

Titin and Myosin, but not Desmin, Are Linked during Myofibrillogenesis in Postmitotic Mononucleated Myoblasts

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Abstract. Monoclonal antibodies specific for the muscle protein titin have been used in conjunction with muscle-specific antibodies against myofibrillar myosin heavy chains (MHCs) and desmin to study myogenesis in cultured cells. Desmin synthesis is initiated in replicating presumptive myoblasts, whereas the synthesis of titin and MHC is initiated simultaneously in their progeny, the postmitotic, mononucleated myoblasts. Both titin and MHC are briefly localized to nonstriated and thereafter to definitively striated myofibrils. At no stage during myofibrillogenesis is either protein observed as part of a sequence of mini-sarcomeres. Titin antibodies bind to the A-I junction, MHC antibodies to the A bands in nascent, maturing, and mature myofibrils. In contrast, desmin remains distributed as longitudinal filaments until well after the definitive myofibrils have aligned laterally. This tight temporal and topographical linkage between titin and myosin is also observed in postmitotic, mononucleated myoblasts and multinucleated myotubes when myofibrillogenesis is perturbed with Colcemid or taxol. Colcemid induces elongating postmitotic mononucleated myoblasts and multinucleated myotubes to round up and form Colcemid myosacs. The myofibrils that emerge in these rounded cells are deployed in convoluted circles. The time required for their nonstriated myofibrils to

transform into striated myofibrils is greatly protracted. Furthermore, as Colcemid induces immense desmin intermediate filament cables, the normal spatial relationships between emerging individual myofibrils is distorted. Despite these disturbances at all stages, the characteristic temporal and spatial relationship observed in normal myofibrils between titin and MHC is observed in myofibrils assembling in Colcemid-treated cells. Newly born postmitotic mononucleated myoblasts, or maturing myotubes, reared in taxol acquire a star-shaped configuration and are induced to assemble "pseudo-striated myofibrils." Pseudo-striated myofibrils consist of laterally aggregated 1.6- μm long, thick filaments that interdigitate, not with thin filaments, but with long microtubules. These atypical myofibrils lack Z bands. Despite the absence of thin filaments and Z bands, titin localizes with its characteristic sarcomeric periodicity in pseudo-striated myofibrils. We conclude that the initiation and subsequent regulation of titin and myosin synthesis, and their spatial deployment within developing sarcomeres are tightly coupled events. These findings are discussed in terms of a model that proposes interaction between two relatively autonomous "organizing centers" in the assembly of each sarcomere.

It is of interest to consider how a myofibril, the assembled product of many developmentally regulated proteins, acquires its invariant structure. In cultured cardiac myoblasts, myofibrillar anti-myosin first localizes to long, narrow, nonstriated filaments. Topographically, these nascent nonstriated myofibrils are co-extensive with long individual stress fiber-like structures (SFLSs).¹ Within hours, the SFLSs disappear and the nascent myofibrils display definitive A, I, M, and, finally, Z bands (12, 28, 30). This spatial

and temporal correlation between a single nascent myofibril and a single transient SFLS is also observed in immature normal multinucleated myotubes and in multinucleated myosheets recovering from the mutagen, ethylmethylsulfonate (3, 5, 28).

The great majority of studies on myofibrillogenesis have focused on the synthesis of myofibrillar isoforms and their assembly into striated myofibrils as it occurs in multinucleated myotubes (references in 15, 17, 18, 39). However, it has long been known that both the initiation of synthesis of most (all?) myofibrillar isoforms and their assembly into ordered interdigitating thick and thin filaments actually occurs before

1. *Abbreviations used in this paper:* IF, intermediate filament; MHC, myofibrillar myosin heavy chain; SFLS, stress fiber-like structure.

fusion into myotubes, and is well advanced in postmitotic, mononucleated myoblasts (9, 10, 23, 26, 29, 36, 37, 50). Recently titin has also been detected in cultured postmitotic, mononucleated myoblasts (22).

The precise location of titin within the sarcomere is still unclear. One epitope of the molecule has been localized by monoclonal antibodies to the A-I junction (22, 35, 49, 51, 52). Some evidence suggests that this long flexible molecule might extend from the H band to the N2 line: other evidence suggests that titin might traverse the entire sarcomere, linking successive Z bands. Given this proposed overlapping between thick and thin filaments by titin and its putative role as an elastic length-limiting filament (35, 49, 51, 52), titin might be expected to be (a) synthesized very early after the birth of the postmitotic, mononucleated myoblast, and (b) rapidly incorporated into nascent myofibrils. Indeed, if there is a temporal sequence such that one protein is first polymerized into a long nonstriated filament to which other myofibrillar proteins are successively added, then titin might be a candidate for such a "core" or "nucleating" myofibrillar protein.

To learn more about when myofibrillar myosin heavy chains (MHCs) and titin are synthesized and assembled first into nonstriated and then into striated myofibrils, Colcemid- and taxol-treated cells in various compartments of the myogenic lineage have been singly and doubly stained with antibodies to titin and MHC. In addition, these cells have been stained with antibodies to the muscle-specific intermediate filament (IF), desmin. Emphasis has been placed on determining the spatial and temporal relationship between the two myofibrillar proteins and the IFs in postmitotic mononucleated myoblasts. We found that the time of appearance and intracellular distribution of titin in postmitotic mononucleated myoblasts is invariably linked to MHC. This tight linkage between titin and myosin is also evident in the atypical myofibrils that are assembled in Colcemid-induced myosacs and in taxol-induced star-shaped, postmitotic, mononucleated myoblasts. This is of interest as in Colcemid-treated cells the orderly integration of thick and thin filaments is often delayed and scattered I-Z-I complexes without associated 1.6- μm long thick filaments are frequent (8, 28, 30, 48). Taxol, on the other hand, induces the assembly of "pseudo-striated myofibrils." These consist of A bands of laterally aligned 1.6- μm long thick filaments that lack thin filaments and Z bands (4, 27, 48). Clearly, the assembly of titin and MHC into sarcomere-like structures does not depend on either the orderly interdigitation of thick and thin filaments or even on the participation of a normally integrated I-Z-I complex. These findings are consistent with the notion that the assembly of each sarcomere involves interaction between two semi-autonomous organizing centers—one involves the assembly of thick filaments and their associated proteins, including titin; the other the assembly of the I-Z-I complex.

Materials and Methods

Cell Culture

Embryonic chick skeletal muscle cultures were established as described (8, 9). Briefly, breast muscle from 12-d-old chick embryos was dissociated in Ca^{2+} - and Mg^{2+} -free salt solution containing 0.25% trypsin (Gibco, Grand Island, NY). Cells were centrifuged and resuspended in MEM (culture

medium) + 10% embryo extract followed by filtration to produce a single cell suspension. Cells were counted and diluted to 0.5×10^6 cells/ml culture medium and plated on collagen-coated Aclar plastic (Allied Chemical Co., Pottsville, PA) or glass coverslips in tissue culture dishes. Colcemid (Sigma Chemical Co., St. Louis, MO) was added to some cell cultures at a concentration of 1 μM at 48 or 72 h after plating followed by fixation after various times in the mitotic inhibitor. Cells were exposed to taxol (National Institutes of Health, Bethesda, MD) 5 h after plating and the drug was replenished each day for 3–4 d before fixation (10 μM). In some experiments, cytosine arabinoside (10) was added at a concentration of 1 $\mu\text{g}/\text{ml}$ to cell cultures 72 h after plating to inhibit the growth of nonmuscle cells. Metaphase-arrested cells were induced by Colcemid (1 μM).

Primary myogenic cultures contain at least four classes of cells: (a) postmitotic, multinucleated myotubes, (b) elongated, postmitotic mononucleated myoblasts, (c) replicating presumptive myoblasts, and (d) replicating fibroblasts. It is likely that there are subclasses of replicating presumptive myoblasts (40). However, it is often difficult to distinguish replicating presumptive myoblasts from fibroblasts (see references 17, 26, 37). Accordingly, in what follows if a cell is negative for desmin, MHC, and titin, it will be simply described as a "fibroblastic" cell. It is to be stressed that some subpopulations of such fibroblastic cells upon subculture readily replicate and give rise to many generations of postmitotic mononucleated myoblasts and multinucleated myotubes (26, 55).

Antibodies

The monoclonal antibodies to titin have been characterized previously (22). Antibodies from the T1 clone were used exclusively in this study. T1 antibodies were purified from mouse ascites fluid by ammonium sulfate fractionation followed by anion exchange chromatography on a Mono-Q column with the use of an FPLC apparatus (Pharmacia, Uppsala, Sweden). They were used at a final concentration of ~ 10 mg/ml. For detecting muscle MHCs, directly conjugated rabbit IgG against chick light meromyosin (10, 25) and a mouse monoclonal antibody against the S1 myosin fragment were used. The polyclonal antibody against light meromyosin was absorbed with purified titin coupled to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) before use. The monoclonal antibody against S1 was a kind gift of Dr. F. Pepe, University of Pennsylvania; it binds to the 25-kD and 20-kD fragment of S1. The polyclonal anti-light meromyosin tended to stain the lateral borders more intensely than the center of the A band; the monoclonal anti-S1 stained the entire A band. They were used interchangeably and both will be referred to as myofibrillar anti-MHC. The properties of the rabbit antibodies against chick desmin and vimentin have been detailed in Bennett et al. (6, 7) and Holtzer et al. (24). To compensate for the more rapid bleaching of fluorescein relative to rhodamine-labeled antibodies, whenever possible the labels were switched from experiment to experiment. If, for example, in a double-label experiment the anti-titin was first visualized by a secondary rhodamine label and the anti-desmin by a fluorescein label, then in the next experiment the anti-titin was visualized by a secondary fluorescein label and the anti-desmin by a rhodamine label.

