

Distributions of Vimentin and Desmin in Developing Chick Myotubes In Vivo. II. Immunoelectron Microscopic Study

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ABSTRACT The distribution of the intermediate filament proteins vimentin and desmin in developing and mature myotubes in vivo was studied by single and double immunoelectron microscopic labeling of ultrathin frozen sections of iliopsoas muscle in 7–21-d-old chick embryos, and neonatal and 1-d-old postnatal chicks. This work is an extension of our previous immunofluorescence studies of the same system (Tokuyasu, K. T., P. A. Maher and S. J. Singer, 1984, *J. Cell Biol.*, 98:1961–1972). In immature myotubes of 7–11-d embryos, significant labeling for desmin and vimentin was found only in intermediate filaments, and these proteins coexisted in the same individual filaments. Each of the two proteins was present in irregular clusters along the entire length of a filament. No exclusively vimentin- or desmin-containing filaments were observed at this stage.

In the early myotubes, the intermediate filaments were essentially all longitudinally oriented, even when they contained three times as much desmin as vimentin. No special relationship was recognized between the dispositions of the filaments and the organization of the myofibrils. Occasionally, several myofibrils were already aligned in lateral registry at this early stage, but labeling for desmin and vimentin was largely absent at the level of the Z bands. Instead, the Z bands appeared to be covered by elements of the sarcoplasmic reticulum. The confinement of intermediate filaments to the level of the Z bands occurred in the myotubes of later embryos after the extensive lateral registry of the Z bands. Thus, intermediate filaments are unlikely to play a primary role in producing the lateral registration of myofibrils during myogenesis, but may be important in determining the polarization of the early myotube and the alignment of its organelles.

Throughout the development of myotubes, desmin and vimentin remained in the form of intermediate filaments, although the number of filaments per unit volume of myotube appeared to be reduced as myofibrils increased in number in maturing myotubes. This observation indicated that the transverse orientation of intermediate filaments in mature myotubes does not result from the *de novo* polymerization of subunits from Z band to Z band, but a continuous shifting of the positions and directions of intact filaments.

The existence of a chemically and morphologically distinct class of intracellular filaments, the intermediate filaments, was first clearly demonstrated by Ishikawa et al. (14) in embryonic skeletal muscle. The intermediate filaments were found to be largely in a longitudinal orientation in the embryonic myotubes and were quite abundant. In adult skeletal muscle, however, intermediate filaments have been difficult to detect, although an early report (18) showed that morphologically characteristic intermediate filaments were present, but in a transverse orientation confined to the region between myofibrils at the level of the Z bands. Immunofluorescent

experiments showed that desmin was present at the periphery of Z bands in adult muscle (10, 32), but for lack of resolution, it could not be distinguished whether the desmin was present in typically elongated intermediate filaments or in some other state. Recent immunoelectron microscopic labeling experiments (28, 30) have, however, firmly established that desmin-containing characteristic intermediate filaments are indeed present, wrapped around the myofibrils at the level of the Z bands in the adult skeletal muscle. Two interesting questions then arise: How does the reorganization of intermediate filaments from the longitudinal to the transverse orientation

occur, and does this reorganization play a critical role in the process of lateral registry of the myofibrils as myotube development proceeds *in vivo*?

The problem is further complicated by the fact that this intramyotube redistribution of intermediate filaments is accompanied not only by a reduction in their total numbers but also by a change in their chemical composition. The protein subunit of the filaments of premitotic myoblasts is vimentin, whereas both vimentin and desmin are present in postmitotic myoblasts and developing myotubes in culture (1, 5, 12). The protein subunit of adult skeletal muscle is, on the other hand, desmin (1, 12, 16, 17, 31, 32), although Granger and Lazarides (11) claimed that vimentin is codistributed with desmin. This raises questions about how desmin-containing intermediate filaments arise during myotube development, whether by the exclusive polymerization of desmin subunits or by the copolymerization with a decreasing concentration of vimentin subunits.

In the preceding paper (32), we studied the iliobtibialis muscle of developing chick embryos *in vivo*, by immunofluorescent labeling for vimentin and desmin on semithin frozen sections of the muscle. To obtain antibodies that were monospecific for either vimentin or desmin, it was necessary to cross-absorb the affinity-purified antibodies on the heterologous antigen column. With these antibodies, we firmly established that by the time of hatching, when immunolabeling of the myotubes for desmin was intense, labeling for vimentin was no longer detectable. We also observed in developing myotubes of 11-d embryos that the distributions of desmin and vimentin showed no relationship with any specific level of the sarcomere (32). However, immunofluorescence studies

provided no definitive information about the directions of individual intermediate filaments, and there remained the possibility that the filaments were more laterally directed at the level of the Z bands than at other levels of the sarcomere.

In this paper, we report studies of the same system at the much higher resolution of immunoelectron microscopy. Single and double indirect immunolabeling of desmin and vimentin were carried out on ultrathin frozen sections of iliobtibialis muscle of 7–21-d chick embryos, and neonatal and 1-d postnatal chicks. The results (*a*) show that vimentin and desmin are localized only in intermediate filaments and coexist in the same individual filaments during myotube development, (*b*) provide no indication that intermediate filaments play a direct role in the lateral registry of the myofibrils, and (*c*) indicate that the transverse orientation of intermediate filaments in mature myotubes results from a continuous shifting of the positions and directions of intact filaments. Concerning point *b*, our results *in vivo* confirm and extend those obtained with myogenic cells cultured *in vitro*, as observed in immunofluorescence studies by Holtzer and co-workers (1, 12).

MATERIALS AND METHODS

Immunochemical Reagents: Guinea pig antibodies against chicken erythrocyte vimentin, and rabbit antibodies against chicken gizzard desmin were affinity-purified, cross-absorbed with the heterologous antigen as described in the first paper of this series (32), and used as the primary antibodies. Affinity-purified and cross-absorbed goat antibodies to guinea pig immunoglobulin G (IgG) and to rabbit IgG were conjugated to ferritin and Imposil (iron-dextran) as described previously (6), and used as the secondary antibodies.

