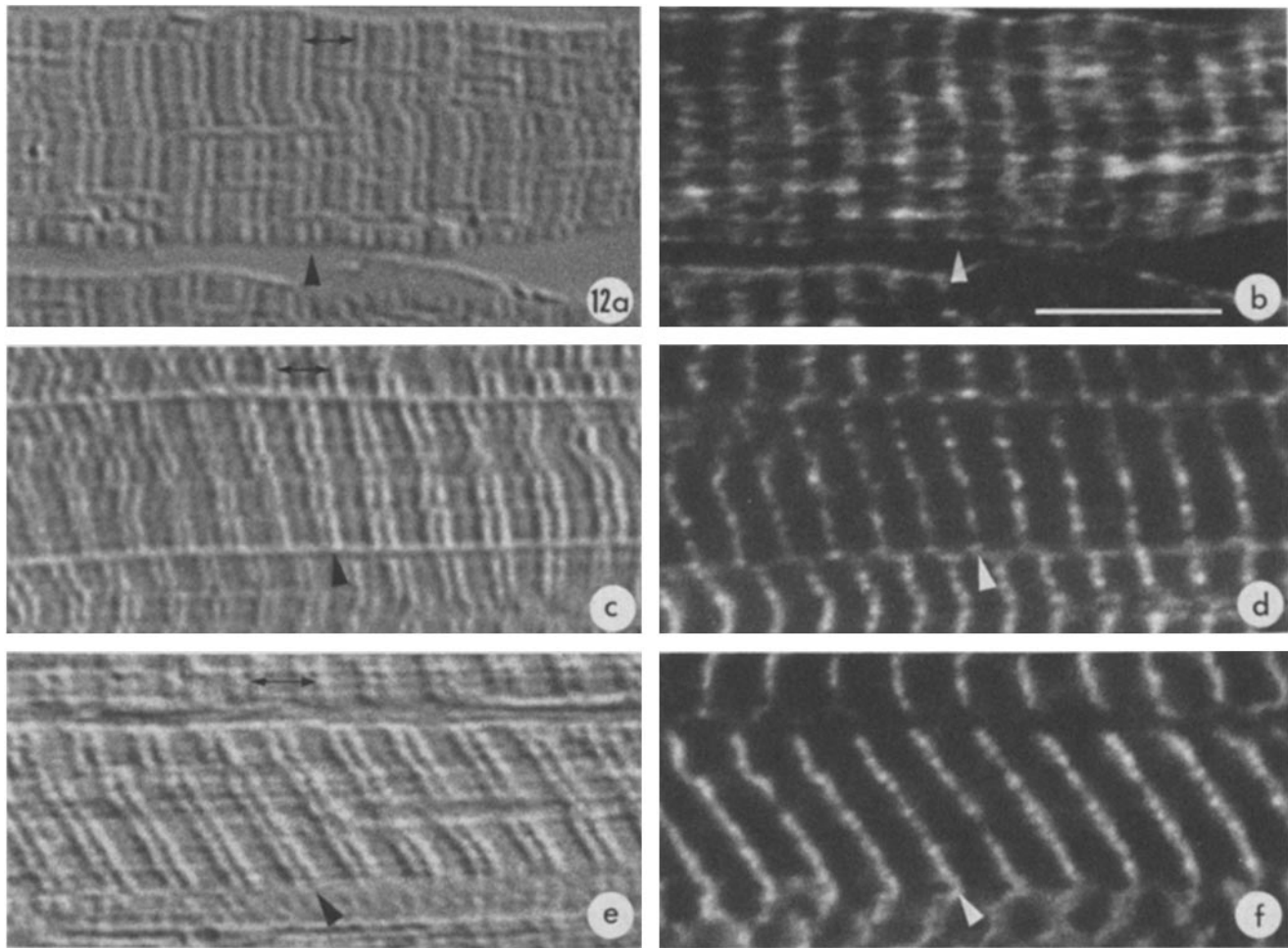


FIGURE 12 Myotubes of prenatal embryo (a and b), neonatal chick (c and d) and adult chicken (e and f), immunostained for desmin. In a, c, and e, Nomarski micrographs, Z lines are laterally in register in a similar manner in all specimens (compare, e.g., the Z line indicated with arrowheads), forming the cross-striated pattern. In b, however, the width of immunofluorescence bands at the levels of the Z lines is $\sim 1 \mu\text{m}$ or greater, much wider than that of the Z lines (compare the Z line and the corresponding fluorescence band indicated with arrowheads in a and b), and filamentous forms of fluorescence are seen to span the length of a few sarcomeres at many places. In d and f, immunofluorescence is confined at the level of the Z line (compare the Z line and the corresponding fluorescence pattern indicated with arrowheads in c and d, and in e and f). The myotubes in a are not stretched more than those in c and e (compare the widths of sarcomeres indicated with double-headed arrows in a, c, and e). Bar, $10 \mu\text{m}$. $\times 2,500$.

data obtained by using the cross-absorbed antibodies unequivocally supported the conclusion reached by Bennett et al. (1) as to the fate of vimentin found in immature myotubes: it disappeared during myogenesis and was not detected in mature myotubes.

Granger and Lazarides (14), on the other hand, presented a two-dimensional gel electropherogram of extracts of adult chicken skeletal muscle as evidence that vimentin existed in adult muscle. However, our one-dimensional gel electrophoresis data clearly indicated that there is a protein which is very similar to vimentin in molecular weight but does not react with our antibody directed to erythrocyte vimentin, and by peptide mapping is distinct from vimentin. These two sets of data ought to be reconciled. A possible explanation is that the vimentin preparation of Lazarides' group from muscle of 14–16-d embryos contained vimentin and another protein that resembles vimentin in both molecular weight and isoelectric point and exists in adult muscle at the level of Z line. Both immature myotubes and adult myofibers would then be positively stained by their antibody but only immature ones by our antibody. This hitherto unidentified protein seems to be