Immunofluorescence Microscopy

Cells were fixed in a solution of 2% formaldehyde in PBS for 2 min followed by incubation in 0.5% Triton X-100 in PBS for 30 min with gentle agitation. Cells were rinsed briefly with PBS followed by staining with antibody. All fixation and permeabilization procedures were performed at room temperature. Standard procedures for indirect immunofluorescence microscopy were used for staining. All secondary antibodies for immunofluorescence were obtained from Cappel Laboratories, Cochranville, PA. Affinity-purified fluorescein-labeled sheep anti-rabbit antibodies and affinity-purified rhodamine-labeled sheep anti-mouse antibodies were mixed before use in double label antibody staining. After the final wash of the antibody staining procedure, cells were stained for 15 min in a solution of 10 μM bisbenzimidazole H33342 (Hoechst Chemical Co., Frankfurt, FRG) to visualize the nuclei.

SDS Gel Electrophoresis and Immune Blot Analysis

SDS gel electrophoresis using 3–12% gradient polyacrylamide slab gels was performed as described (22). Electrophoretic transfer onto nitrocellulose was done for a period of 48 h at 4°C. After incubation of the nitrocellulose sheets with primary antibody, decoration was visualized by treatment with ^{125}I -labeled rabbit anti-mouse antibody conjugated by the Iodogen technique (Pierce Chemical Co., Rockford, IL) followed by autoradiography.

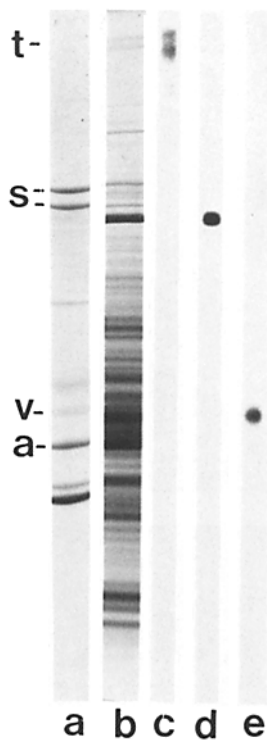


Figure 1. Immunoblot analysis of day-4 cultures. Whole cell homogenates were subject to electrophoresis on a 3–12% SDS gradient gel (b) and then electrophoretically transferred to nitrocellulose paper and reacted with antibodies. (a) Coomassie-stained standards. s, Spectrin doublet; v, vimentin; a, actin. (b) Coomassie-stained day-4 culture homogenate. t, titin doublet. (c–e) Immunoblots stained with (c) T1 antibodies, (d) anti-light meromyosin antibodies, and (e) anti-desmin antibodies. The monoclonal antibody against S1 reacted only with myosin heavy chains (data not shown).

Results

Immunoblots Prepared from Day-4 Myogenic Cultures

To support the reliability of our immunofluorescence staining reagents, whole cell homogenates were prepared from day-4 cultures. Such cultures consist of many thousands of multinucleated myotubes, each of which contains numerous definitively striated myofibrils as well as many fibroblastic cells and small numbers of postmitotic, mononucleated myoblasts (see Figs. 2 and 3). The homogenates were subjected to SDS PAGE followed by transfer to nitrocellulose paper and stained with antibodies to desmin, MHC, and titin. Immunoblots of day-4 cultures stained with anti-titin (Fig. 1 c). This confirms that our titin antibodies recognized only titin polypeptides in the culture homogenates. The polyclonal antibody against light meromyosin and the monoclonal against S1 reacted exclusively with the MHC band at 200 kD while the anti-desmin reacted only with the desmin band at 53 kD (Fig. 1, d and e). Chicken desmin migrates just ahead of mammalian desmin (24).

Staining Patterns of Cells in Day-4–8 Myogenic Cultures

To relate and interpret the distribution of our antibodies in postmitotic, mononucleated myoblasts to that observed in maturing myotubes, we first stained day-4–8 cultures. Fig. 2, a–f, shows triple-stained fluorescence micrographs that illustrate the distribution of desmin, MHC, and titin in normal multinucleated myotubes and their absence in adjacent fibroblastic cells. Both myotubes and fibroblastic cells are vimentin positive (7, 16, 24), but only the myotubes in Fig. 2 are desmin positive (see below). In day-4 myotubes, the lateral alignment of individual striated myofibrils is well advanced and in EM sections every sarcomere displays a prom-

inent Z band (30, 32). The pattern of binding of anti-titin along the myofibril varies greatly depending on the degree of contraction. In very relaxed myofibrils, it binds to the A-I junction; in moderately contracted myofibrils the distance between adjacent A bands becomes so narrow that the two fluorescent lines appear as a single band in the fluorescence microscope (Figs. 2 b and 3 a). In contrast, the anti-MHC stains the broad A band (Figs. 2 e and 3 b). These distinct and characteristic staining patterns are observed both in thick, mature myofibrils and in thin nascent ones as well. Fig. 2, a and d, illustrates that in day-4 myotubes, desmin IFs are still oriented longitudinally. Fig. 3, a and b, shows micrographs of cultures double stained to detect both MHC and titin. They demonstrate that if a myofibril is positive for titin, irrespective of its diameter, it is positive for myosin and vice versa. Most frequently, the staining with the directly conjugated anti-MHC is not as intense or distinct as is the indirect staining with anti-titin.

In addition to thousands of multinucleated myotubes and modest numbers of elongated, postmitotic mononucleated myoblasts (see below) day-4–8 primary cultures contain $3\text{--}5 \times 10^6$ fibroblastic cells per 35-mm petri dish. These cells do not bind either anti-titin or anti-MHC (e.g., Figs. 2–4). Double staining of these fibroblastic cells with anti-desmin plus either anti-MHC or anti-titin reveals that roughly 7% of the cells were desmin positive, but myosin or titin negative. The converse, namely cells that were titin or myosin positive but desmin negative, has not been observed. The desmin-positive but MHC- and titin-negative cells most probably are the replicating presumptive myoblasts or immediate precursors to the postmitotic mononucleated myoblasts (9, 13, 28).

Staining Patterns of Elongated, Postmitotic Mononucleated Myoblasts in Primary Cultures

Primary cultures contain modest numbers of “new-born,” as yet unfused, postmitotic mononucleated myoblasts. Owing to their characteristic density and unusual elongated morphology—often $>400 \mu\text{m}$ —they can be recognized while living under the phase-contrast microscope. 15–20 h after their last mitosis, these rapidly elongating, bipolar postmitotic myoblasts invariably stain with antibodies to desmin, MHC, and titin. Combined EM and cytoimmunofluorescence studies have shown that postmitotic myoblasts contain numerous aligned interdigitating thick and thin filaments, vimentin and desmin IFs, and a very dense population of longitudinally oriented microtubules of indefinite length (4, 23, 26, 45). Fig. 4, a–e, illustrates how rapidly in these postmitotic, mononucleated myoblasts the binding pattern of both anti-titin and anti-MHC changes from a generalized fluorescence, to staining of a nonstriated, and then typically striated, myofibril. The earliest postmitotic myoblasts appear to bind both myofibrillar antibodies throughout the cytoplasm (Fig. 4, a and b). From observations of living cells under the phase-contrast microscope we estimate that 10–15 h separates the myoblast in Fig. 4, a and b, from that in Fig. 4, c and d. Another 10–15 h separates the myoblast in Fig. 4, c and d, from that in Fig. 4, e and f. In this period the myoblasts elongate considerably and their myofibrils assume a definitive periodicity of $\sim 2.3 \mu\text{m}$ (Fig. 4, e and f). Only in one detail is the precise localization of titin in the nascent myofibrils in postmitotic myoblasts different from that ob-