Specimen Preparation and Immunoelectron Microscopy: Iliobtibialis muscle of 7–21-d chick embryos, and neonatal and 1-d

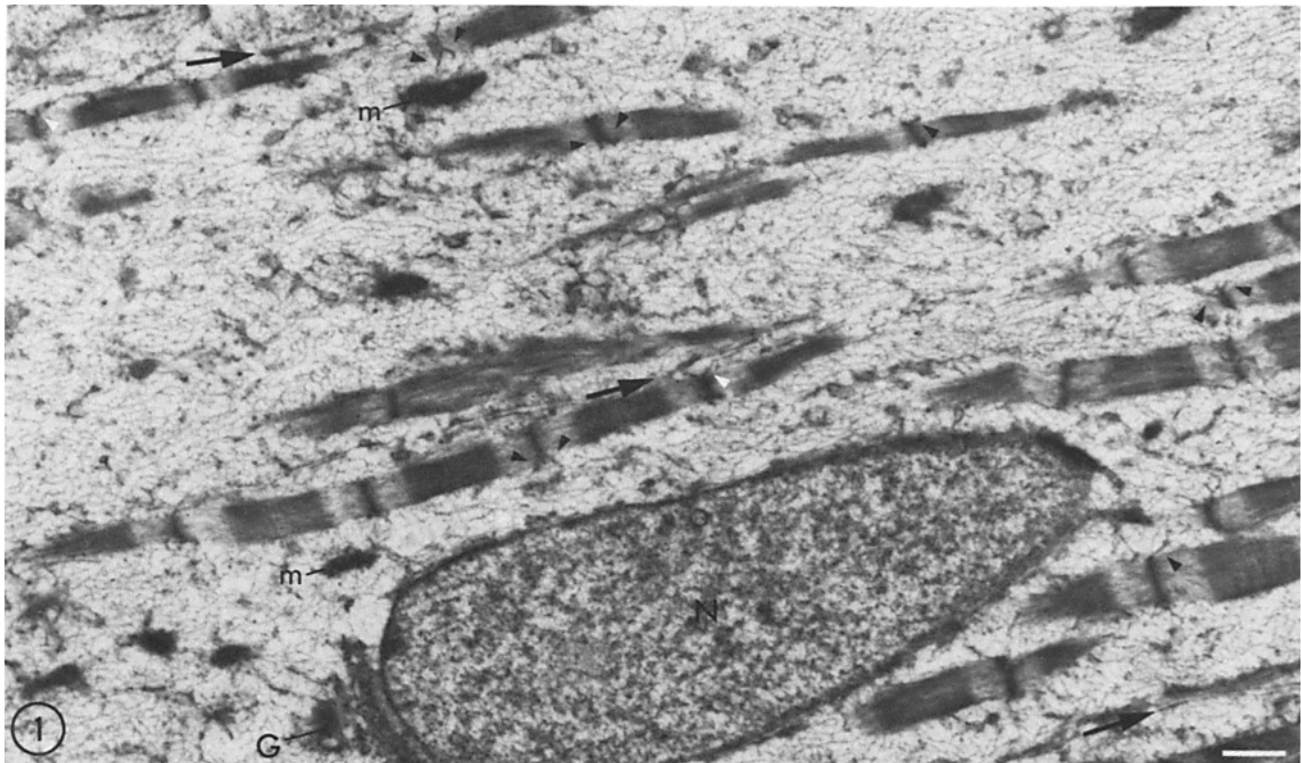
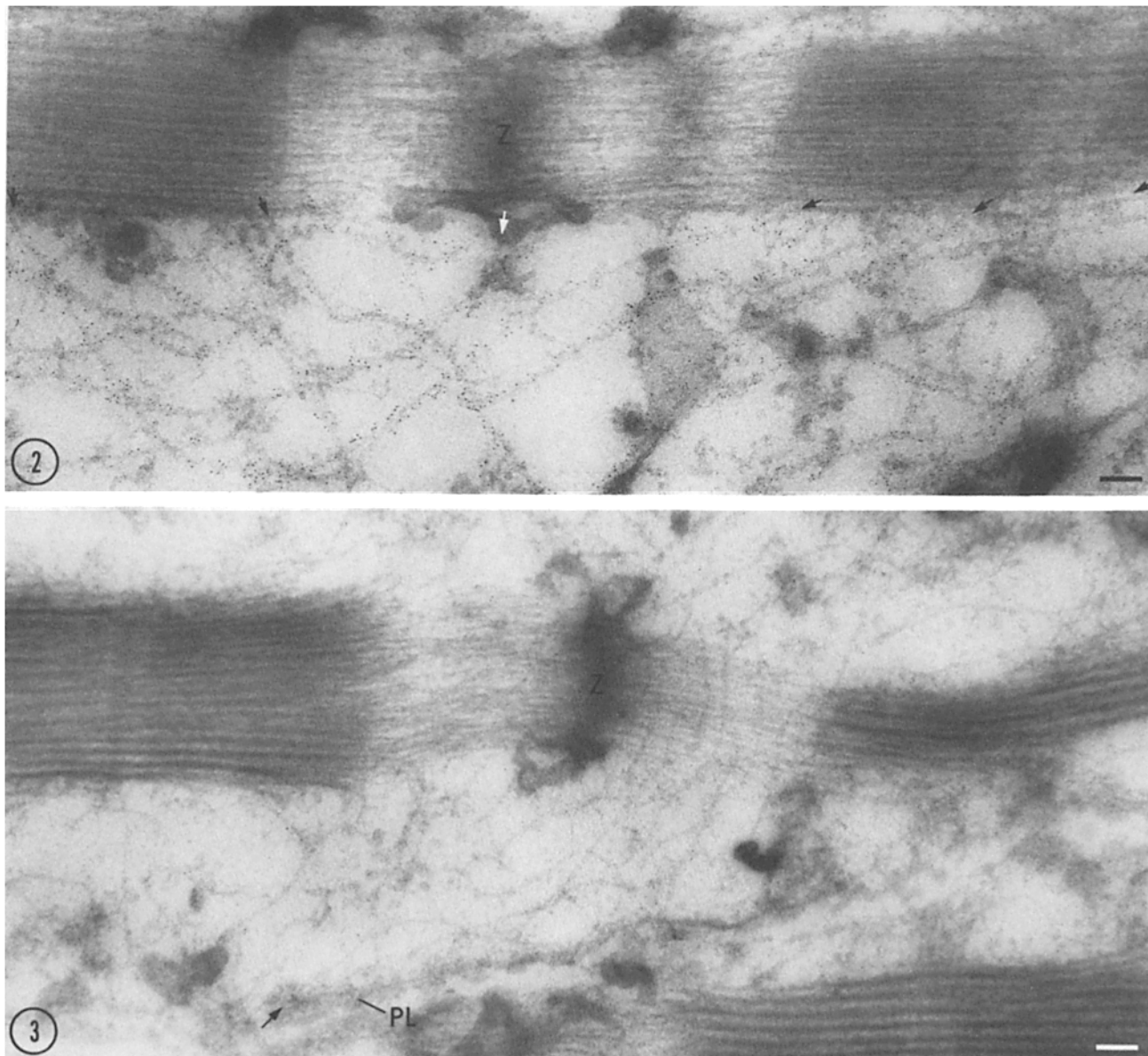