the same protein as detected in our one-dimensional gel electrophoresis but the proof that this is the case awaits further studies. This protein should be isolated and characterized and its distribution in developing myotubes studied independently from that of vimentin. There is a possibility that this protein is an isoform of vimentin which shows little or no cross-reaction with the vimentin of myoblasts or erythrocytes.

In 7–11-d embryos, the vimentin-specific antibody stained both myotubes and mononucleated cells in the intermyotube spaces (Fig. 8a and b). On the other hand, the desmin-specific antibody stained myotubes but not mononucleated cells which included fibroblasts and myoblasts (Fig. 8c and d). These findings were consistent with the notion that fibroblasts contain vimentin but not desmin (20, 23). On the other hand, Gard et al. (7) and Ip et al. (18) observed positive immunostaining of desmin in embryonic skeletal muscle fibroblasts and cardiac fibroblasts in primary culture, respectively. Whether the difference is due to the cross-reactivity of anti-desmin antibodies with vimentin or whether there are two populations of fibroblasts that differ in this respect needs to be clarified.

Adult skeletal muscle contracted when fixed under the present conditions and, to preserve the stretched state, it was necessary to take some measure to counteract the contraction. Many myofibrils of 11-d embryos did not contract in the same fixative (~3 μm of sarcomere length in Figs. 8 and 9), which seemed to indicate that they were not yet capable of contraction. In 17-d embryos, however, the same fixation did cause contraction. This stage was coincidental with the emergence of cross-striations, that is, the occurrence of in-register lateral association of myofibrils in many myotubes. Lateral registry of sarcomeres across the myotubes would be necessary to cause a uniform and efficient contraction of the myotube as a whole.