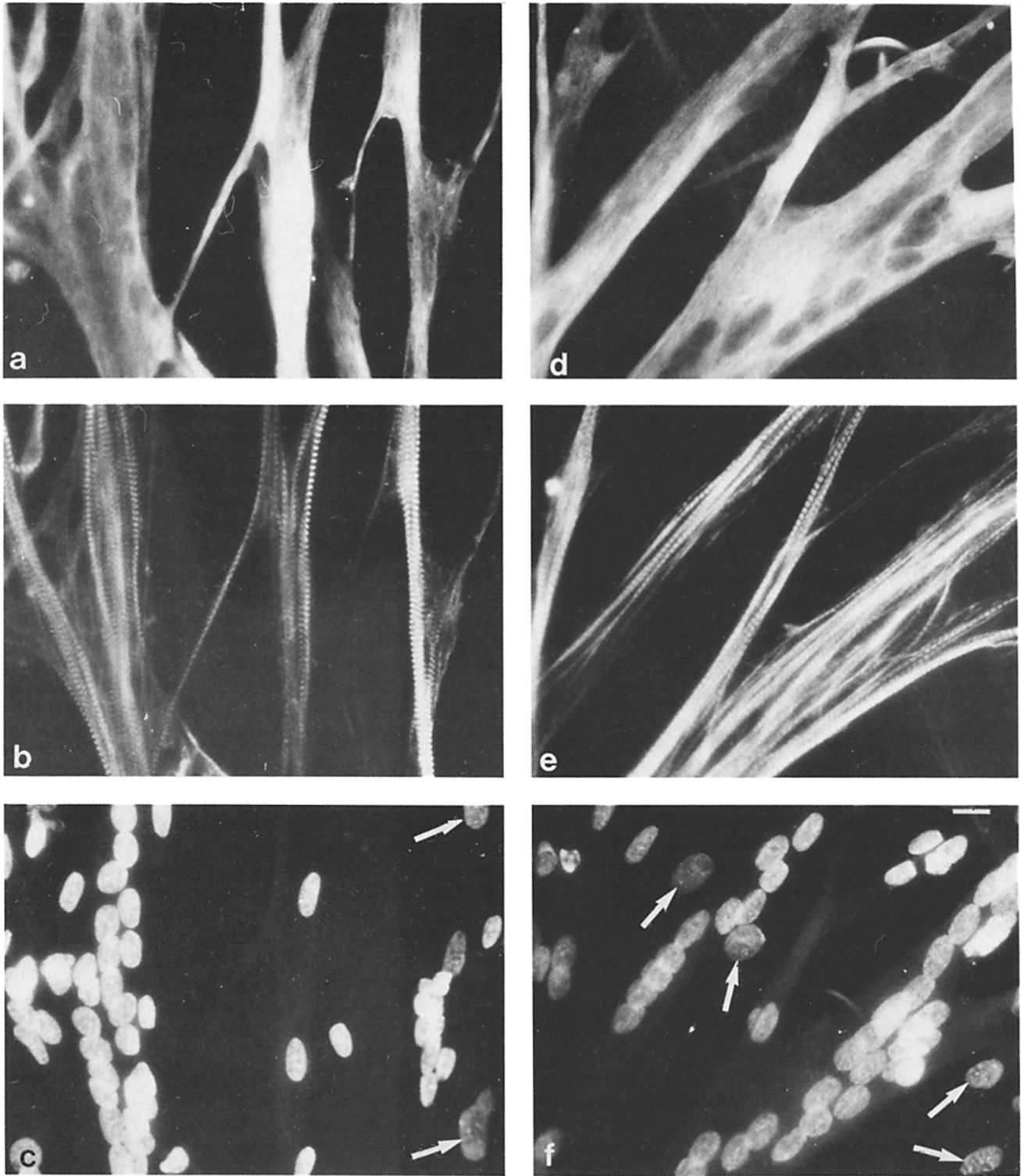


Figure 2. Triple-stained micrographs of two different microscopic fields from day-4 cultures. *a-c* are of the same cluster, while *d-f* are of another cluster of cells. Both multinucleated myotubes and fibroblastic cells bind the nuclear stain bisbenzimidide (*c* and *f*) but only the myotubes bind anti-titin (*b*, fluorescein label) and anti-myosin (*e*, fluorescein label). Though dispersed vimentin and desmin IFs (*a* and *d*, rhodamine label) ramify throughout the myotubes, some regions appear to be more densely packed than others. There is no obvious correlation between this irregular distribution of vimentin and desmin IFs and the number or degree of maturation of the myofibrils. The borders of the myotubes are outlined after treatment with anti-desmin (*a* and *d*), but difficult to visualize with anti-titin or anti-myosin (*b* and *e*). The nuclei in myotubes (*c* and *f*) can line up in long columns or can overlap. Arrows in *c* and *f* point to nuclei of fibroblastic cells. The density of fibroblastic cells in both of these microscopic fields is lower than usual due to the addition of cytosine arabinoside to the medium. Bar, 10 μm .

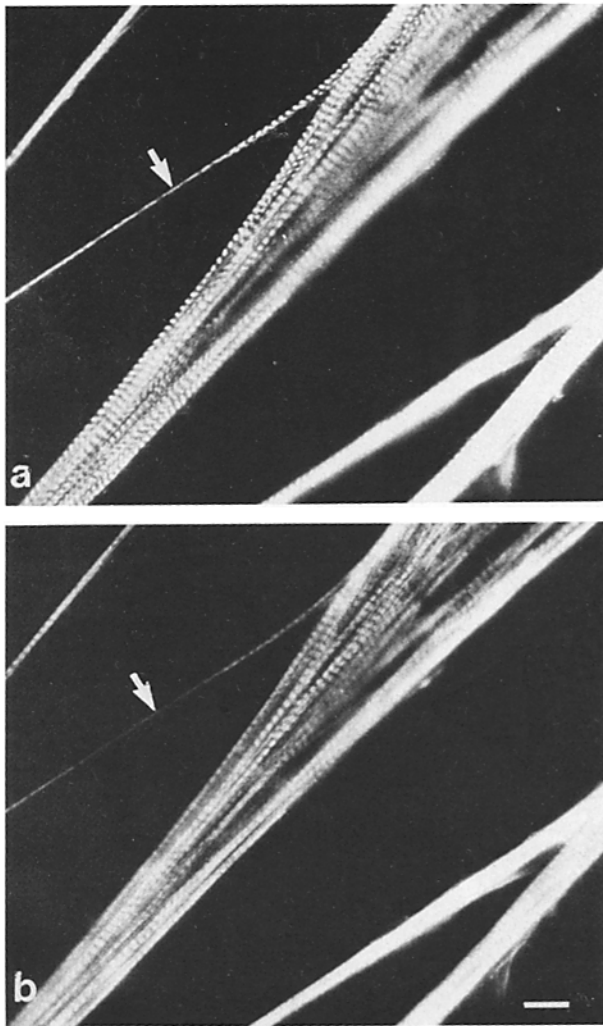


Figure 3. Identical cluster of day-4 myotubes double-stained with anti-titin (*a*, rhodamine label) and anti-myosin (*b*, fluorescein label). Note that the titin-positive regions invariably are associated with nearby MHC-positive regions. Bar, 10 μ m.

served in more mature myofibrils. Though clearly distributed in a sarcomeric periodicity (Fig. 4 *e*), the titin-positive bands in nascent myofibrils rarely form a doublet bordering the region where the Z band should be assembling. The significance of this observation regarding either the assembly of the myofibrils or possible contraction is not clear. A more precise determination of the localization of titin with respect to emerging A, I, and Z bands will require the use of immunoelectron microscopic procedures. Staining of postmitotic mononucleated myoblasts with either anti-titin or anti-myosin has not revealed at any stage of development closely spaced strings of minisarcomeres.

That normal titin-positive cells, like normal MHC-positive cells, never reach mitosis was shown by the following experiments. Colcemid was added to day-2 cultures, 4 h before the cells were fixed. The cells were stained with either antibodies to desmin, MHC, or titin, and then double-stained with bisbenzimidazole. Of over 300 Colcemid metaphase-arrested cells, 8% were desmin positive; none bound the antibodies to MHC or titin to fine filaments. These findings suggest that the same developmentally regulated controls that

initiate MHC synthesis in postmitotic, mononucleated myoblasts coordinately initiate titin synthesis (10, 26, 30, 37, 41). Desmin synthesis, initiated in replicating presumptive myoblasts, continues to be synthesized in postmitotic myogenic cells.

The Temporal and Spatial Coupling of Myosin and Titin Is Maintained in Colcemid-disturbed Postmitotic Myogenic Cells

Colcemid perturbs, in a reversible fashion, both the postmitotic mononucleated myoblasts and the multinucleated myotubes. It induces the former to round up into mononucleated spherical cells and the latter to retract into thick isodiametric multinucleated myosacs (8, 24, 25, 28, 30, 32). While not detectably blocking their assembly, Colcemid delays the orderly interdigitation of thick and thin filaments into precisely structured, tandem sarcomeres. In addition, Colcemid induces (*a*) the assembly of randomly oriented and isolated I-Z-I complexes that lack associated thick filaments, and (*b*) the aggregation of the cell's vimentin and desmin IFs into massive, meandering cables (11, 24, 28, 30, 32). Accordingly, we asked (*a*) whether the delay induced by Colcemid in the transformation from nonstriated to striated myofibrils would alter the characteristic spatial and temporal linkage between titin and MHC, and (*b*) whether the induced aggregation of desmin into IF cables would preclude the lateral alignment of myofibrils, or their attachment to, the sarcolemma via their Z bands as might be expected if desmin functioned as a "mechanical integrator" (34).