FIGURE 1 Myotubes of an 11-d embryo, boundaries of which are indicated with large arrows. Individually separated myofibrils, mitochondria (*m*), and a nucleus (*N*) capped by a Golgi apparatus (*G*) are all longitudinally aligned. Intermediate filaments, thickened by indirect ferritin immunolabeling of desmin, show a wavy feature but run longitudinally as a whole and fill the interorganellar spaces uniformly. Short segments of sarcoplasmic reticulum are associated with the edges of the Z bands (dark arrowheads). Some Z bands appear to be attached to the plasmalemma (white arrowheads). Bar, 1 μm . $\times 8,300$.

postnatal chicks were fixed in the manner as described previously (32), except that a mixture of 3% paraformaldehyde and 1% glutaraldehyde was used as the fixative instead of a 3% paraformaldehyde solution. Fixed muscles of 7–17-d embryos were embedded in 5% polyacrylamide gel, as previously described (32), whereas those of 21-d embryos and the young chicks were not. Cryoultramicrotomy, indirect single- and double-immunolabeling of ultrathin frozen sections with ferritin- and Imposil-conjugates, and adsorption staining of the immunostained sections were carried out by published methods (6, 23, 26, 29). Muscles of 17-d embryos were also observed by the conventional method. They were first treated in a Triton X-100-containing chemical skinning solution (34) for 60 min at room temperature, to reduce the background density of the cytosol so that intermediate filaments are recognized more clearly than in the intact state. The muscles were then fixed in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h at room temperature, postfixed in 1% OsO₄ in the same buffer for 1 h, and after dehydration in ethanol, embedded in Epon 812.

RESULTS

The overall morphological aspects of myotubes of 11-d embryos in ultrathin frozen sections were similar to those of 12-d embryos previously observed in conventional plastic-embedded sections (4). Myofibrils were longitudinally aligned and still sparsely distributed in the cytoplasm. Most of them were individually separated and not yet in lateral registry (Fig. 1). Sarcomeres in many myotubes, however, already showed the characteristic adult morphology and when fixed, often remained in a stretched state of ~3 μm long (Fig. 1). Nuclei and mitochondria were also elongated in the longitudinal direction, and one end of the nucleus was often capped by a



FIGURES 2 and 3 Fig. 2: A myotube of an 11-d embryo. Indirect ferritin immunolabeling of desmin is seen to have increased the apparent diameter of intermediate filaments to 20–40 nm. Some intermediate filaments are found in close contact with the myofibril at random positions along the sarcomere (black arrows), as well as with a segment of sarcoplasmic reticulum (white arrow) on the edge of the Z band (Z). Bar, 0.1 μm. × 60,000. Fig. 3: A myotube of an 11-d embryo. A control for Fig. 2, with the anti-desmin antibody replaced with normal rabbit IgG. Little ferritin labeling is seen. Several intermediate filaments cross over an I band area (right side of the Z band [Z]). Intermediate filaments appear to be associated with the plasmalemma (PL) at many sites such as the one indicated with a small black arrow. Bar, 0.1 μm. × 60,000.

Golgi apparatus (Fig. 1).

The cytoplasm of myotubes of this stage was full of intermediate filaments. They were extensively and specifically labeled when the frozen sections were immunostained for desmin (Fig 2). This labeling often increased the diameter of the filaments to 20–40 nm due to the added thickness of the antibodies and ferritin particles (Fig. 2; compare with a control in Fig. 3), and made the filaments easily recognizable even at low magnification (Fig. 1). The intermediate filaments were also immunolabeled for vimentin (Fig 4). This labeling occurred in patches intermittently along all the filaments, with the overall density of labeling lower than that of desmin on similar specimens (compare Fig. 4 with Fig. 2). The suggestion from these single labeling experiments, therefore, was that both desmin and vimentin were present in the same intermediate filaments with desmin at a higher density than vimentin at this stage. To establish this point firmly, we did double immunolabeling experiments on the frozen sections. With the combination of indirect ferritin-antibody labeling for desmin and Imposil-antibody labeling for vimentin (Fig. 5), or with the reverse combination (Fig. 6), the electron-dense markers for vimentin and desmin were found on the same single filaments. No extended lengths of single filaments were observed labeled with only one of the two markers. The number of markers for vimentin was about a tenth that for desmin in the first combination, as determined from a number of fields such as Fig. 5, but was about equal in the second (Fig. 6), reflecting a somewhat lower sensitivity of Imposil-antibody as compared with ferritin-antibody labeling under equivalent conditions. Taking this differential sensitivity into account, we calculated from these results that the ratio of desmin to vimentin subunits in the filaments at this stage was ~3:1, and that the efficiency of the labeling with ferritin conjugates was about three times that of Imposil in these experiments (see the legend of Fig. 6 for details of the calculation). Both types of double-labeling experiments, furthermore, showed that desmin and vimentin were not uniformly distributed along the same filaments, but instead were often found in clusters alternating all along a filament (for example,

vimentin clusters designated by arrows in Fig. 5, and arrowheads in Fig. 6).

In the 11-d developing myotubes, low magnification pictures showed that the labeled intermediate filaments were wavy but longitudinally oriented on the whole (Fig. 1), corresponding to our earlier immunofluorescence observations (32) of this stage. These filaments show no apparent relationship with any level of the sarcomere, either in distribution or in direction. At higher magnification, the relationships of individual intermediate filaments to myofibrils and organelles in the myotubes could be examined. Some filaments appeared to be in contact with myofibrils (black arrowheads, Fig. 2) or sarcoplasmic reticulum (white arrowhead, Fig. 2), but it was not clear that these represented actual contacts, and there was no regularity to their distribution along the myofibrils. In some specimens, intermediate filaments were observed to be threaded across a myofibril (bracket, Fig. 3), although there was no indication that these crossing filaments were associated with the myofibrils in any regular arrangement.

The overall impression gained from these morphological observations is that in 11-d myotubes, the intermediate filaments are largely in a longitudinal but wavy array, loosely and irregularly intertwined among and around the individual myofibrils. Some of the filaments appear to form contacts with organelles in the myotube, and perhaps with individual myofibrils, although in the latter case, there is no spatial regularity to such possible contacts.