Concomitantly at this stage, both desmin and vimentin began to disappear from the level of the M line, thus initiating the process of confining these proteins to the level of the Z line. In fact, both Bennett et al. (1) and Gard and Lazarides (8) observed that with cultured myotubes the confinement of desmin (without [1] or together with [8] vimentin) took place after the formation of cross-striations. Nevertheless, Lazarides and coworkers (8, 20) proposed that desmin- and vimentin-containing intermediate filaments have an important role not only in the maintenance of the lateral organization of myofibrils in mature myotubes but also in the registration of myofibrils in developing myotubes. It is possible that the association of intermediate filaments with I bands occurs at the time of their dissociation from the level of M line and causes the lateral registry of I bands. If this were the case, however, the process of intermediate filament rearrangement should exhibit as much order as the process of cross-striation formation. Actually, at any given stage of development, the state of cross-striation was always much more highly developed than that of the arrangement of the structures containing desmin or vimentin. This was most clearly seen in prenatal embryos. In some myotubes at this stage cross-striations were as precisely defined as in adult myotubes (Fig. 12a) but filamentous structures containing desmin were still widely distributed around the Z line in an irregular manner (Fig. 12b). We also showed that the lateral registry of several myofibrils took place in 7–11-d embryos, that is, much earlier than hitherto observed in whole-mount cultured cells (1, 8). These observations suggest that in vivo the process of confining intermediate filaments to the Z line level does not precede but follows the process of lateral registry of myofibrils. Nevertheless, there remains a possibility that a small number of intermediate filaments undetectable by immunofluorescence measurements are associated with the Z lines throughout myogenesis and contribute to the registration of the myofibrils.

A cytoskeletal function of intermediate filaments has been amply demonstrated by Erickson and Thornell (2) in Purkinje fibers of cow heart that contained a large number of intermediate filaments: an extensive extraction of other organelles including myofilaments did not alter the shape of individual cells and the assemblies of cell bundles. One might therefore propose a similar role for intermediate filaments in developing myotubes. In immature myotubes, longitudinally arranged intermediate filaments might be required to maintain the cylindrical form of the myotubes and to allow emerging myofibrils to be aligned in parallel to themselves, that is, in the longitudinal direction. When myofibrils increase in number, fill the myotubes, and are laterally associated in register, they would gain sufficient rigidity to maintain the cellular

asymmetry by themselves. The initial scaffolding of intermediate filaments could then be rearranged to reinforce the lateral alignment of Z disks. It is of interest to note that, as judged from immunofluorescence intensities, the number of intermediate filaments needed for the maintenance of the organization of adult skeletal muscle fibers appears to be considerably smaller than that which we suggest is required for the maintenance of the asymmetry of immature myotubes.

We observed that when several myofibrils were brought into lateral registry in 7–11-d embryos, the concentrations of both desmin and vimentin were often lower at the level of the Z line than along the rest of the sarcomere (Fig. 9a–d). Ezerman and Ishikawa (3) reported that in several day-old cultures of chicken myotubes sarcoplasmic reticulum was associated with myofibrils at the level of the Z line. In our subsequent immunoelectron microscopic study, (K. T. Tokuyasu, P. A. Maher, and S. J. Singer; manuscript in preparation) we will show that such association does occur in vivo in myotubes of 11-d embryos, and that the exclusion of intermediate filaments from the vicinity of Z line by sarcoplasmic reticulum is the likely cause of the reduced concentration of desmin and vimentin at the Z line level.

On the basis of the above observations and considerations, we conclude the following: (a) during embryonic development in vivo, desmin and vimentin co-distribute in developing myotubes, but vimentin gradually disappears toward the threshold of hatching—this provides evidence for the physiological relevance of similar results previously obtained in vitro with cultures of myogenic cells (1); and (b) the process of lateral registry of myofibrils, which is recognized as early as in 7-d embryos, precedes by a substantial time the process of confining desmin and vimentin to the level of Z line, which is vaguely identifiable in 17-d embryos, i.e., only 4 d before hatching.

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