The rate of myosin synthesis and assembly into myofibrils peaks in day-4 myotubes (10). There are relatively few myofibrils in the newly fused myotubes in late day-2 cultures. Accordingly, Colcemid was added to the medium (1 μ M) of early day-2 cultures, the cells were incubated for another 48 h in the presence of the drug, and then fixed and stained. Fig. 5, *a-d*, shows micrographs of Colcemid myosacs from early day-4 cultures stained with anti-titin and anti-MHC (Fig. 5, *a* and *b*) and with anti-titin and anti-desmin (Fig. 5, *c* and *d*). Emerging myofibrils in early Colcemid myosacs are considerably more disorganized and remain nonstriated for longer periods than emerging myofibrils in control myotubes (cf. Fig. 5, *a-c*, with Figs. 2 and 3). Nevertheless, the tight spatial linkage of titin and MHC observed in normal myofibrils at this stage of maturation is maintained in the corresponding myofibrils in Colcemid myosacs. If reared for another 24-48 h in Colcemid, the number of normally striated myofibrils in all Colcemid myosacs increases considerably (Fig. 5, *e-h*). By what mechanism Colcemid delays the transition from nonstriated to striated myofibril is unknown. It is worth stressing that during the later period in Colcemid, the earlier dispersed myofibrils associate laterally. This lateral alignment of striated myofibrils takes place despite the fact that the cell's desmin is tied up into immense IF cables (Fig. 5 *d*). If removed from Colcemid, the desmin IF cables disperse, the myosacs elongate, and in 24-48 h are anisodiametric structures with long, striated myofibrils.

EM studies (28, 30, 48) have demonstrated that large numbers of free, scattered I-Z-I complexes with appropriately polarized, inserted, thin filaments, but without associated thick filaments, assemble in Colcemid myosacs. There is no obvious spatial relationship between these scattered I-Z-I complexes and nearby thick and thin filaments, or the in-

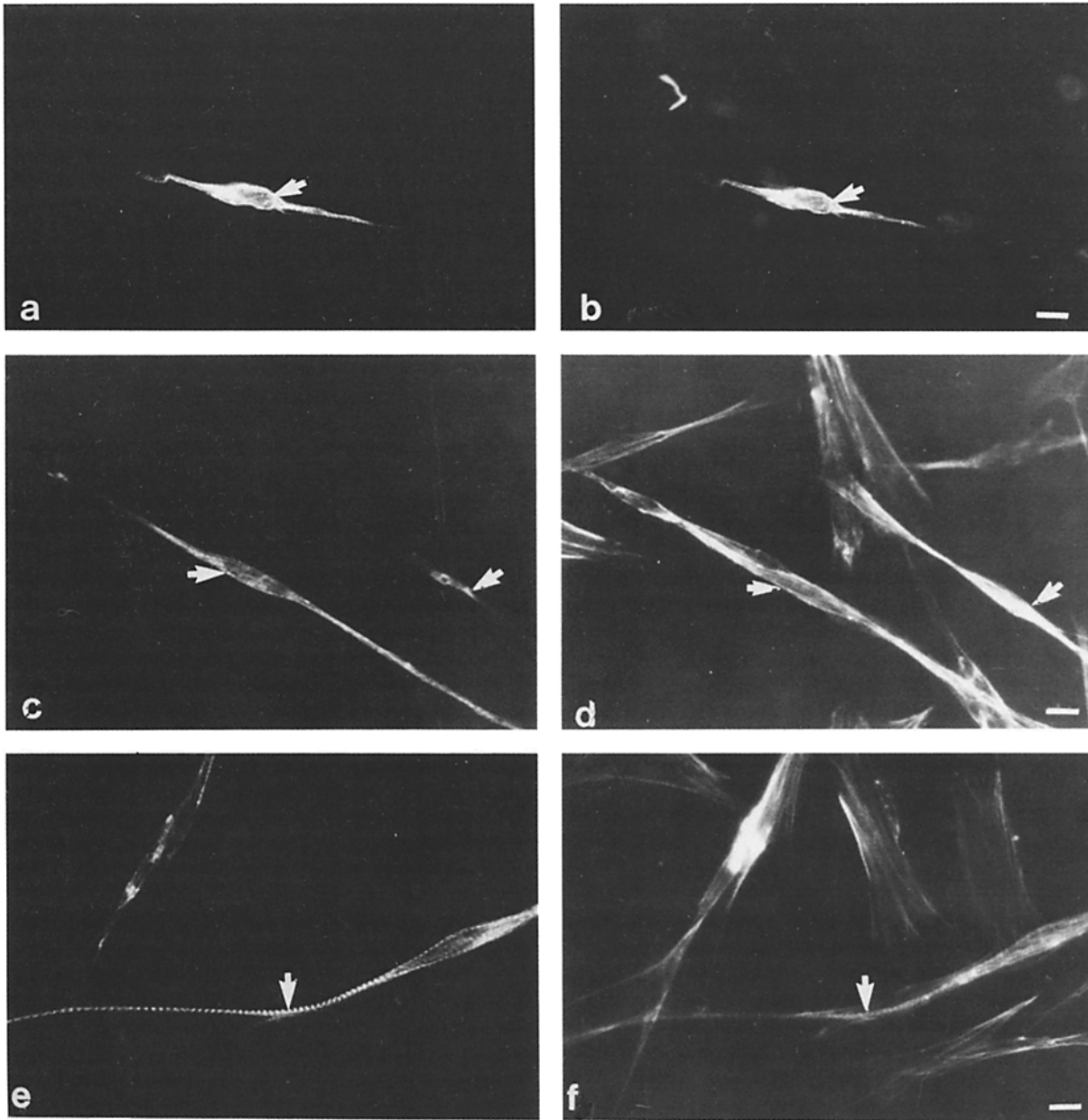


Figure 4. Different microscopic fields of a low density day-3 culture illustrating three postmitotic, mononucleated myoblasts in different stages of maturation. *a* and *b* are double-stained with antibodies to titin (rhodamine) and MHC (fluorescein). The nuclei of six fibroblastic cells that did not bind either antibody are barely detectable in *b*. *c* and *d* show cells double-stained with anti-MHC (fluorescein) and rhodamine-phalloidin. Many of the cells that are MHC negative, but rhodamine-phalloidin positive, are replicating presumptive myoblasts. Note in *d*, the juxtaposition of several mononucleated cells that are beginning to elongate. This intimate pre-alignment of still MHC-negative bipolar myoblasts precedes fusion by several hours. *e* and *f* illustrate a mature postmitotic, mononucleated myoblast double-stained with anti-titin (fluorescein) and rhodamine-phalloidin. The clear sarcomeric periodicity of the single long myofibril revealed by staining with anti-titin is less distinct after staining with rhodamine-phalloidin. Bar, 10 μ m.

duced desmin IF cables. Extrapolating from Fig. 5, *a-h*, it is not likely that the anti-titin binds to these I-Z-I complexes. However, this important point can only be resolved by using immunoelectron microscopy procedures.

The many rounded postmitotic, mononucleated myoblasts (8, 11, 26) found in these Colcemid-treated cultures are particularly instructive. They are readily distinguished from rounded, replicating, metaphase-arrested cells by their interphase nuclei and the fact that they are desmin, MHC, and titin positive. Without exception, they display what under the

fluorescence microscope appear to be MHC- and titin-positive nonstriated myofibrils. These are deployed in a convoluted, circular fashion. In EM sections (Lin, Z., and H. Holtzer, unpublished observations), these myofibrils prove to consist of scrambled arrays of hexagonally interdigitating thick and thin filaments. Scattered free I-Z-I complexes are also common in these cells. Colcemid-induced, rounded, myoblasts are very similar to the rounded, postmitotic, mononucleated myoblasts induced by cytochalasin B (11, 26). Clearly, myofibrils that appear nonstriated under the light

microscope can consist of poorly aligned, but nevertheless interdigitating, thick and thin filaments.

The Temporal and Spatial Coupling of Myosin and Titin Is Maintained in Taxol-induced Pseudo-striated Myofibrils

In the presence of taxol, postmitotic mononucleated myoblasts fail to elongate or fuse, but form star-shaped cells that assemble pseudo-striated myofibrils. These intriguing myofibrils consist of laterally aligned, 1.6- μm long thick filaments that, instead of interdigitating with thin filaments, interdigitate with long microtubules. These thick filament-microtubule arrays, in addition to generally excluding thin filaments, also lack Z bands, as determined in EM sections (4, 27, 28, 48).

Taxol was added to day-1 cultures and the cells incubated for an additional 3–5 d in the presence of the drug. Many of the cells were arrested and killed in mitosis. Postmitotic mononucleated myoblasts, however, continued to increase in size and remained relatively constant in number over the last 2–3 d of culture. These postmitotic myoblasts did not translocate, but gradually acquired the characteristic star-shaped configuration of taxol-treated myogenic cells. Why taxol-treated muscle, but not other types of taxol-treated cells, become star-shaped, or why taxol blocks fusion of postmitotic myoblasts into myotubes, is not known.

Day-4 taxol-treated, postmitotic, mononucleated myoblasts were double stained in various combinations with anti-titin, anti-myosin, and anti-desmin (Fig. 6, *a–f*). The anti-titin stains sharp bands with a sarcomeric periodicity similar to that observed in postmitotic mononucleated myoblasts and control myotubes; e.g., $\sim 2.3 \mu\text{m}$. The width of the titin-positive bands varies considerably, but never achieves the width of A bands revealed by either the polyclonal or monoclonal anti-MHC. EM sections of these pseudo-striated myofibrils reveals that the distance separating adjacent A bands varies more than observed in controls, and also that the lateral alignment of the 1.6- μm long thick filaments is also less rigorous than in controls (Lin, Z., and H. Holtzer, unpublished observations). We currently are determining by means of immunoelectron microscopy techniques whether titin binds to the same site at the distal ends of the thick filaments in both normal and in these abnormal sarcomeres.