Occasionally, several myofibrils were seen to be already laterally associated in register in the 11-d myotubes (Fig. 7), as previously described in our immunofluorescence study (32). When such myofibrils were tangentially sectioned, we found sarcoplasmic reticulum superimposed over all the laterally aligned Z band regions (Figs. 7 and 8) with desmin immunolabeling absent or very low in these areas (Fig. 8). Correspondingly, in individual isolated myofibrils, small fragments of sarcoplasmic reticulum were often found associated with the peripheries of the Z bands (Fig. 1, black arrowheads, and Fig. 2). It was noted that the level of the Z bands in the myofibrils aligned in register (large arrows, Fig. 7) was often

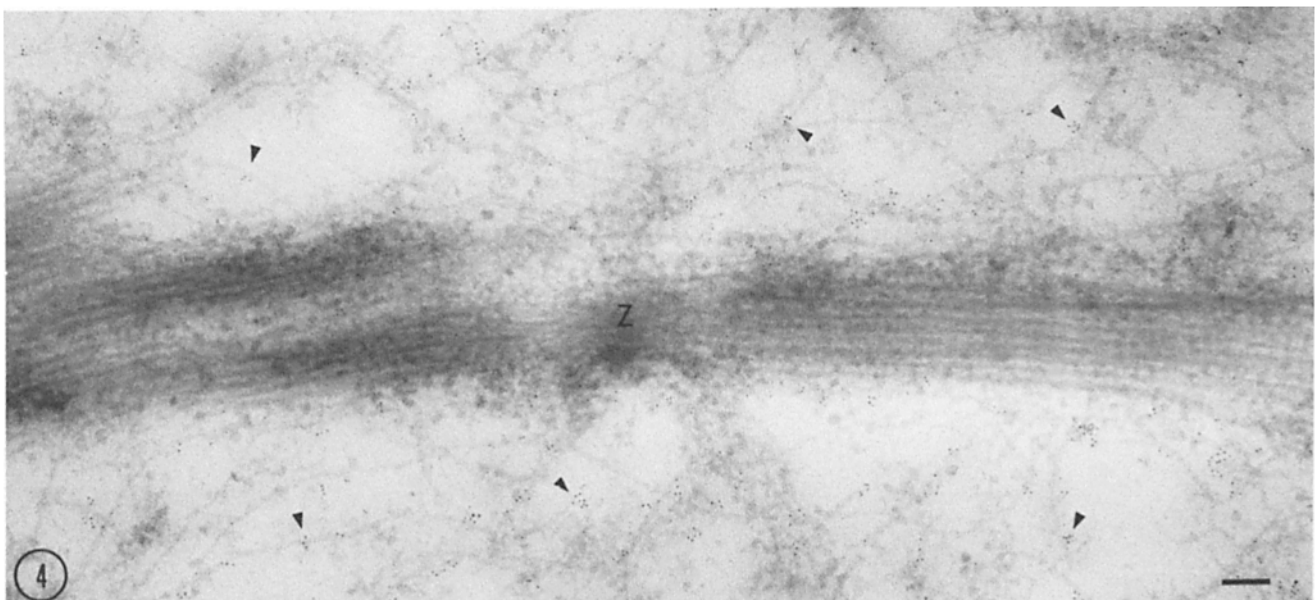


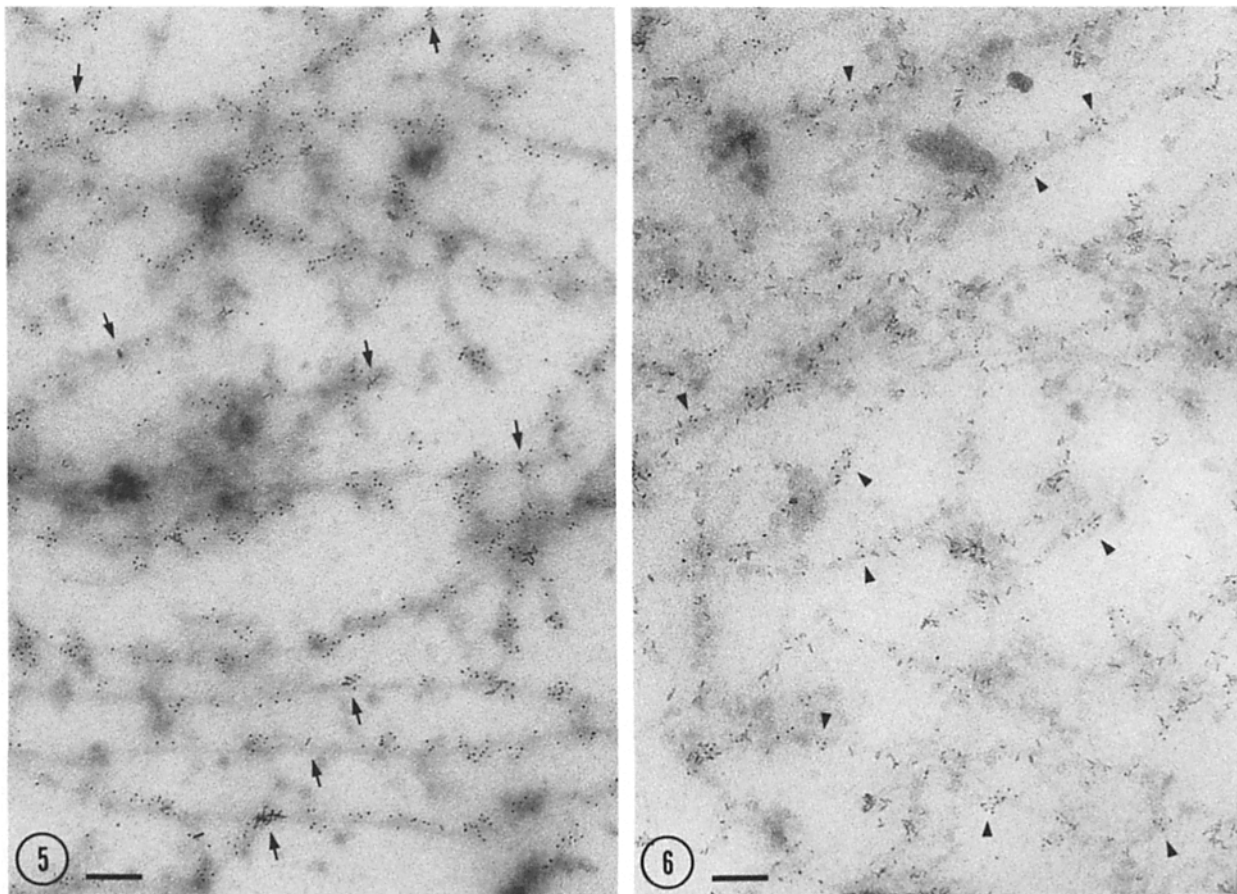
FIGURE 4 A myotube of an 11-d embryo, indirectly immunolabeled for vimentin with ferritin. The labeling appears to occur in all intermediate filaments but in a nonrandom and partially clustered manner (arrowheads). Bar, 0.1 μ m. \times 60,000.

completely out of phase with the Z band of an individual myofibril (small arrow, Fig. 7) that was in the immediate vicinity.