How new sarcomeres are added to the distal ends of myofibrils is of interest (5, 12, 23, 24, 26, 29, 30). In normal, elongating, postmitotic myoblasts the most distal sarcomere, as well as those more proximal, are roughly 2.3 μm in width. Distal to this terminal sarcomere, there often is a nonstriated MHC-positive filament that varies considerably in length. In normal postmitotic mononucleated myoblasts no MHC-positive minisarcomeres are observed distal to this terminal sarcomere. With these considerations in mind, the distal tips of the pseudo-striated myofibrils as revealed by titin and MHC labeling were closely inspected. As illustrated in Fig. 6, *a*, *c*, and *e*, the titin-positive bands are separated by distances of $\sim 2.3 \mu\text{m}$. The most distal titin staining structures are a single band. Titin-positive structures approximating a doublet are generally found more proximally. No titin-positive structures that could be described as strings of minisarcomeres are observed at any stage. The length of the MHC-

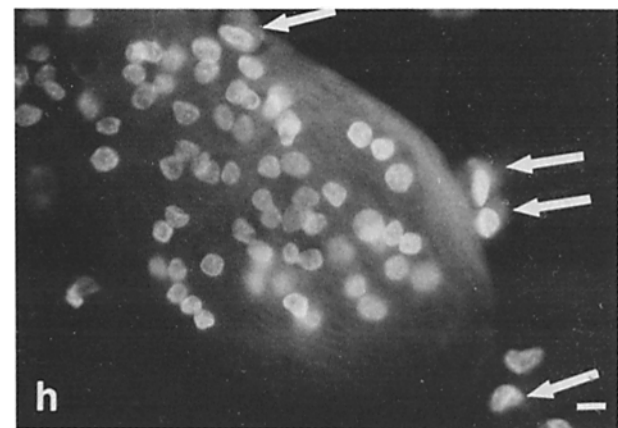
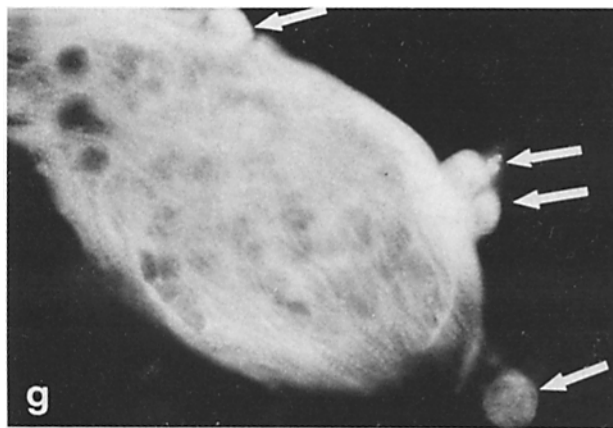
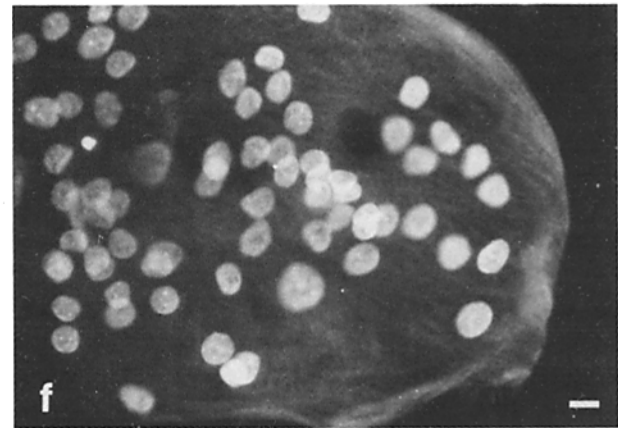
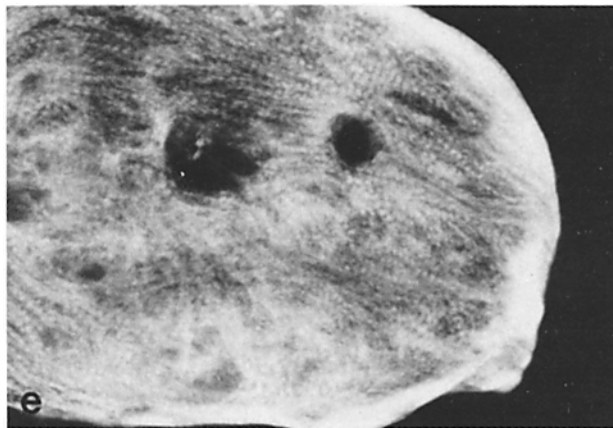
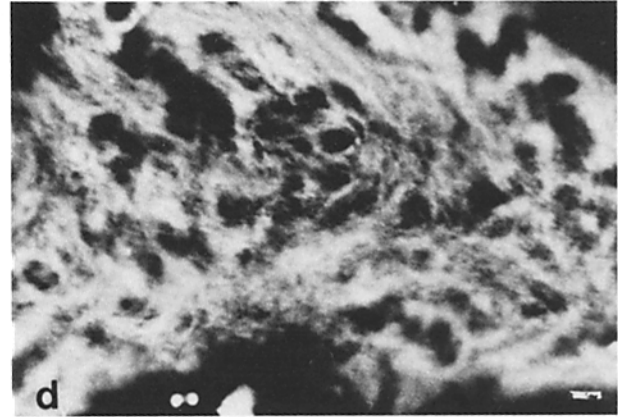
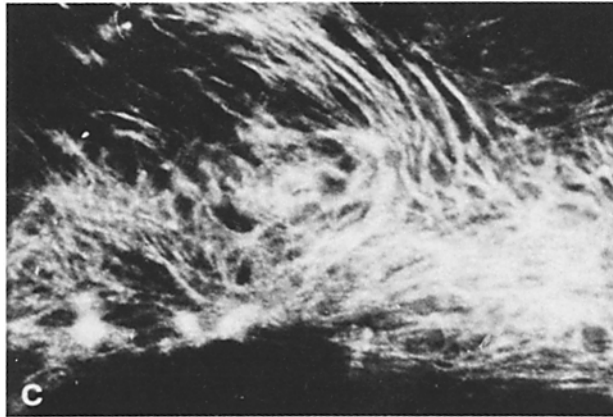
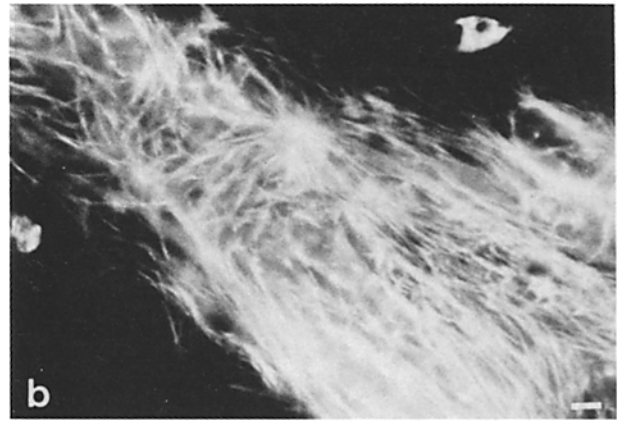
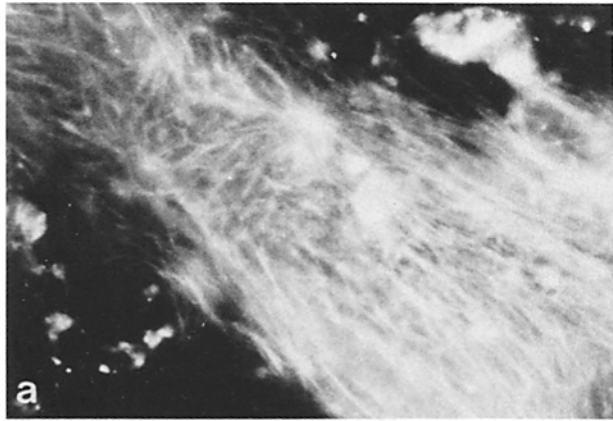
positive nonstriated filament at the ends of these elongating myofibrils varies as it does in normal elongating myofibrils.

Double-stained combinations of anti-titin or anti-myosin with anti-desmin demonstrated that desmin is always present in these taxol-treated, postmitotic mononucleated myoblasts. Of interest is that rather than being uniformly dispersed throughout the sarcoplasm as in control postmitotic myoblasts, desmin in taxol-treated cells is often induced to aggregate into fluorescent “hot spots” (Fig. 6 *f*). EM sections of these “hot spots” reveal IF skeins of a kind rarely observed in control myoblasts, but common in taxol-treated muscle fibroblasts (19).