In 17-d embryos, myotubes were nearly full of myofibrils, as expected from the previous immunofluorescence observations (32). There was a variation among myotubes or even among different areas within single myotubes in the extent of packing of the myofibrils as well as in the degree of lateral registry of the fibrils. Nevertheless, myotubes as a whole were similar to the adult myofibers, and sarcoplasmic reticulum was often seen to cover the entire sarcomere (not shown). In the areas where myofibrils came into register, a reduction of immunolabeling at the mid-region of the sarcomere became recognizable (not shown), as observed in our previous immunofluorescence study (32). Interfibrillar spaces were reduced in width at this stage, and it became difficult to recognize intermediate filaments even in conventional, plastic-embedded sections of osmicated preparations (Fig. 9). This difficulty was reminiscent of the difficulty encountered in the

adult muscle (30). In tangential sections of the interfibrillar spaces, however, intermediate filaments were clearly recognized, often more abundantly near the level of the Z band than near the mid-region of the sarcomere (Fig. 10). Correspondingly, in the frozen sections that tangentially included the surface of myofibrils, linear arrays of labeling were often observed (Fig. 11). In such fields, it was seen that desmin and vimentin coexisted in the same arrays, just as in the previous stage, although the amount of vimentin relative to desmin is greatly reduced at this stage (Fig. 11).

In 21-d embryos, just before hatching, filling of the myotubes with myofibrils in lateral register was nearly as complete as in adult muscle, but the distribution of desmin immunolabeling between the myofibrils was not uniformly as closely confined to the level of the Z bands as in the adult tissue (32). In myotubes at this stage, ferritin labeling showed linear arrays that dispersed longitudinally away from the Z bands often as much as $1\ \mu\text{m}$ or more (Fig. 12). The longitudinal spread of labeling persisted in scattered areas in some



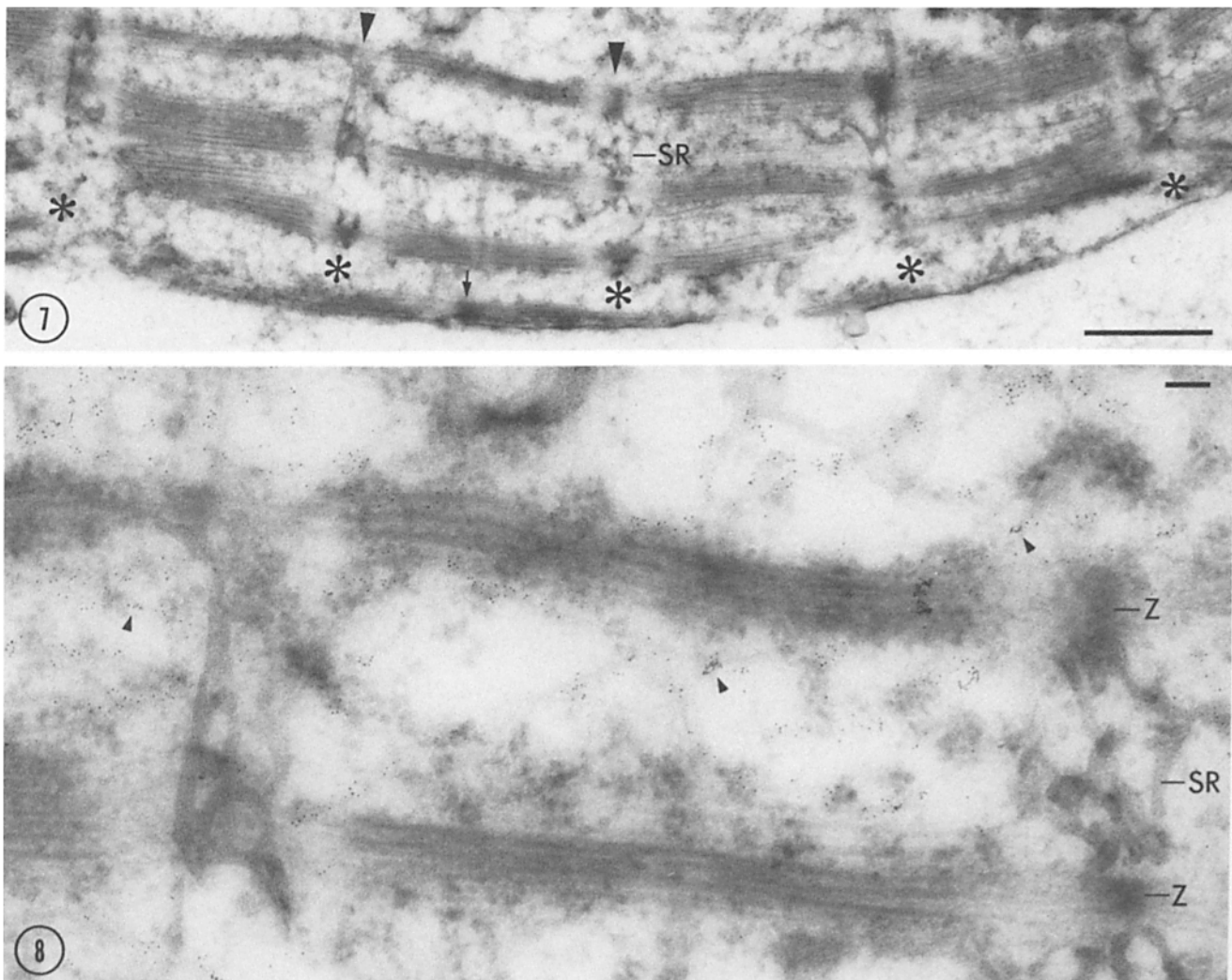
FIGURES 5 and 6 Fig. 5: A myotube of an 11-d embryo, doubly immunolabeled for desmin (ferritin) and vimentin (Imposil). Individual filaments can be discerned that are labeled with both markers. Vimentin labeling occurs sparsely and discretely (arrows), but often without superposition with desmin labeling. Bar, $0.1\ \mu\text{m}$. $\times 72,000$. Fig. 6: A myotube of an 11-d embryo, doubly immunolabeled for desmin (Imposil) and vimentin (ferritin). The exchange of the markers made vimentin labeling (arrowheads) relative to desmin labeling much more abundant in this figure than in Fig. 5. Individual filaments are now clearly seen to be labeled with both markers in a clustered manner. Vimentin labeling is seen to be largely excluded from regions of desmin labeling. If Imposil conjugates are $1/x$ of ferritin conjugates in the sensitivity of antigen detection, and the quantity of vimentin relative to desmin in intermediate filaments is $1/y$, then in the combination of Imposil labeling of vimentin and ferritin labeling of desmin, the ratio of the number of Imposil particles to that of ferritin particles will be $1/xy$ to 1 (R_1). On the other hand, in the case of ferritin labeling of vimentin and Imposil labeling of desmin, the ratio of ferritin particles to Imposil particles in number will be $1/y$ to $1/x$ (R_2). If R_1 is about $1/10$ as seen in Fig. 5 and R_2 is nearly 1 as seen in Fig. 6, then $1/xy = 1/10$ and $x/y = 1$. From these two equations, x^2 or $y^2 = 10$. Therefore, x or y is calculated to be ~ 3 . Bar, $0.1\ \mu\text{m}$. $\times 72,000$.

myotubes of 1-d postnatal chicks (Fig. 13) but in other myotubes, the labeling was largely confined to the level of the Z band (not shown). These findings are the high resolution correlates of related results obtained in our immunofluorescence study (32). Labeling for vimentin at this stage was either very low or undetectable (not shown), in agreement with the previous immunofluorescence observations (32). Labeling in the form of linear arrays was appreciably greater in intensity in myotubes of 21-d embryos or 1-d postnatal chicks (Figs. 12 and 13) than in those of 11- or 17-d embryos (Figs. 2 and 10). This is believed to occur partly because myotubes of 11-d and 17-d embryos were embedded in a polyacrylamide gel, whereas those of the later stages were not; we reported previously (27) that the polyacrylamide gel embedding causes lowering of the immunolabeling intensity.

DISCUSSION

The immunoelectron microscopic labeling techniques that

have been developed in this laboratory (23, 26) for the specific high resolution single and double labeling of antigens on ultrathin frozen sections of biological specimens, have allowed us to address a number of problems of *in vivo* embryonic skeletal muscle development. One set of significant findings relates to the process of the chemical transformation from vimentin to desmin in developing myotubes. In 11-d myotubes, when most of the myofibrils are still individual and isolated, and the intermediate filaments are mostly longitudinally arrayed in the cytoplasm, we have shown directly that each individual intermediate filament contains both vimentin and desmin subunits (Figs. 5 and 6). The possible coexistence of vimentin and desmin in the same intermediate filaments has been inferred previously from several types of observations: (a) under appropriate *in vitro* conditions, solubilized vimentin and desmin subunits can be reconstituted into morphologically characteristic intermediate filaments with solubility properties intermediate to those of the homopolymer

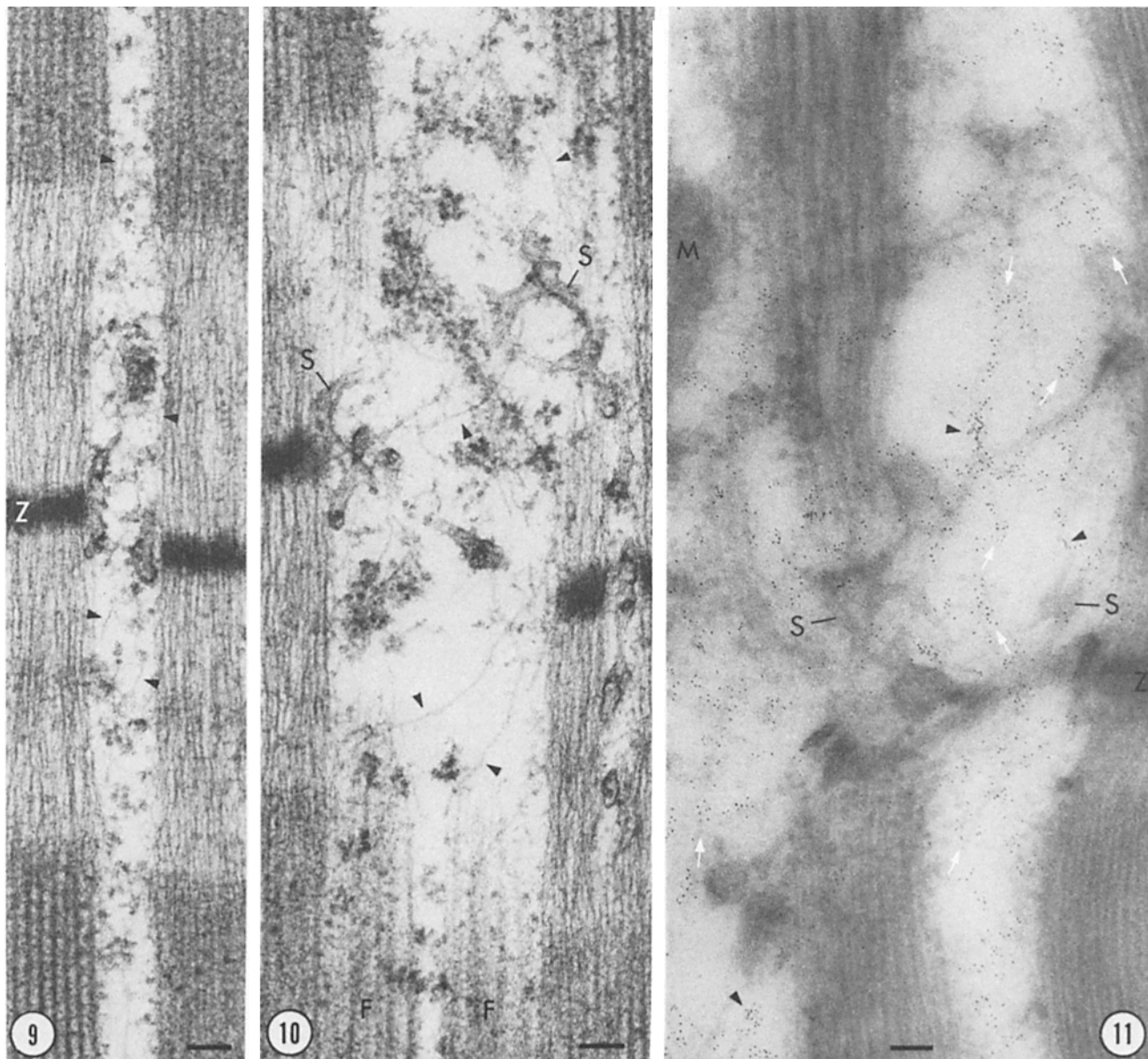


FIGURES 7 and 8 Fig. 7 is a portion of a myotube of an 11-d embryo; sectioning has skimmed three myofibrils in lateral registry at their surfaces and five levels of Z bands (asterisks) are seen to be covered by sarcoplasmic reticulum (SR). A Z band (small arrow) of a myofibril running along the plasmalemma is found near the mid-sarcomere level of the adjacent myofibrils that are themselves in lateral registry. The upper portion of the two levels of the Z bands indicated with two large arrowheads is enlarged in Fig. 11. SR is in alignment with the tangentially skimmed Z bands (Z) of the two myofibrils. Both desmin labeling (ferritin) and vimentin labeling (Imposil, indicated with arrowheads) are virtually absent in the areas occupied by SR (both over the Z bands and in the intermyofibrillar spaces), but show uniform distributions in the adjacent intermyofibrillar areas. Bars in Figs. 7 and 8, 1 μm and 0.1 μm , respectively. Fig. 7, $\times 18,000$. Fig. 8, $\times 60,000$.