Discussion

The emphasis in this report is on the assembly of striated myofibrils in postmitotic, mononucleated myoblasts. In vivo and in vitro replicating presumptive myoblasts normally do not synthesize myofibrillar isoforms, nor do they fuse to form multinucleated myotubes (9, 10, 23, 26, 30, 37). However, in addition to synthesizing vimentin, a small subset of replicating presumptive myoblasts initiate the synthesis of desmin. The progeny of these, as mononucleated cells, withdraw from the cell cycle, coordinately initiate the synthesis of many myofibrillar protein isoforms, and acquire the capacity to fuse with other postmitotic myoblasts (9, 10, 18, 30, 41). These cells rapidly elongate, and irrespective of whether or not they fuse, start to assemble long, fine myofibrils. For a brief period these nascent myofibrils appear nonstriated, but this quickly changes to that of tandem full-sized sarcomeres. These early myofibrils, as well as binding antibodies to myofibrillar MHC and to titin, have been shown to bind antibodies to tropomyosin (30), C-protein (18), alpha-actinin (14, 33), and myomesin (20). These proteins are detected in association first with nonstriated, and only subsequently, with striated myofibrils. Finding that all these proteins are synthesized in the postmitotic mononucleated myoblasts does not prove, however, that all are required for or must participate simultaneously in the assembly of the myofibril. The significance of the transient fine, long, nonstriated nascent myofilaments is unclear. It is probable, however, that even at this early stage of myofibrillogenesis, typical polarized 1.0- μm long, thin alpha-actin and bipolar 1.6- μm long, myosin thick filaments are present. Longitudinal and lateral overlap between such definitive-sized filaments would give the impression of a nonstriated myofibril in the fluorescence microscope (23, 27, 30, 37, 43).

Though much is known about the biochemistry and molecular biology of the myofibrillar proteins, there is no information as to how they assemble into ordered myofibrils and/or sarcomeres. For example, do some stages require extra-myofibrillar spatial information provided transiently by pre-existing SFLSs, IFs, or microtubules? What in the process is largely self-assembly? Is there an ordered time course of expression for certain myofibrillar proteins thus allowing for some crucial intermediates in the structure to be accumulated? We considered the possibility that titin might function to integrate thick and thin filaments as various models view titin interacting with thick filaments (21, 49, 51–54), and the N₂ line in the I band (52) or the Z band (34). If titin did



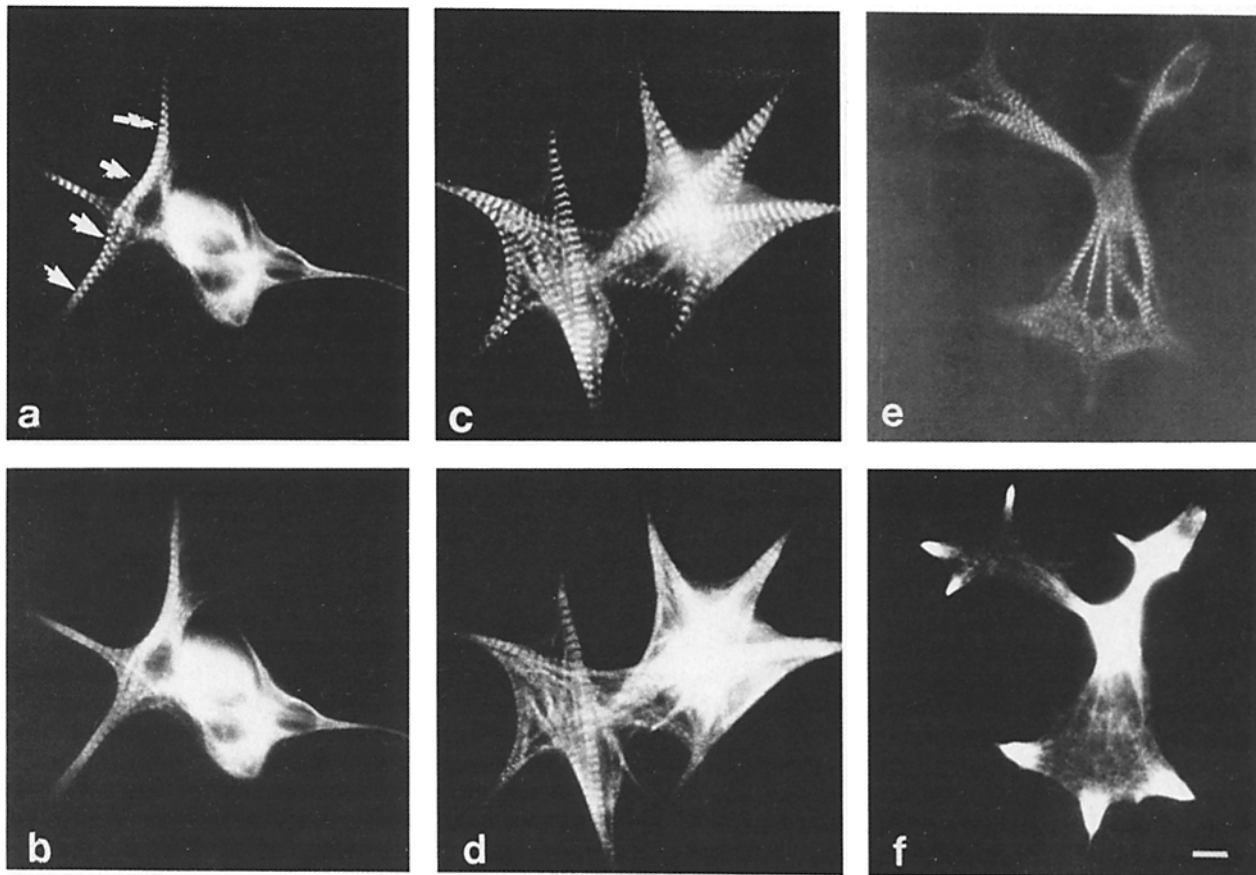


Figure 6. *a–d* illustrate, respectively, a tight cluster of four overlapping, nonfusing, and two adjacent, nonfusing, postmitotic, mononucleated myoblasts reared in taxol for 3 d. *a* and *c* show cells stained with anti-titin (rhodamine); *b* and *d* show cells stained with anti-myosin (fluorescein). The tapering of the myofibrils in the arms of star-shaped myoblasts that conform to the cells overall shape is unique to taxol-treated cells. Note, as indicated by the arrows, the definitive-sized sarcomeres that have assembled directly under the cell membrane. This intimate association of emerging pseudo-striated myofibrils with the sarcolemma occurs in spite of the absence of Z bands in many of these pseudo-sarcomeres. Staining with both anti-titin and anti-MHC reveals the characteristic complex criss-crossing of individual myofibrils in cells that do not elongate properly. Taxol does not induce fibroblastic cells or other cell types, including cardiac myocytes or smooth muscle cells, to assume a star-shaped configuration. *e* illustrates the binding of anti-titin (fluorescein), whereas *f* illustrates the unusual distribution of anti-desmin “hot spots” in these cells (rhodamine). Minisarcromeres have not been observed at any stage during the assembly of pseudo-striated myofibrils.

function as a core or scaffold protein around which thick and thin filaments and their associated proteins were sequentially added, then there should be a period when nascent myofibrils were titin positive but MHC negative. Instead, in normal newly born postmitotic, mononucleated myoblasts, as well as in similar cells treated with Colcemid or taxol, the presence of titin invariably marked the presence of MHC. Given the limitations of the microscopic techniques used, it is obvi-

ous that we cannot exclude the possibility that for brief periods—e.g., minutes—small numbers of longitudinally oriented titin molecules exist, and to these pre-existing elongated polypeptides, thick and thin filaments are added in a rigorously prescribed temporal sequence. But if such a temporal and spatial sequence is obligatory, our data places severe constraints on the time intervals in this putative multi-stage assembly process.

Figure 5. Four different double-stained, Colcemid-induced, multinucleated myosacs. *a* and *b* illustrate the binding of anti-titin (rhodamine) and anti-myosin (fluorescein) to numerous emerging, unstriated myofibrils in early day-4 myosacs. This Colcemid myosac contains 18 nuclei as determined under the phase-contrast microscope. This myosac should be compared with a control early day-4 myotube such as illustrated in Figs. 2 and 3. *c* and *d* show an early day-4 myosac with 26 nuclei double stained with anti-titin (fluorescein) and anti-desmin (rhodamine). Incipient striations are observed in a minority of the intertwining, as yet unstriated, myofibrils stained with anti-titin (Fig. 2). The distribution of the tortuous induced IF cables (*d*) bears no obvious relationship with the forming myofibrils. *e* and *f* show a late day-4 Colcemid myosac stained with anti-MHC (rhodamine) and bisbenzamide (Hoescht 33250). The improved lateral alignment of the now clearly striated myofibrils contrasts with Colcemid myosacs from earlier cultures. Note the irregular distribution of nuclei in these myosacs and the lack of an obvious correlation between nuclei and myofibrils. Not infrequently striated myofibrils will encircle a cluster of nuclei. *g* and *h*, also from a late day-4 myosac, are stained with anti-titin (rhodamine) and with bisbenzamide (Hoescht 33250). Due to the thickness of the myosac and the superimposition of myofibrils, their striated nature is obscured. The numerous scattered I-Z-I complexes evident in EM sections do not appear to stain with antibodies to titin or desmin. Arrows point to four rounded postmitotic, mononucleated myoblasts. The two mononucleated cells negative for titin in the lower cluster are, by definition, fibroblastic cells that have not cycled during the period Colcemid was present. Bar, 10 μ m.

Although the taxol-induced pseudo-striated myofibrils are aberrant structures, they provide basic information regarding normal myofibrillogenesis. They demonstrate that in the absence of recognizable thin filaments and Z bands, A band-like structures can assemble. The 1.6- μm long thick filaments that comprise the A bands of these curious myofibrils demonstrates that the length-determining mechanism for thick filaments does not require the participation of intact thin filaments or Z bands. These observations, coupled with studies of myosin monomers in solution (31), suggest that the assembly of 1.6- μm long thick filaments *in vivo* and *in vitro*, might be relatively autonomous. Studies in progress reveal that in both untreated and taxol-treated postmitotic, mononucleated myoblasts, the earliest discernable A bands bind antibodies to C protein and myomesin, while EM sections of these thick filament-microtubule arrays reveal numerous bridges connecting the 1.6- μm long thick filaments to one another. Whether the bridges between thick filaments observed in pseudo-striated myotubes are related to those described by Suzuki and Pollock (44) is unknown.

The emergence of ordered A bands and isolated I-Z-I complexes in Colcemid myosacs and in taxol-treated cells (4, 26-28, 30, 48) suggests that each sarcomere may require the integrated interaction of two partially independent "organizing centers." One center would organize the A band and involves the lateral alignment of bipolar thick filaments and their stoichiometric complexing with C protein and myomesin. The other center would organize the I-Z-I complex and involves the insertion of oppositely polarized thin alpha-actin filaments—including the associated tropomyosins and troponins—into either face of the Z band. Molecules like titin might integrate these two relatively autonomous "organizing centers" leading to the characteristic interdigitation of the two major sets of filaments. Even then it is not likely that titin serves as a rigid template, for the molecule is thought to be an elastic string rather than a rigid rod. The length of this string-like molecule has been estimated as 1 μm and thus could stretch nearly half the length of a myofibril (51). If this long molecule was not attached at both ends to two or more points within the myofibril, it would be expected to collapse in elastic recoil to one attachment site. Since the thick and thin filaments either directly or indirectly serve as attachment sites for titin, it is likely that the molecule would have to be incorporated into the myofibril either simultaneously, or immediately after the appearance of thick and thin filaments rather than serving as a pre-existing template for their alignment. Our studies suggest that titin appears simultaneously with MHC and, as in adult muscle, is attached to the ends of the thick filaments from the time of its first appearance in the myofibril. This coupling of titin to the thick filaments occurs independently of its attachment to other putative sites within the I band such as the N_2 line or the Z band since it occurs in both Colcemid- and taxol-treated cells. The typical sarcomeric coupling of titin and MHC in taxol-induced pseudo-striated myofibrils is of interest for these structures are deficient in thin filaments and Z bands. Immunoelectron microscopy will be required to determine where titin links to the thick filaments in normal and drug-treated cells.

Though striations are most conspicuous in the vicinity of the nucleus, not infrequently they are first observed in the cell's processes. If there is a gradient of soluble myofibrillar

monomers with a high point around the nucleus and diminishing toward the cell's growth tips, it might be reflected in a gradient of maturation along the length of the myofibril, or a tapering in the diameter of a myofibril distally. However, the diameters of the myofibrils illustrated in Figs. 3 and 4 are remarkably constant at distances considerably removed from the nuclei. This observation would be consistent with the notion that the myofibrillar proteins are synthesized in proximity to where they are assembled into myofibrils, and account for the fact that pools of myofibrillar proteins are not found.

Sanger et al. (42) have microinjected cardiac myocytes and myotubes with labeled anti-alpha-actinin and monitored the cells with video intensifying optics. They claimed to have observed a series of fluorescent Z bands separated by progressively narrower nonfluorescent zones. Close inspection of their micrographs does not, in fact, reveal a progressive sequence of minisarcomeres as described in their text. Other studies (3, 5, 12) using a similar anti-alpha-actinin emphasized that such antibodies stain both the closely spaced dense bodies of SFLSs as well as Z bands. What Sanger et al. (42) identified as a string of closely spaced Z bands supposedly marking the boundaries of a series of minisarcomeres were probably the dense bodies of the SFLSs that extend into the cell's growth tip (for details see reference 12). The findings described in this paper regarding pseudo-striated myofibrils are germane to this issue. In control and drug-treated cells, the binding of the anti-titin in a sarcomeric periodicity extends into the finest distal tips of all myofibrils (Fig. 6, *a*, *c*, and *e*). The sarcomeric periodicity at the ends of elongating myofibrils after staining with antibodies to titin or myosin is difficult to reconcile with the notion of a series of minisarcomeres consisting of aligned, progressively foreshortened A bands, reflecting, in turn, correspondingly foreshortened thick filaments.

Earlier double-staining experiments with anti-MHC and anti-desmin revealed that as myotubes matured, desmin IFs shifted from a longitudinal to a more transverse orientation (6, 7, 16, 24, 25). There was no evidence from these studies that this striking redistribution reflected the involvement of desmin IFs in integrating adjacent myofibrils by way of their Z bands, or that this shift resulted in desmin IFs connecting Z bands to the sarcolemma. Essentially similar conclusions have been reached by Tokuyasu et al. (46, 47) using immunoelectron microscopy. Further evidence of a secondary role for desmin during myofibrillogenesis is the assembly of pseudo-striated myofibrils in taxol-treated cells. The typical sarcomeric periodicity of both titin and MHC is maintained in spite of the fact that the cell's desmin was tied up in IF skeins (see also Fig. 11 in reference 28).

Earlier findings (6, 7, 13, 16) and those reported here would identify desmin as the earliest known marker for cells in the myogenic lineage. This observation on normal myogenic cells is consistent with, and relevant to, interpreting the finding that human and rat rhabdomyosarcoma cells are mostly desmin positive, irrespective of whether they are positive for other myofibrillar proteins such as titin (1, 2, 38). Our finding that replicating presumptive myoblasts have initiated the synthesis of desmin suggests that the cell of origin of rhabdomyosarcomas is not derived from mature, postmitotic muscle, but from the normally quiescent satellite cells. This view would be consistent with the observation that

cardiac tissue lacks satellite cells and heart-derived rhabdomyosarcomas seem rare. It will be interesting to determine whether in immortalized and partially transformed myogenic cell lines there is a breakdown in the controls that normally regulate, separately, the synthesis of desmin and that of titin and myosin.