filaments (25), (b) vascular smooth muscle and baby hamster kidney cells in culture that contain both vimentin and desmin yield intermediate filaments that, upon chemical cross-linking and solubilization, produce desmin-vimentin cross-links (20), and (c) in cardiac fibroblasts, a low level of immunoelectron microscopic labeling for desmin was found intermittently along intermediate filaments, from which it was inferred that the unlabeled regions of the same filaments were occupied by vimentin subunits (13). Nevertheless, our observations provide the first direct evidence for such coexistence of desmin and vimentin in embryonic skeletal muscle intermediate fil-

aments *in vivo*. It has also been demonstrated by immunoelectron microscopy that the glial fibrillary acid protein and vimentin coexist in the same intermediate filaments in a cultured human glioma cell (21).

An interesting feature of this coexistence is its clustered character. The vimentin and desmin subunits are not uniformly distributed along the same filament, but appear in alternating clusters all along the filament (Figs. 4–6). A similar clustered character was found for the glial fibrillary acid protein–vimentin distribution in the glioma cell intermediate filaments (21).



FIGURES 9–11 Figs. 9 and 10: Longitudinal sections of plastic-embedded myotubes of a 17-d embryo. An interfibrillar space is cut nearly across in the section in Fig. 9, whereas such a space is tangentially included in the section of Fig. 10, as judged by the tapering of two myofibrils (*F*) toward the Z band (*Z*) and the presence of sarcoplasmic reticulum (*S*) near the level of the Z band. In Fig. 9, structures indicated with arrowheads are difficult to identify with certainty as segments of intermediate filaments. In Fig. 10, however, intermediate filaments are quite clearly recognizable (arrowheads). They are seen to be more concentrated near the level of the Z band than near the mid-region of the sarcomere. Bar, 0.1 μm . Figs. 9 and 10, $\times 65,000$. Fig. 11: Frozen section of a myotube of a 17-d embryo. In this area, sarcoplasmic reticulum (*S*) covering a myofibril (*F*) is tangentially included in the section. Ferritin-labeling for desmin is seen to form linear arrays (white arrows) that are believed to correspond to individual intermediate filaments. Imposil particles (dark arrowheads) indicating the presence of vimentin are recognized in the same desmin-labeled filaments. Bar, 0.1 μm . $\times 60,000$.

These results provide some insight into the processes involved in the conversion from vimentin to desmin intermediate filaments in developing myotubes. It should first be noted that characteristic highly elongated intermediate filaments appear to be present throughout the process; there is, for example, no indication that stable short strands of exclusively desmin-containing filaments are formed *de novo* between the Z bands of the myofibrils in the maturing skeletal muscle. On the contrary, the intermediate filaments in adult muscle appear to be characteristically highly elongated, and to be wrapped around the myofibrils at the level of the Z bands (28, 30, 31). Our results also eliminate two other conceivable processes for the vimentin-to-desmin conversion. One is the *de novo* polymerization of desmin subunits into longitudinally oriented, homopolymeric but typical multi-stranded (7, 24) intermediate filaments, whereas independently vimentin filaments were degraded. There was no evidence for vimentin- or desmin-containing longitudinal filaments exclusively in the 11-d myotubes. The second process eliminated is the *de novo* polymerization of a single strand of desmin starting from one end of a preexisting multistranded vimentin filament; this would have led to an apparently uniform codistribution of desmin and vimentin labeling along the filaments at the stage when the amount of desmin in the myotube exceeded vimentin.



FIGURE 12 A myotube of a 21-d embryo. The surface of a myofibril is skimmed by the sectioning at the level of a Z band (Z). Some of the ferritin labels for desmin form linear arrays. Labeling is seen to be more nearly confined to the Z band level in the area above the Z band, but in the area below, the labeling extends 1 μm or more (arrow) from the Z band. Bar, 0.1 μm . $\times 53,000$.

Although our results are therefore sufficient to rule out certain mechanisms for the vimentin-to-desmin conversion, they are insufficient to establish the correct one. The presence of both desmin and vimentin clusters all along individual intermediate filaments suggests the possibility that existing filaments are remodeled in the course of the vimentin-to-desmin conversion, but earlier stages of myotube development, when the myotubes contain lower desmin/vimentin ratios, need to be studied to get more information on this point. The widespread occurrence of Ca^{++} -activated proteases highly specific for vimentin and desmin (16, 33) may be relevant in connection with possible filament remodeling processes. Since the absolute numbers of intermediate filaments appear to be fewer in adult muscle than in the developing myotubes, it is likely that intermediate filament turnover is also occurring during myogenesis.

We turn now from the discussion of the chemical transformation of the intermediate filaments, to the spatial distribution of the filaments and the changes in their distribution in developing myotubes. In 11-d embryos, although most of the myofibrils are still individual and isolated in the cytoplasm, the bulk of the intermediate filaments are longitudinally oriented as wavy filaments loosely surrounding the myofibrils (Figs. 1–3). Associations of at least some of the filaments with nuclei and mitochondria (Fig. 1) that appear to exist at this stage may correspond to similar associations previously observed in adult skeletal and cardiac muscle (30, 31). There also appear to be irregularly spaced attachments of intermediate filaments to myofibrils (Fig. 2). The general longitudinal orientation of the intermediate filaments, along with their attachments to organelles and myofibrils, may therefore serve to orient and polarize the contents of the myotube in the longitudinal direction at this stage. Since longitudinally oriented microtubules are present in the developing myotube (2, 4, 35), microtubules may, together with the intermediate filaments, participate in this polarization.

The lateral registry of the myofibrils in the developing myotubes does not appear to be mediated by the intermediate filaments. This conclusion was inferred from our earlier immunofluorescence study (32), but it was still possible that a minority of the intermediate filaments might be laterally associated with a specific level of the early developing myofibrils. On the other hand, at the higher resolution of immunoelectron microscopy, individual filaments can be recognized. In the 11-d embryos, some of the myofibrils are already in register, but there is no regular association of individual intermediate filaments with these myofibrils. This is also true of the more extensively in-register myofibrils in the 17-d (Figs. 9–11) and 21-d (Fig. 12) myotubes. In particular, at the 17-d stage, the labeling for desmin and vimentin is largely excluded from the intermyofibrillar spaces surrounding the mid-regions of the in-register sarcomeres as was observed in our previous immunofluorescence study (32). The belt of tightly coordinated intermediate filaments that is wound around the in-register Z bands of each myofibril in adult skeletal muscle (30) does not show its characteristic compact form until hatching, after all of the myofibril registry has already occurred. The overall conclusion is that intermediate filaments do not have a direct role in the production of the lateral registry of myofibrils in developing skeletal myotubes. These *in vivo* results lend physiological significance as well as greater precision to similar conclusions that had been derived from earlier immunofluorescence studies of *in vitro* cultures of

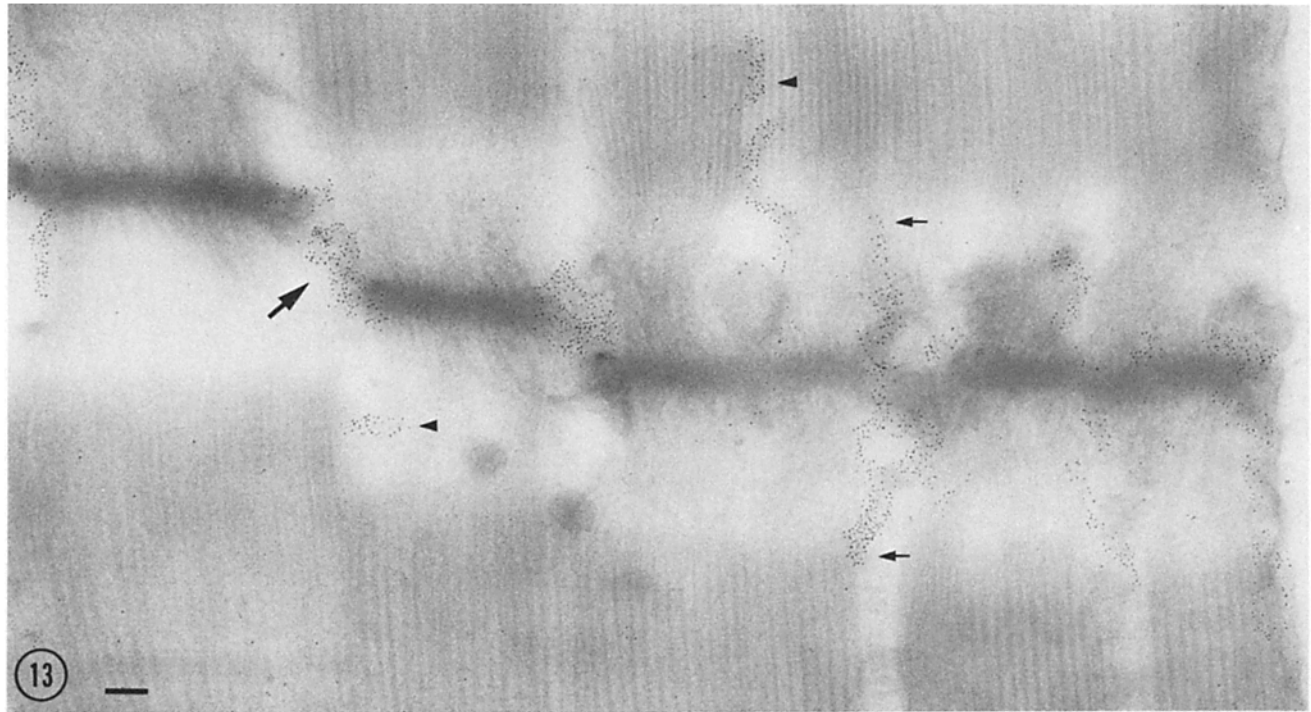


FIGURE 13. A myotube of a 1-d postnatal chick. Z bands are not exactly in register, which is believed to have been caused during the artificial stretching of muscle pieces at fixation. The range of ferritin immunolabeling for desmin around the Z band varies from a divergence of $0.5 \mu\text{m}$ (the pair of small arrows) to a nearly complete confinement to the Z band level (the large arrow). Isolated arrays of labeling (arrowheads) are probably due to segments of filaments that are continuous with those emanating from the Z band level. Bar, $0.1 \mu\text{m}$. $\times 53,000$.

myogenic cells (1, 12). Such findings of ours are at variance with those of Kelly (15) and Price and Sanger (19). This difference seems to be due largely to differences in the methods used for the observation of intermediate filaments. A difficulty in the purely morphological approach taken by these authors is that a relatively high magnification is required to recognize the tracks of the 10-nm thick filaments, but a low magnification is needed to evaluate their overall distribution in relation to myofibrils distributed sparsely in a large area. In the present study, the thickening of the filaments due to the association of antibodies and labeling markers made it possible to observe both the tracks of the filaments and the sarcomeres of myofibrils at low magnification.

How then do the desmin-containing intermediate filaments come to surround the myofibrils at the level of the Z bands in adult skeletal muscle? Our results indicate that even after the myotubes become largely filled with myofibrils in extensive lateral registry, the intermediate filaments are mostly oriented in the longitudinal or oblique direction within the intermyofibrillar spaces. If during the process of development, part of the increase in numbers of myofibrils occurs by a longitudinal splitting of myofibrils after they achieve a certain thickness from the lateral addition of myofilaments and other sarcomeric components (8, 9, 22), the longitudinal intermediate filaments might then diffuse laterally into the spaces generated between the newly split myofibrils. These intermediate filaments might be sporadically attached to myofibrils (Fig. 2), as well as loosely wound around them (Fig. 3), while maintaining a largely longitudinal orientation. Only very late in development, near hatching, would these loosely wound filaments become condensed around the Z bands during a relatively short time interval. This condensation step would

most likely be mediated by some type(s) of molecular interaction(s) between components linking the desmin-containing filaments to one another and to the Z bands, with the conditions favoring such interactions being temporally regulated to occur around the time of hatching.

If there is no evidence that intermediate filaments are involved in causing myofibril registry, a possible role for elements of the sarcoplasmic reticulum is suggested by our results. There is a remarkably persistent and uniform association of sarcoplasmic reticulum with the Z band regions of each myofibril in the 11-d myotubes, not only with the laterally registered myofibrils (Figs. 10 and 11), but also with the isolated individual myofibrils (Fig. 1, arrowheads). These observations *in vivo* probably represent the physiological counterpart of the findings of Ezerman and Ishikawa (3) who reported that *in vitro* in several 1-d-old cultures of chicken myotubes, sarcoplasmic reticulum was associated with myofibrils at the level of the Z band. This association may be responsible for the apparent exclusion of desmin and vimentin immunolabeling around the Z bands in early in-register myofibril assemblies (32; Fig. 11). It is possible that the lateral registry of the myofibrils is induced by a lateral collection of elements of the sarcoplasmic reticulum to which the Z bands of individual myofibrils are attached.

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