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References

1. Altmannsberger, M., M. Osborn, J. Treuner, A. Holscher, K. Weber, and A. Schauer. 1982. Diagnosis of human childhood rhabdomyosarcoma by antibodies to desmin, the structural protein of muscle specific intermediate filaments. *Virchows Arch. B Cell Pathol.* 39:203-215.
2. Altmannsberger, M., K. Weber, B. Droste, and M. Osborn. 1985. Desmin is a specific marker for rhabdomyosarcoma of human and rat origin. *Am. J. Pathol.* 118:85-95.
3. Antin, P. B., S. Forry-Schaudies, S. Duran, J. Eshleman, and H. Holtzer. 1986. Using a co-carcinogen (TPA) and carcinogen (EMS) to probe myofibrillogenesis. In *Molecular Biology of Muscle Development*, UCLA Symposium of Molecular and Cellular Biology. C. Emerson, D. Fischman, B. Nadal-Ginard, M. A. Q. Siddiqui, editors. Alan R. Liss, New York. 709-723.
4. Antin, P., S. Forry-Schaudies, T. Friedman, S. Tapscott, and H. Holtzer. 1981. Taxol induces postmitotic myoblasts to assemble interdigitating microtubule-myosin arrays that exclude actin filaments. *J. Cell Biol.* 90:300-308.
5. Antin, P. B., S. Tokunaka, V. T. Nachmias, and H. Holtzer. 1986. Role of stress fiber-like structures in assembling nascent myofibrils in recovering ethyl methanesulfonate myosheets. *J. Cell Biol.* 102:1464-1479.
6. Bennett, G. S., S. A. Fellini, and H. Holtzer. 1978. Immunofluorescent visualization of 100 Å filaments in different cultured chick embryonic cell types. *Differentiation.* 12:71-82.
7. Bennett, G. S., S. Fellini, Y. Toyama, and H. Holtzer. 1979. Redistribution of intermediate filament subunits during skeletal myogenesis and maturation in vitro. *J. Cell Biol.* 82:577-584.
8. Bischoff, R., and H. Holtzer. 1967. The effect of mitotic inhibitors on myogenesis in vitro. *J. Cell Biol.* 36:111-128.
9. Chi, J. C., S. A. Fellini, and H. Holtzer. 1975. Differences among myosins synthesized in non-myogenic cells, presumptive myoblasts, and myoblasts. *Proc. Natl. Acad. Sci. USA.* 72:4999-5003.
10. Chi, J. C. H., N. Rubinstein, K. Strahs, and H. Holtzer. 1975. Synthesis of myosin heavy and light chains in muscle cultures. *J. Cell Biol.* 67:523-537.
11. Croop, J. M., and H. Holtzer. 1975. Response of fibrogenic and myogenic cells to cytochalasin B and to colcemid. I. Light microscopic observations. *J. Cell Biol.* 65:271-285.
12. Dlugosz, A. A., P. B. Antin, V. T. Nachmias, and H. Holtzer. 1984. The relationship between stress fiber-like structures and nascent myofibrils in cultured cardiac myocytes. *J. Cell Biol.* 99:2268-2278.
13. Dlugosz, A. A., S. J. Tapscott, and H. Holtzer. 1983. Effects of phorbol-12-myristate 13-acetate on the differentiation program of embryonic chick skeletal myoblasts. *Cancer Res.* 43:2780-2789.
14. Endo, T., and T. Masaka. 1984. Differential expression and distribution of chicken skeletal- and smooth muscle-type alpha-actinins during myogenesis in culture. *J. Cell Biol.* 99:2322-2332.
15. Eppenberger, H., and J. C. Perriard. 1984. Developmental processes in normal and diseased muscle. *Exp. Biol. Med.* 9:1-293.
16. Fellini, S., G. Bennett, and H. Holtzer. 1978. Selective binding of antibody against 10 nm filaments to different cell types in myogenic cultures. *Am. J. Anat.* 153:451-457.
17. Fischman, D. A. 1972. The synthesis and assembly of myofibrils in embryonic muscle. In *The Structure and Function of Muscles*, G. H. Bourne, editor. Academic Press, New York. 75-148.
18. Fischman, D. A. 1986. Morphogenesis and myofibrillogenesis in skeletal muscle. In *Myology*, A. Engel and B. Banker, editors. 1-75.
19. Forry-Schaudies, S., J. M. Murray, Y. Toyama, and H. Holtzer. 1986. Effects of Colcemid and taxol on microtubules and intermediate filaments in chick embryo fibroblasts. *Cell Motility and Cytoskeleton.* 6:324-338.
20. Grove, B. K., L. Cerney, J. C. Perriard, and H. M. Eppenberger. 1985. Myomesin and M-protein: expression of two M-band proteins in pectoral muscle and heart during development. *J. Cell Biol.* 101:1413-1421.
21. Gruen, L. C., N. L. King, L. Kurth, and I. J. McKenzie. 1982. Studies on the structure of connectin in muscle. *Int. J. Pept. Protein Res.* 20:401-407.
22. Hill, C., and K. Weber. 1986. Monoclonal antibodies distinguish titins from heart and skeletal muscle. *J. Cell Biol.* 102:1099-1108.
23. Holtzer, H., and J. W. Sanger. 1972. Myogenesis, old views rethought. In *Research in Muscle Development and the Muscle Spindle*. B. Banker, R. Przyblski, J. Vander Moulen, and M. Victor, editors. *Excerpta Med. Int. Congr. Ser.* 122-133.
24. Holtzer, H., G. S. Bennett, S. J. Tapscott, J. M. Croop, A. Dlugosz, and Y. Toyama. 1981. Changes in intermediate-sized filaments during myogenesis and neurogenesis. In *International Cell Biology 1980-81*. H. G. Schweiger, editor. Springer-Verlag, Berlin. 293-305.
25. Holtzer, H., G. S. Bennett, S. J. Tapscott, J. M. Croop, and Y. Toyama. 1982. Intermediate-sized filaments: changes in synthesis and distribution in cells of myogenic and neurogenic lineages. *Cold Spring Harbor Symp. Quant. Biol.* 46:317-329.
26. Holtzer, H., J. Croop, H. Ishikawa, and A. P. Somlyo. 1975. Effects of cytochalasin B and colcemid on myogenic cultures. *Proc. Natl. Acad. Sci. USA.* 72:513-517.
27. Holtzer, H., S. Forry-Schaudies, P. Antin, Y. Toyama, M. Goldstein, and D. Murphy. 1982. Taxol induces myosin-microtubule arrays in postmitotic myoblasts and myotubes. In *Muscle Development: Molecular and Cellular Control*. M. Pearson and H. Epstein, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. 383-396.
28. Holtzer, H., S. Forry-Schaudies, A. Dlugosz, P. Antina, and G. Dubyak. 1985. Interactions between IFs, microtubules, and myofibrils in fibrogenic and myogenic cells. In *Intermediate Filaments*. E. Wang, D. Fischman, R. K. H. Liem, T.-T. Sun, editors. New York Academy of Sciences, New York. 455:106-125.
29. Holtzer, H., J. Marshall, and H. Finck. 1957. An analysis of myogenesis by the use of fluorescent antimyosin. *J. Biophys. Biochem. Cytol.* 8:705-723.
30. Holtzer, H., J. Sanger, H. Ishikawa, and K. Strahs. 1972. Selected topics in skeletal myogenesis. *Cold Spring Harbor Symp. Quant. Biol.* 37:549-566.
31. Huxley, H. E. 1963. Electron microscopic studies on the structure of natural and synthetic protein filaments from striated muscle. *J. Mol. Biol.* 7:287-308.
32. Ishikawa, H., R. Bischoff, and H. Holtzer. 1968. Mitosis and intermediate-sized filaments in developing skeletal muscle. *J. Cell Biol.* 38:538-555.
33. Jokusch, H., and B. M. Jokusch. 1980. Structural organization of the Z-line protein, alpha-actinin, in developing skeletal muscle cells. *Dev. Biol.* 75:231-238.
34. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature (Lond.)*. 283:249-256.
35. Maruyama, K., T. Yoshioka, H. Higuchi, K. Ohashi, S. Kiumra, and R. Natori. 1985. Connectin filaments link thick filaments and Z-lines in frog skeletal muscle as revealed by immunoelectron microscopy. *J. Cell Biol.* 101:2167-2172.
36. Moss, P., and R. Strohmman. 1976. Myosin synthesis by fusion-arrested chick embryo myoblasts in cell culture. *Dev. Biol.* 48:431-437.
37. Okazaki, K., and H. Holtzer. 1965. An analysis of myogenesis in vitro using fluorescein-labelled antimyosin. *J. Cytol. Histochem.* 13:726-739.
38. Osborn, M., C. Hill, M. Altmannsberger, and K. Weber. 1986. Monoclonal antibodies to titin in conjunction with antibodies to desmin separate rhabdomyosarcomas from other tumor types. *Lab. Invest.* In press.
39. Pearson, M. L., and H. F. Epstein, editors. 1982. *Muscle Development: Molecular and Cellular Control*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 1-581.
40. Quinn, L. S., H. Holtzer, and M. Nameroff. 1985. Generation of chick skeletal muscle cells in groups of 16 from stem cells. *Nature (Lond.)*. 313:692-694.
41. Rubinstein, N., J. Chi, and H. Holtzer. 1976. Coordinated synthesis and degradation of actin and myosin in a variety of myogenic and non-myogenic cells. *Exp. Cell Res.* 97:387-393.
42. Sanger, J. M., B. Mittal, M. Pochapin, and J. W. Sanger. 1986. Myofibrillogenesis in living cells microinjected with fluorescently labeled alpha-actinin. *J. Cell Biol.* 102:2053-2066.
43. Shimada, Y., and J. Obinata. 1977. Polarity of actin filaments at the initial stage of myofibril assembly in myogenic cells in vitro. *J. Cell Biol.* 72:777-785.
44. Suzuki, S., and G. H. Pollack. 1986. Bridge-like interconnections between thick filaments in stretched skeletal muscle fibers observed by the freeze-fracture method. *J. Cell Biol.* 102:1093-1098.
45. Tapscott, S. J., G. S. Bennett, Y. Toyama, F. A. Kleinbart, and H. Holtzer. 1981. Intermediate filament proteins in the developing chick spinal cord. *Dev. Biol.* 86:40-54.
46. Tokuyasu, D. T., P. A. Maher, A. H. Dutton, and S. J. Singer. 1985. Intermediate filaments in skeletal and cardiac muscle tissue in embryonic and adult chicken. In *Intermediate Filaments*. E. Wang, D. Fischman, R. K. H. Liem, and T.-T. Sun, editors. New York Academy of Sciences, New York. 455:200-212.
47. Tokuyasu, K. T., P. A. Maher, and S. J. Singer. 1985. Distributions of vimentin and desmin in developing chick myotubes in vivo. II. Immunoelectron microscopic study. *J. Cell Biol.* 100:1157-1166.
48. Toyama, Y., S. Forry-Schaudies, B. Hoffman, and H. Holtzer. 1982. Effect of taxol and colcemid on myofibrillogenesis. *Proc. Natl. Acad. Sci. USA.* 79:6556-6566.
49. Trinick, J., P. Knight, and A. Whiting. 1984. Purification and properties of native titin. *J. Mol. Biol.* 180:331-356.
50. Vertel, B., and D. Fischman. 1976. Myosin accumulation in mononucleated cells of chick muscle cultures. *Dev. Biol.* 48:438-446.
51. Wang, K. 1985. Sarcomere-associated cytoskeletal lattices in striated muscle. In *Cell and Muscle Motility*. J. W. Shay, editor. Plenum Publishing

Corp., New York. 6:315-369.

52. Wang, K., J. McClure, and A. Tu. 1979. Titin: major myofibrillar components of striated muscle. *Proc. Natl. Acad. Sci. USA.* 77:3254-3258.

53. Wang, S.-M., and M. L. Greaser. 1985. Immunocytochemical studies using a monoclonal antibody to bovine cardiac titin on intact and extracted myofibrils. *J. Muscle Res. Cell Motil.* 6:293-312.

54. Wang, S.-M., E. Schultz, and M. L. Greaser. 1984. Immunofluorescence studies with a monoclonal anti-titin antibody on skeletal muscle myofibrillogenesis. *J. Cell Biol.* 99(5,Pt.2):436a. (Abstr.)

55. Yeoh, G. C., and H. Holtzer. 1977. The effect of cell density, conditioned medium, and cytosine arabinoside on myogenesis in primary and secondary cultures. *Exp. Cell Res.* 104:63-78.